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Potential Regulation of AMP-Activated Protein Kinase Pathway in Stress Induced Synaptic Plasticity in the Basolateral Complex of the Amygdala

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Abstract

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By Wei Liu

The basolateral complex of the amygdala (BLC) is known to be involved in the regulation of emotion, and over excitation of the BLC is thought to underlie the development of mood disorders. An imbalance in the activity of two neuronal subpopulations in the BLC, namely excitatory, glutamatergic, principal neurons and inhibitory, GABAergic, interneurons can cause the amygdala to become hyper-excitable. Chronic stress is known to cause such a change and facilitate a type of synaptic plasticity, long term potentiation (LTP) in the BLC. Moreover, LTP formation in the BLC is calcium-dependent, and requires activation of dopamine D1 receptors that are coupled to the adenylate cyclase, cAMP, and protein kinase A second messenger cascade. Similar to stress, unpublished *in vitro* patch clamp studies in the Rainnie lab showed that rolipram, a phosphodiesterase type 4 (PDE4) inhibitor that blocks the hydrolysis of cAMP to 5'AMP, decreased the threshold for LTP induction and action potential generation, thereby increasing the excitability of BLC principal neurons. Significantly, increasing intracellular cAMP levels did not mimic the effect of rolipram, whereas increasing 5'AMP levels did. Thus, we reasoned chronic stress disrupts a baseline homeostatic regulatory system that detects 5'AMP levels and shifts induction thresholds to maintain homeostasis in the BLC. We proposed that AMP-activated protein kinase (AMPK), which is known to maintain homeostasis and conserve energy in the periphery, acts as a central metabolic sensor by detecting AMP:ATP levels in BLC neurons. Here, we used a multidisciplinary approach employing RT-PCR, Western blots, and immunohistochemistry to create a baseline profile of the AMPK signaling cascade in the BLC, and examined protein expression level changes following repeated restraint stress (RRS) and food restriction (FR) protocols. We report that principal neurons of the BLC express two upstream activators of AMPK, LKB1 and CaMKKβ, as well as AMPK itself, and downstream target Kv2.1, which is known to alter the threshold for action potential firing upon activation. Moreover, using selective AMPK activators we further confirmed the role of AMPK as a functional regulator for energy conservation in neurons under baseline conditions, and showed RRS can cause a persistent perturbation of neuronal homeostasis.

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Introduction

The basolateral complex of the amygdala: Anatomy and its role in fear memory formation.

The amygdala is an almond-shaped structure located deep in the medial temporal lobe and is divided into over 10 subnuclei, where extensive intra- and internuclear connections are found (Sah et al., 2003). Specifically, the basolateral complex of the amygdala (BLC) is divided into the lateral nucleus (LA), the basolateral nucleus (BLA) and the accessory basal nucleus. Additional subnuclei of the amygdala include cortical-like nuclei, the medial and central nuclei (CeA). Functionally, the amygdala is known to play a central role in emotional and sensory information processing. Of all the amygdala subnuclei, the BLC is considered to be the primary point of convergence for sensory input while the CeA is thought to be the primary output structure, and both are involved in the initiation of the physiological responses to fearful stimuli (Pape & Pare, 2010).

Although the BLC lacks the laminar organization of the overlying cortex, it has similar neuronal composition, morphology, synaptology, electrophysiological and pharmacological properties and, as a consequence, is regarded as a pseudo-cortical structure. The BLC contains two distinct types of neuron: the predominant subgroup (>80%) are the glutamatergic principal neurons that have extensive dendritic trees covered by spines, as well as axons that have multiple local collaterals in addition to targeting output structures. The other subgroup are the local GABAergic interneurons, which have relatively short aspiny dendrites, but extensive local axon collaterals. These GABAergic interneurons can be further subdivided into different subgroups based on their expression of the calcium-binding proteins parvalbumin (PV), calbindin (CD) and calretinin (CR) and/or the neuropeptides somatostatin (SOM), vasoactive intestinal peptide,

cholecystokinin (CCK), or neuropeptide Y (NPY). Importantly, these subgroups also have distinct electrophysiological characteristics (McDonald & Mascagni, 2006), and will therefore be used as markers for identifying interneuron subpopulations in this study. Even though interneurons only constitute approximately 20% of the total population, they play a major role in regulating the overall excitability of the BLC. Evidence also suggests that different subtypes of BLC interneurons synapse onto different compartments of the BLC principal neurons. For example axon collaterals of parvalbumin interneurons form "pericellular baskets" around the soma of large pyramidal neurons (McDonald & Mascagni, 2006) where they are thought to play a key role in regulating the firing activity of these neurons (Ryan et al., 2012).

Previous studies using a fear conditioning model of fear memory formation, illustrated in **Figure 1** (Sigurdsson et al., 2007) have clearly shown that the amygdala plays an important role in fear-related circuitry (LeDoux, 2003). Fear allows animals to avoid dangerous situations and is evolutionarily conserved in all species, due to its crucial role in survival. Classic Pavlovian fear conditioning is a type of memory formation where animals learn to associate an emotionally neutral stimulus, a conditioned stimulus (CS) like a tone, to an aversive stimulus, an unconditioned stimulus (US) like a footshock. After several pairings, the CS can now elicit the behavioral fear response when presented alone, for example freezing in rats. The neural circuitry underlying fear conditioning initially involves somatosensory and auditory information of CS and US input converging onto neurons of the BLC from cortical and thalamic sources. Subsequently, convergence of CS and US signals takes place within the BLC at a cellular level and then the BLC relays the information received to the output nuclei of the amygdala such as the CeA (Royer et al., 1999). The CeA then projects to downstream targets including the brainstem and hypothalamus to initiate defense behaviors both hormonally and through

activation of the autonomic system (LeDoux et al., 1998). Hence, the BLC and CeA have been proposed as potential sites for the formation and storage of conditioned fear memories (Le Doux, 2000; Pare et al., 2004).

Increased interest in fear conditioning stems from the realization of the tight connection between fear memory formation regulation and human anxiety disorders. Abnormal regulation and hyperactivity of the amygdala is thought to underlie certain psychiatric and anxiety disorders, e.g. posttraumatic stress disorder (PTSD), panic disorder, and phobias. Human functional magnetic resonance imaging (fMRI) studies have shown that amygdala activation is elevated in response to aversive external stimuli during fear acquisition and also during associative emotional learning tasks (Whalen et al., 1998; LaBar et al., 1998). In addition, subjects with PTSD show abnormal activation of the amygdala during conditioned fear acquisition and extinction (Linnman et al., 2008). Together these data suggest that abnormal patterns of activity observed in the human BLC are directly related to subjective states of anxiety.

It is thought that the hyper-excitability in the BLC that results in abnormal fear memory formation may be mediated via alterations in second messenger signaling cascades, e.g. the calcium-calmodulin and cAMP/PKA signaling cascades, which then disrupt the cellular mechanisms that encode fear memory formation, namely long term potentiation (LTP). The following section is a more detailed description of this current view.

The molecular mechanism underlying fear memory formation: Long term potentiation (LTP) and the involvement of second messenger signaling cascades.

Numerous studies have tried to elucidate the molecular and physiological mechanisms underlying fear memory formation. Nowadays, long-term potentiation (LTP) is one of the most widely accepted substrates for learning and memory due to the nature of changes in synaptic strength that occur during this process. In support of this hypothesis, evidence suggests that fear conditioning causes LTP-like changes in synaptic strength at a cellular level, which are crucial for fear memory formation (Sigurdsson et al., 2007). The cellular LTP hypothesis for fear conditioning states that before fear conditioning the synaptic strength of the CS input onto BLC principal neurons is fairly weak, and cannot trigger the fear response. In contrast, the US triggers stronger and more robust responses in the BLC. After fear conditioning, due to the convergence of the CS and US inputs, the CS input alone becomes stronger and more effective at triggering downstream areas that control fear response. Hence, the induction of LTP in BLC principal neurons is thought to act as a substrate for fear memory formation (Sigurdsson et al., 2007).

Previous studies have shown that the induction of LTP requires activation of postsynaptic N-methyl-D-aspartate (NMDA) receptors, and subsequent calcium influx (Huang & Kendal, 1998). Additionally, Fadok et al showed that fear memory formation is critically dependent on release of dopamine in the BLC from afferents arising from the ventral tegmental area (VTA). Hence, fear memory formation was prevented in transgenic mice in which the ability of VTA neurons to synthesize dopamine was inhibited. However, when dopamine release in the pathway from the VTA to the BLC was selectively restored animals fear conditioned normally (Fadok et al., 2010). Consistent with this observation, human imaging studies have shown that dopamine

binding to D1 receptors correlated to signal changes in the amygdala in response to fearful faces (Takahashi et al., 2010). Furthermore, D1 receptors are found in close proximity to NMDA receptors in the spines of BLC principal neurons, which are the sole site of excitatory synaptic contacts in these neurons (Muly et al., 2009). Moreover, in vitro whole-cell patch clamp recording studies from our lab have also shown that application of the D1 receptor antagonist, SCH23390, blocked the induction of LTP in BLC principal neurons, whereas application of the D1 receptor agonist, SKF38393, facilitated LTP (Li et al., 2011). Together this evidence indicates that spines of BLC principal neurons are a potential site for LTP induction and, hence, fear memory formation.

D1 receptors are known to be G protein coupled receptors (GPCRs), which are coupled to a second messenger cascade that includes adenylate cyclase (AC), cyclic AMP (cAMP), and protein kinase A (PKA). GPCRs have seven membrane spanning regions and are associated with trimeric G-proteins at their cytosolic domains. It is the identity of the G-protein α-subunit that determines the coupled second messenger cascade. Significantly, the D1 receptor associates with Gαs, where GDP bound to the G protein is exchanged for a GTP when dopamine binds to the receptor. This exchange activates the Gα subunit, causing it to dissociate from the Gβ and Gγ subunits. Gα in turn activates AC, which catalyzes the conversion of ATP into cAMP, which then activates PKA by freeing its catalytic domain. This PKA catalytic domain acts to phosphorylate multiple downstream target proteins, including one that plays a major role in consolidating short term memory into long term memory, cAMP-response element binding protein (CREB). CREB regulates the transcription of many genes encoded in learning and memory, specifically brain-derived neurotrophic factor (BDNF), a neurotrophic factor that is involved in neuronal growth and survival (Jessel & Sanes, 2000). Normally cellular cAMP levels

are rapidly returned to baseline levels by the activity of phosphodiesterase 4 (PDE4), which cleaves cAMP into 5'-AMP, thus preventing further activation of PKA. The schematic for cAMP/PKA pathway is illustrated in **Figure 2a.**

Previous studies have shown that BDNF is highly expressed in the BLC, and mRNA levels of BDNF are temporarily elevated immediately after fear conditioning, indicating its role in amygdala-dependent learning and memory (Rattiner et al.,2004). In addition, Rattiner and colleagues also showed that activation of the cognate receptor for BDNF, tyrosine kinase receptor B (TrKB) was necessary for fear memory acquisition and consolidation (Rattiner, Davis, French, and Ressler 2004). Consistent with this observation, work from our lab has shown that the TrKB inhibitor, genistein, could block LTP induction, whereas TrKB agonists facilitated LTP induction in BLC principal neurons (Li et al.,2011). Significantly, this study also showed that inhibitors of adenylate cyclase or PKA inhibitor could also attenuate LTP induction in BLC principal neurons. Together this evidence suggests that fear memory formation via LTP induction in the BLC depends on dopamine and TrKB receptor activation and subsequent activation of the AC-cAMP-PKA second messenger cascades. Consequently, the activity of the PDE4 family of phosphodiesterases can be viewed as a potential homeostatic mechanism which returns cAMP to basal levels following fear conditioning.

Recently, homeostatic mechanisms that regulate neuronal, and circuit excitability to maintain experience-dependent stability in fear conditioning have gained much attention. Homeostatic regulatory mechanisms potentially underlie adaptive responses generated in the presence of a threat e.g. after fear conditioning, or during fear memory consolidation. Below is presented a general overview of the potential molecular mechanisms and signaling pathways involved in homeostatic plasticity in complex microcircuits.

Homeostasis: potential homeostatic mechanisms underlying stress induced fear and anxiety.

In neuronal circuits, homeostatic plasticity is thought to represent a mechanism by which neurons maintain a proper balance between excitation and inhibition. Under normal circumstances, homeostatic negative feedback mechanisms most likely allow neurons to detect their own activity and adjust their intrinsic excitability to within a baseline range (Turrigiano, 2011). However, even small perturbations of homeostasis, e.g. fear and anxiety or learning experiences that cause changes in synapse number and strength, can upset the balance and trigger seizure-like activity.

Cell culture studies have shown that, on a circuit level, homeostasis involves maintaining firing rates and patterns within a certain target range. For example, if neuronal cultures are induced to fire more/less than normal, neurons actively compensate for the perturbation and over time firing returns to the baseline level (Turrigiano et al., 2008). Hence, in *ex vivo* cultures homeostatic regulatory mechanisms appear to maintain neuronal firing around a stable set-point, however there is less evidence for a similar process occurring *in vivo*.

Two mechanisms have been proposed that may mediate in vivo homeostatic plasticity; 1) homeostatic synaptic mechanisms that target both excitatory and inhibitory synapses, and 2) neuronal intrinsic excitability (Turrigiano, 2011). Synaptic scaling at excitatory synapses is a cell-autonomous process whereby neurons sense changes in their own firing activity and adapt by adjusting (scaling) postsynaptic strength either up or down to accommodate for the altered firing activity (Turrigiano, 2011). Additionally, neurons are also thought to homeostatically regulate their intrinsic excitability using intracellular calcium signal cascades to change the balance of inward and outward ion currents that maintain membrane potential and action

potential threshold (Marder & Goaillard, 2006). However, despite the involvement of calcium signaling in each of these processes, little is known about the mechanisms and signaling pathways mediating intrinsic and synaptic homeostatic plasticity.

Fear and anxiety states are thought to be controlled by a hierarchy of neural systems, e.g. the amygdala, prefrontal cortex, and hypothalamus to ensure appropriate emotional and behavioral responses as well as maintain baseline activity after threat. Stress is a major predicting factor in the etiology and expression of many affective disorders (Pittenger et al., 2008), and it has been shown to alter synaptic plasticity and enhance amygdala-dependent fear learning (Rau, 2005). We reasoned that cell-autonomous plasticity in neurons can maintain homeostasis in the amygdala in response to acute stress but under conditions of chronic stress, homeostatic processes become imbalanced resulting in neuronal over-excitation, and ultimately contributing to pathological conditions such as schizophrenia, PTSD, and epilepsy. Work in the Rainnie lab has shown that repeated restraint stress in rats leads to a long lasting increase of the excitability of BLC principal neurons and a concomitant increase in anxiety-like behavior, suggesting that perturbation of homeostatic processes in neural systems may lead to persistent states of fear and anxiety. However, no study to date has examined whether, or not, there is an internal homeostatic sensor in the BLC principal neurons that may control homeostatic excitability in the fear response circuitry.

The role of second messenger systems in stress-induced synaptic plasticity in fear and anxiety circuits.

In vitro whole-cell patch clamp recording technique is widely used to measure synaptic plasticity in neural circuits. Here, according to the protocol published (Li et al.,2011), LTP can

be induced in afferent pathways, e.g. external capsule onto BLC principal neurons using two different electrical stimulation protocols; 1) theta burst stimulation (TBS) and 2) high frequency stimulation (5x100Hz for 1second; 5xHFS). In addition, treatment-induced alterations in the sensitivity of BLC neurons to LTP induction can be determined using a weaker stimulation protocol. For example, after certain behavioral and pharmacological manipulations 2xHFS, which normally does not induce LTP in BLC principal neurons, can now induce robust LTP because the threshold for induction is lowered.

Previous studies from our lab have shown that exogenous application of either D1 or TrkB receptor agonists can successfully induce LTP in BLC principal neurons using 2xHFS by lowering the threshold for LTP induction (Li et al., 2011). Consistent with this observation, previous studies in the Rainnie lab have shown that intracellular application of the non-selective PDE4 inhibitor, rolipram, lowered the threshold for LTP induction in BLC principal neurons, as illustrated in **Figure 3a**, which mimicked the effects of D1 and TrKB receptor in BLC principal neurons. In addition, rolipram also lowered the threshold for action potential generation in BLC principal neurons and caused an increase in input membrane resistance, illustrated in **Figure 3b**.

In neurons, PDE4 is solely responsible for metabolizing cAMP to 5'-AMP, suggesting that either elevated cAMP or reduced 5'-AMP may contribute to the response to rolipram. As mentioned above D1 and TrkB receptors are coupled to activation of the AC, cAMP, and PKA signaling pathway and rolipram may mimic activation of the same pathway by blocking the hydrolysis of cAMP to 5'AMP. Therefore the shift in the induction threshold for LTP is potentially due to the activation of AC-cAMP PKA pathway. However, intracellular application of a non-hydrolysable form of cAMP, 8Br-cAMP, failed to mimic the rolipram effect, suggesting that elevating cAMP levels were not responsible for the change in induction threshold.

Significantly, inclusion of AMP in the recording pipette increased the threshold for action potential generation. Since elevated AMP level corresponds to the opposite effect of rolipram, it is reasonable to see the reverse effect on action potential generation threshold shift compared to rolipram. Hence, fluctuations in the level of cytosolic AMP appear critical for regulating the intrinsic excitability of BLC principal neurons by setting the threshold for action potential generation and we reasoned that the reduction of the threshold for LTP induction may be due to a change in basal 5'AMP levels.

Notably, inclusion of the calcium chelator, BAPTA, in the patch recording pipette also blocked LTP induced by 2xHFS in the presence of D1 agonists, further suggesting that the regulation of LTP induction threshold also involves calcium as part of the second messenger signaling cascade (Li et al., 2011). Finally, unpublished observations from our lab have also demonstrated that chronic stress caused a similar reduction in the threshold for LTP induction in BLC principal neurons, as illustrated in **Figure 4**. Stress is known to elevate intracellular calcium levels in many brain regions, hence similar mechanisms may contribute to the alteration in the LTP induction threshold seen with chronic stress and neurotransmitter agonists.

Upon examination of existing literature one candidate enzyme stood out as a potential metabolic sensor in BLC principal neurons due to its known role in regulating energy homeostasis in the periphery, namely the AMP activated protein kinase (AMPK). AMPK detects changes in the metabolic state of cells by sensing alterations in the cAMP to 5'AMP ratio, and its activity is enhanced by two upstream kinases, one of which is itself activated by elevated calcium levels. As reviewed in detail below, we reasoned alterations in AMPK activity may be involved in the observed changes in LTP induction threshold following chronic stress.

AMP kinase: the potential homeostatic sensor in the amygdala that regulates synaptic plasticity and fear and anxiety.

As reviewed by Ramamurty and Ronnett (2012), AMP kinase is a heterotrimeric enzyme, containing a catalytic α subunit and regulatory ß and γ subunits. To activate AMPK, phosphorylation must take place at the α subunit threonine-172 by its upstream kinases, and it is thought that stress may be a trigger for this phosphorylation process. Moreover, two upstream kinases have been identified, liver kinase B1 (LKB1) (reviewed in Hardie, 2004), and calcium calmodulin-dependent kinase kinase ß (CaMKK ß) (Woods et al., 2005) which is highly expressed in neurons, and both facilitate AMPK activation. The presence of high levels of CaMKKβ in neurons, and the requirement for elevated calcium levels to facilitate AMPK activation makes AMPK a good candidate as a potential metabolic sensor and thus a homeostatic regulator of BLC principal neurons. In addition, as mentioned above AMPK is a sensor of AMP/ATP levels, where elevation of AMP or ADP levels and suppression of ATP levels can act as triggers to activate enzymatic activity. Upon activation, AMPK phosphorylates a large number of target proteins, however the common goal is to stimulate catabolic processes, inhibit anabolic processes and restore cellular energy homeostasis. Uncoupling protein 2 (UCP2), a mitochondrial protein that modulates energy expenditure, as well as UCP5/BMCP1, which is found to be highly expressed in the brain (Kim-Han et al., 2001), have been proposed and identified as downstream targets of AMPK (Verhulst et al., 2012). Importantly, one of the target proteins of AMPK is an inwardly rectifying potassium channel Kv2.1 (Hardie et al., 2012). Phosphorylation of Kv2.1 lowers the voltage threshold for activation and, hence, acts to raise the threshold for action potential generation similar to the threshold change observed with intracellular AMP infusion in BLC principal neurons. The schematic for the AMPK system is

shown in **Figure 2b.** Finally, AMPK is also involved in chronically altering gene transcription, suggesting that AMPK may be involved in some of the epigenetic consequences of long-term stress exposure (Ramamurthy & Ronnett, 2012).

As expected for an energy sensor, AMPK is expressed widely in the periphery, in particular in the liver and skeletal muscles, but it is also reported to be present in the hypothalamus where it is considered to be an "energy integrator" to monitor whole-organism energy balance and regulate food intake behavior (Ramamurthy & Ronnett, 2012). Recent studies have shown that hypothalamic AMPK activity is inhibited by peripheral or central hyperglycemia, and activated by hypoglycemia or 2-deoxy-D-glucose (2DG)-induced glycopenia, and is independent of activation by CaMKKs (Kawashima et al.,2012). However, no study to date has reported the presence, function or related cascades of the AMPK pathway in the BLC.

We reasoned that conservation of energy balance is a ubiquitous cellular process and that similar control systems would be present in the central nervous system (CNS) as are found in the periphery. As mentioned above the BLC is a key component of central circuits that regulate emotion and fear memory formation, and in many people mood can change quite dramatically depending on energy levels. Consequently, we postulated that AMPK may play a key role in regulating energy balance in BLC principal neurons and thereby regulate affect. We consider anxiety-like behaviors in rodents to be equivalent to emotions/moods in humans and, hence, rodents are a good model system to examine the role of AMPK in regulating affective behavior.

Specific aims

As mentioned above the BLC is a key component of the fear circuit and regulates emotions, which also suggests a homeostatic regulatory role in the CNS. Although our preliminary data strongly suggest that an AMPK-like enzyme may be present in BLC principal neurons, no study to date has directly examined the presence, or absence, of AMPK in the BLC. Significantly, as described previously, the activity of AMPK is enhanced as a result of phosphorylation by a calcium-dependent protein kinase, CaMKKβ, or a tumor suppressor, liver kinase B1 (LKB1). CaMKKβ not only phosphorylates AMPK but also CaMKIV, a kinase that also plays a critical role in LTP induction and anxiety-like behavior (Shum et al., 2005). Therefore we hypothesized that AMPK activity in the BLC would be regulated by a calcium-dependent second messenger cascade that requires upstream kinase activation of CaM, CaMKKβ, and possibly LKB1. Additionally, we were also interested in investigating CaMKIV in the BLC as an alternative potential route related to homeostatic regulation in the BLC besides the AMPK pathway. Furthermore, we were interested in downstream effectors of AMPK, which include UCP2 and UCP5/BMCP1, as well as Kv2.1. Therefore we consider the AMPK and related cascade system to include CaMKKβ, LKB1, AMPK, CaMKIV, UCP2, UCP5/BMCP1, and Kv2.1.

We further proposed that these cascades would be present in BLC principal neurons and also interneurons where AMPK acts as a key metabolic sensor that regulates cellular excitability and alters anxiety-like behavior by detecting changes in energy levels and/or stress induced calcium levels. Hence, the specific aims of this study were to examine the distribution and expression pattern of these cascades in principal neurons and interneurons within the BLC of control animals, as well as the function of AMPK in BLC principal neurons. Then we sought to

determine if the AMPK pathway is disrupted by chronic stress using a repeated restraint stress paradigm. To answer these questions, we used a multidisciplinary approach employing electrophysiological, molecular, Western blot and immunohistochemical techniques to elucidate the expression pattern and function of the AMPK cascades in both control and stressed rats.

Finally previous studies have shown that hypoglycemia can be induced by intracerebroventricular administration of 2-deoxyglucose (2-DG) in rats, which then activates AMPK in neurons of the hypothalamus (Kawashima et al., 2012). Significantly, in our restraint stress protocol we normally observe a weight loss of around 20% of normal weight. Hence, we reasoned that 2-DG application in our in vitro electrophysiology studies as well as a food restriction protocol, which mimics the effect of hypoglycemia, might alter AMPK activity in BLC principal neurons and thereby alter the relative expression of AMPK and related signaling cascades. We will examine the effect of 2-DG/food restriction on these cascades using both electrophysiological and Western blot techniques.

Given that the amygdala demonstrates a hyperactive profile in most psychiatric disorders and given that we propose AMPK as the main metabolic regulator of neuronal homeostasis, we predict that the AMPK pathway would be activated and the cascade levels would be up-regulated in the BLC following repeated restraint stress and food restriction protocols.

Methods

Animals

Sprague-Dawley rats were group housed except those under the food restriction protocol, which will be described in detail below. Rats were kept on an artificial 12 : 12 h light cycle, kept at 22°C, and given access to water and food ad libitum. All experimental protocols conform to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of Emory University.

Whole tissue RNA isolation

Total RNA was isolated from BLC tissue by homogenizing each sample in Trizol (Invitrogen, Carlsbad, CA). The isolated RNA was then reverse-transcribed using a cocktail containing 5 μl of 10× RT buffer, 10 mM dNTP mix, 10× random hexanucleotide, Multiscribe RT 5 U/μl and RNAase free water. The mixture was incubated in a thermal cycler at 25°C for 10 min and then at 37°C for 120 min, the resulting cDNA samples were stored at −20°C. All reagents were obtained from Applied Biosystems (Foster City, CA).

Qualitative PCR.

The cDNA was amplified using 10× PCR buffer (Qiagen, Germantown, MD), 3 mM MgCl2 (Qiagen), 10 mM dNTPs, 2.5 U of Taq DNA Polymerase (Qiagen) and 100 nM primers. PCR primers used for each of the AMPK pathway cascades were developed from GenBank sequences with commercially available Oligo software (IDT Tools, Coralville, IA, USA). The housekeeping gene 18S rRNA was used in all experiments as a positive control. All of the oligonucleotide primers used in this study are given in **Table 1**. Standard PCR was performed on

a PTC-200 Peltier thermal cycler (MJ Research) using the following program: 94°C for 40 s, 56°C for 40 s and 72°C for 1 min for 40 cycles. PCR products were visualized by staining with ethidium bromide and separated by electrophoresis in a 1% agarose gel.

Single Cell RT-PCR (sc RT-PCR)

Single cell RT-PCR was performed according to the protocol previously published by our lab (Hazra et al.,2011). Whole-cell patch clamp recordings were obtained from visually and electrophysiologically identified BLC principal neurons as illustrated in **figure 5**. After establishing that cells had the physiological properties consistent with their being BLC principal neurons, the cytosolic contents of the cells were aspirated into the recording electrode and then expelled into a microcentrifuge tube containing 5 µl of a reverse transcription (RT) cocktail by applying positive pressure. The mRNA was reverse-transcribed using 50 U of Multiscribe RT and was performed in a final volume of 10 µl. The cDNA was stored at −20°C before further processing. Subsequently, 3 µl of each RT product was amplified in triplicate in 0.2 ml thin-walled PCR tubes. Here, 19 µl of PCR mixture I [1× Buffer, dNTP mix, 1 µg/µl primer V3 (dT)24] and 0.05 U/µl Taq DNA Polymerase (Qiagen) were added to each tube for the first round of PCR. Cycling conditions for the first round of PCR were: 95°C for 3 min, 50°C for 2 min and 72°C for 3 min. The tubes were immediately put on ice for 1 min, and 19 µl of PCR mixture II was added with a composition the same as that of PCR mixture I but with primer V1 (dT)24 replacing primer V3(dT)24 (Kurimoto et al., 2006). A 20-cycle PCR amplification was then performed with the following cycling conditions: 95°C for 30 s, 67°C for 1 min and 72°C for 3 min with a 6 s extension per cycle. The total amount of cDNA amplified per sample was 123 µl. Amplified cDNA sample was quantified using a Nanodrop 1000 Spectrophotometer (Thermo

Scientific, Wilmington, DE), and the concentration per sample ranged from 500 to 1000 ng/µl, and had OD values ranging from 1.9 to 2.0.

The amplified cDNA from each cell was analyzed for the expression of 18S rRNA as a positive control marker. Only those cell samples that were positive for 18S rRNA were subjected to amplification with primers. Using cDNA obtained from 7 18S positive cells, we performed qualitative PCR using standard procedures on cascades of interest as described above. The resultant cDNA was subjected to another amplification step using 2 µl of cDNA from each cell as a template and 100nM of each of the primers for CaMKKß, LKB1, AMPK and CaMKIV, described in **Table 1.** ScRT-PCR was performed under the supervision of Dr. Rimi Hazra, Ph.D. Electrophysiological recording and single cell mRNA aspiration was performed by Dr. Jidong Guo, Ph.D.

Western Blot

BLC tissue from 16 animals (control handled N=4, repeated restraint stress N=4; vivarium ad libitum food N=4, vivarium food restriction N=4) were dissected as outlined for RNA isolation, and then homogenized in buffer containing protease inhibitors, 5mM HEPES, and 0.32 M sucrose (pH=7.4) according to the protocol described in (Dabrowska & Rainnie, 2010). Protein concentration was determined using a bicinchoninic acid assay, and18µg of protein per sample was loaded onto polyacrylamide-SDS mini-gels (Biorad, Hercules, CA, USA) separated electrophoretically, blotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), and blocked for 1 hour in blocking buffer containing 2% nonfat dry milk, 0.1% Tween 20, 0.05 M NaCl, and 1 M HEPES (pH 7.4). The membranes were incubated overnight at 4ºC with the following primary antibodies: a goat polyclonal anti-CaMKKß (1:500, Santa Cruz

Biotechnology, Santa Cruz CA), a rabbit polyclonal anti-LKB1 (1:500 Novous Biologicals), a mouse monoclonal anti-CaMKIV(1:500, Santa Cruz Biotechnology, Santa Cruz CA), a rabbit monoclonal anti-AMPK ß1,2 (1:1000, Cell Signaling Technology), a goat polyclonal anti-UCP2 (1:500, Santa Cruz Biotechnology, Santa Cruz CA) and a goat polyclonal anti-BMCP1 (1:500, Santa Cruz Biotechnology, Santa Cruz CA). On the following day, the samples were incubated with HRP-labeled specific secondary antibody for 1 hour at room temperature. The proteins in the BLC samples are detected using SuperSignal West Chemiluminescence (Pierce Biotechnology) and visualized with an Alpha Innotech Fluorochem imaging system (Alpha Innotech, San Leandro, CA, USA). After signal development, nitrocellulose membranes were stripped by incubating with Restore Plus Western blot stripping buffer (Thermo Scientific) for 20 minutes, washed with 0.05 M PBS and probed again with a mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primary antibody (10R-G109a, Fitzgerald Industries International INC, Concord, MA, USA 1:2000) as above. Finally, the relative integrated intensity values for each sample was analyzed using an Alpha Innotech Fluorochem imaging system and calculated by comparing individual samples bands. Western blot analysis was performed under the supervision of Dr. Joanna Dabrowska, Ph.D.

Immunohistochemistry

Dual immunofluorescence was performed according to the protocol previously published by Dabrowska and Rainnie (2010). Dual-immunofluorescence experiments were performed on 4% paraformaldehyde-fixed rat brain sections derived from control adult (45–60-days old) rats. Coronal brain sections (50 μm) were cut on a Leica CM 3050S cryostat and stored at −20 °C in a cryoprotective medium consisting of 25% glycerol and 30% ethylene glycol in 0.05 M phosphate buffer until needed.

To examine the relative expression of the cascades of interest in the BLC, we performed dual immunofluorescence on free-floating serial sections of the rat BLC. Representative sections were rinsed 3× for 10 min in PBS, permeabilized with 0.5% Triton-X 100 in PBS, and incubated for 48 h at 4 °C with the primary antibodies as described previously except we used a rabbit polyclonal anti-AMPKα1,2 (1:500, Santa Cruz Biotechnology, Santa Cruz CA) and used an additional mouse monoclonal anti-Kv2.1 (1:1000, Neuromab) here, and 0.5% Triton-X/PBS solution with either one of the marker antibodies, including a mouse polyclonal or rabbit polyclonal anti-calcium/calmodulin-dependent protein kinase α II (CaMKIIα) (1:1000, LifeSpan Biosciences, Seattle WA), a rabbit polyclonal anti-parvalbumin (PARV) (1:1000, Swant, Bellinzona, Switzerland), a mouse monoclonal anti-calbindnin (1:1000, Sigma-Aldrich), a goat polyclonal anti-calretinin (1:1000, Swant, Bellinzona, Switzerland) and a rabbit polyclonal Neuropeptide Y(1:1000, Chemicon-Millipore, Billerica MA). CaMKIIα is exclusively expressed in the BLC principal neurons (McDonald et al.,2002) and parvalbumin, calbindin, calretinin and NPY are markers for GABAergic interneurons in the BLC (McDonald & Mascagni,2006) .

Sections were then rinsed 3× for 10 min in PBS and incubated at room temperature for 2 h with the corresponding secondary antibodies, including Alexa-Fluor 488 goat anti-mouse IgG, Alexa-Flour 488 donkey anti-goat IgG, Alexa-Flour 488 goat anti-rabbit IgG, Alexa-Fluor 568 goat anti-rabbit IgG, Alexa-Fluor 568 donkey anti-mouse IgG (1:500, Molecular Probes, Invitrogen, Carlsbad, CA, USA). Following incubation, sections were rinsed 3× for 10 min in PBS, and 1× in phosphate buffer (PB), mounted on gelatin-coated glass slides and coverslipped using Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA).

Dual-immunofluorescence analysis was accomplished by focusing on neurons throughout the thickness of the section and by maintaining the same focal plan switching between filter sets

to ascertain if the cell shows dual immunofluorescence. Confocal spinning disk laser microscopy was used to obtain high-resolution photomicrographs (63× magnification) using an Orca R2 cooled CCD camera (Hammamatsu, Bridgewater, NJ, USA) mounted on a Leica DM5500B microscope (Leica Mircosystems, Bannockburn, IL, USA). Cell counting was performed by labeling immunoreactive neurons for each specific marker from representative sections of the entire BLC region using the simple PCI analysis program.

Immunoreactive intensity measurement

Immunofluorescence staining was performed according to the protocol described above, except rat brain sections were derived from 8 adult (45–60-days old) rats (control N=4, RRS N=4). As described previously, antibodies against LKB1, AMPK and CaMKIV were used in this experiment. Unfortunately, for immunohistochemistry the antibody against CaMKKß showed a very high non-specific background staining, so we decided not to include immunoreactive intensity measurements for this antibody. Specific primary antibodies include a rabbit polyclonal anti-LKB1 (1:500 Novous Biologicals), a mouse monoclonal anti-CaMKIV (1:500, Santa Cruz Biotechnology, Santa Cruz CA) and a rabbit polyclonal anti-p-AMPKα1,2 (1:500, Santa Cruz Biotechnology, Santa Cruz CA), and the secondary antibodies used were Alexa-Fluor 568 goat anti-rabbit IgG, Alexa-Fluor 488 goat anti-mouse IgG, and Alexa-Flour 488 goat anti-rabbit IgG (1:500, Molecular Probes, Invitrogen, Carlsbad, CA, USA) respectively.

Confocal spinning disk laser microscopy was used to obtain high-resolution photomicrographs (40× magnification) in Z stacks. Photomontages for each stain were developed for a maximum projection for each Z stack, and the mean intensity level of immunolabeling was obtained using simple PCI program intensity measurement tool.

Repeated restraint stress

Repeated restraint stress (RRS) paradigm was utilized to mimic neurochemical and behavioral effects of chronic stress. During the RSS procedure, 4 rats were subjected daily to 1-hour restraint stress in wire-mesh restrainers for 4 consecutive days and then were left undisturbed until behavioral testing on day 10 after the onset of the stress protocol, while 4 control rats were tested for startle response (pre- and posttest) and were left undisturbed in the test room. On day 10 following the RRS protocol, all animals were tested for their acoustic startle response (posttest) and then BLC samples from the 4 control and 4 stressed animals were collected and assayed for protein expression (Western Blot) and immunofluorescence.

Food restriction protocol

On arrival at the vivarium Sprague Dawley rats were housed individually and maintained on a standard 12 h light/12 h dark cycle, with ad libitum access to water. Starting no earlier than one week after arrival and continuing until the day of sacrifice, food was restricted to a level that maintained the rats at approximately 85% of the vendor's standard growth curve. After 12 weeks of age, the rats are considered fully grown, and the additional weight put on by freely fed rats is mostly fat. Thus, rats over the age of 12 weeks were maintained at the same weight level as 12-week-old rats (around 300g). Rats were weighed daily.

In vitro whole-cell patch clamp electrophysiology studies

In vitro patch clamp electrophysiology studies were performed by Dr. Chen-Chen Li according to the protocol previously published by Li et al (Li et al., 2011). The following drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA): AICAR, 5-Aminoimidazole-4-carboxamide ribonucleotide and 2-DG, 2-deoxy glucose. Drugs were applied in the ACSF using

a continuous gravity fed bath application unless specifically stated.

Statistics

For immunoreactive intensity level analysis, all data was collected from the Simple PCI program. Means of the immunolabeling intensity level were compared between controls and repeated restraint stress brain slices using a two-sample equal variance student's T-test.

For Western Blot analysis, all data were normalized to GAPDH expression levels, which served as a control for total protein levels within a sample. Following normalization, means of the protein expression level were compared between controls and stressed/food restricted animals using a two-sample equal variance student's T-test for both RRS and food restriction protocols.

For *in vitro* patch clamp electrophysiology studies, a paired t test was used for the LTP induction threshold analysis and an unpaired t-test for the analysis of normalized EPSP level at 30 min after high frequency stimulation (HFS).

Results

Baseline profile for AMPK and related cascades in the BLC.

Although there has been extensive research on the role of AMP activated protein kinase as a metabolic sensor in the periphery, the few studies that have looked at AMPK in the CNS have primarily focused on its role in hypothalamic regulation of feeding. No study to date has examined the cellular expression of AMPK in the BLC, or any of its upstream/ downstream effector systems. Consequently, we first used whole tissue RT-PCR and western blot analysis to determine the presence of AMPK and related signaling cascades in the BLC. We then used single cell RT-PCR from electrophysiologically identified BLC principal neurons and immunohistochemistry to examine the relative expression of the AMPK signaling pathway in the different subpopulations of BLC neurons.

Whole tissue RT-PCR

As illustrated in **Figure 6**, RT-PCR analysis of mRNA recovered from BLC tissue slices revealed that both of the upstream activators of AMPK, CaMKKß, and LKB1 were expressed at high levels in this tissue in addition to AMPK and its down stream target Kv2.1, and another target of CaMKKß, CaMKIV. A typical gel picture showing the respective bands is presented in **Figure 6.**

Single Cell RT-PCR (scRT-PCR)

Previous electrophysiological studies in our lab have shown that intracellular application of rolipram, a PDE4 inhibitor, lowered the threshold for action potential generation (illustrated in **Figure 3b**) and facilitated LTP in BLC principal neurons (illustrated in **Figure 3a**). Subsequent

studies suggested that this response may result from the activity of the AMP-activated protein kinase AMPK. Consequently, we were interested in elucidating whether, or not, each of the components of the AMPK signaling cascades identified in our RT-PCR results were expressed by principal neurons of the BLC. Here, we performed scRT-PCR on neurons that had been classified as BLC principal neurons based on their electrophysiological profile (details shown in **Figure 5**). Our scRT-PCR revealed the presence of several key members of the AMPK signaling cascades in BLC principal neurons. Specifically 5 out of 7 principal neurons tested expressed CaMKKß mRNA transcripts, 4 out of 7 expressed LKB1, 4 out of 7 expressed AMPK, 5 out of 7 expressed Kv2.1 and 7 out of 7 expressed CaMKIV. The data is summarized in **Table 2**. Importantly, 2 out of 7 principal neurons examined expressed all five mRNA transcripts, suggesting that activation of AMPK can occur through two distinct pathways in BLC principal neurons.

Western Blot Analysis

Although our scRT-PCR results showed that principal neurons of the BLC can express several permutations of mRNA transcripts for CaMKKß, LKB1, AMPK, Kv2.1 and CaMKIV, it is not definitive evidence that these neurons can also express mature proteins. Hence, we performed a Western blot analysis to examine mature protein expression of components of the AMPK signaling cascades in the whole BLC tissue samples. As shown in **Figure 7**, our Western blot analysis revealed single bands for all proteins at the predicted molecular weights, CaMKKß (66kDa), LKB1 (48kDa), AMPK ß1,2 (30kDa), and CaMKIV (60kDa), thus confirming the presence of mature protein expression of the AMPK signaling cascades in the BLC. However due to time limitations, we did not perform Western blot analysis on Kv2.1. In addition, Western

blot analysis using antibodies against UCP2 and UCP5 showed unspecific bands, indicating the antibodies were not isoform-specific and could not be used in our study.

Immunohistochemistry

Next we used dual-immunohistochemistry on rat BLC brain slices to determine the relative expression of the mature peptides of AMPK and related cascades in principal neurons and interneurons, respectively. Previous studies have established that calmodulin-dependent protein kinase II alpha (CaMKIIα) as a cytosolic marker for principal neurons in the BLC (McDonald et al., 2002). Conversely, previous studies have shown that GABAergic interneurons in the BLC can be grouped into functional subunits based on their expression of calcium-binding proteins like parvalbumin, calbindin, and calretinin, as well as neuropeptides like neuropeptide Y (NPY) (McDonald & Mascagni,2006). Hence, we also examined co-expression of the AMPK signaling cascades in BLC interneurons using dual-immunohistochemical staining for parvalbumin, calbindin, calretinin and NPY. All neurons were dual labeled with antibodies against CaMKKß, LKB1, AMPK and CaMKIV in all brain sections containing the BLC.

As shown in **Figure 8**, our dual-immunofluorescence experiments revealed that in principal neurons labeled with CaMKIIα expression was mainly observed throughout the neuron, including the soma and dendrites, but not in the nucleus. Similarly, CaMKKβ was also observed throughout the neuron, in the soma and dendrites, and was often found to co-localize with CaMKIIα. Indeed, analysis of representative Z stacks showed that 87.8% of CaMKIIα positive neurons co-express CaMKKβ. In addition, CaMKKβ signal was also observed in the soma and dendrites of parvalbumin-labeled interneurons, as well as in the soma of calbindin- and NPY-labeled interneurons. Analysis of representative Z stacks showed that 50% of parvalbumin- and

NPY-labeled interneurons expressed CaMKKβ, whereas 100% of calbindin-labeled interneurons expressed CaMKKβ.

We next examined whether the mature protein for the second serine/threonine kinase, LKB1, was also expressed in principal neurons of the BLC. As shown in **Figure 9** our dual-immunofluorescence experiments revealed that LKB1 is highly expressed in the soma and dendrites of presumed principal neurons, where it was observed to co-localize with CaMKIIα. Moreover, analysis of representative Z stacks revealed that 100% of CaMKIIα positive neurons co-expressed LKB1. In addition, like CaMKKβ, LKB1 was also observed in the soma of parvalbumin interneurons, and showed 100% co-expression in parvalbumin-labeled interneurons. Unfortunately, the antibodies against CaMKKβ and LKB1 were raised in the same host and, hence, we could not determine the degree of overlap of these two AMPK activators in the same cells. However, our scRT-PCR data suggest that a subpopulation of principal neurons have the potential to co-express both CaMKKβ and LKB1.

Next we looked to see if AMPK was differentially expressed in principal neurons versus interneurons. As illustrated in **Figure 10**, our dual-immunofluorescence experiments revealed that p-AMPKα1,2 expression was observed mainly in the nucleus and cytoplasm of principal neurons. Furthermore, analysis of representative Z stacks showed that 100% CaMKIIα-positive neurons also co-expressed p-AMPKα1,2. In addition, like its upstream activators, p-AMPKα1,2 expression was also observed in parvalbumin labeled interneurons, where it was found at highest levels in the nucleus and cytoplasm. Interestingly, AMPKα1,2 was also expressed in calbindin and calretinin labeled interneurons where it was seen to localize in the neuronal membrane and dendrites. Analysis revealed 100% of parvalbumin and calbindin labeled interneurons and only 42.9% of calretinin labeled interneurons expressed AMPKα1,2.

In **Figure 11**, our dual-immunofluorescence experiments revealed that CaMKIV expression was mainly in the nucleus and cytoplasm of neurons that co-localized with CaMKIIα. Analysis of representative Z stacks showed 100% CaMKIIα positive neurons expressed CaMKIV. Additionally, 75% of parvalbumin labeled interneurons expressed CaMKIV in the nucleus and cytoplasm. However, only a very small amount (36.4%) of NPY labeled interneurons expressed CaMKIV in the cytoplasm, and no calretinin labeled interneurons expressed CaMKIV. All the results are summarized in **Table 3**.

Finally, we also performed immunofluorescence staining for one of the downstream targets of AMPK, namely Kv2.1. Although due to time limitations we did not perform dual-immunofluorescence staining to look at co-localization of Kv2.1 and the different markers of neuronal subpopulations. However as illustrated in **Figure 12**, Kv2.1 was expressed in the majority of neurons in the BLC, and hence is most likely found in both principal neurons and interneurons. According to Hardie et al., (2012), AMPK can activate the outwardly rectifying Kv2.1 potassium channel by shifting its voltage gating to a more hyperpolarized membrane potential and they have proposed that in doing so AMPK can regulate action potential firing patterns. We reasoned the activation of Kv2.1 by AMPK might also play a role in the shift of action potential threshold generation.

To sum up, the majority of principal neurons in the BLC express CaMKKß, LKB1, AMPK and CaMKIV. CaMKKß, LKB1, AMPK and CaMKIV are also expressed in interneurons, but the distribution within each subpopulation of interneuron is different for each cascade. In addition, our results also strongly indicate the expression of Kv2.1 in both principal neurons and interneurons, and that this may represent a common target substrate in both cell types.

Electrophysiological study: the effect of AMPK activation on synaptic plasticity in BLC principal neurons from control animals.

After confirming the presence of several members of the AMPK signaling pathway in BLC principal neurons, we next sought to examine the functional properties of these cascades in BLC principal neurons. Therefore we employed an *in vitro* whole cell patch clamp recording technique to study the effects of application of AICAR, a selective AMPK activator, on the intrinsic properties of BLC principal neurons in brain slices obtained from control animals. The results of this study are summarized in **Table 4.** Notably, application of AICAR caused a depolarizing shift in the threshold for action potential generation in BLC principal neurons ($n=8$, $p<0.001$), suggesting a key role for AMPK in regulating homeostatic intrinsic excitability of BLC principal neurons in control animals.

Effect of repeated restraint stress on protein expression levels of AMPK and related cascades in the BLC

Since the electrophysiological recording data from our lab strongly suggest a functional role of AMPK activation in BLC principal neurons from control animals, we investigated whether repeated restraint stress would interrupt homeostasis in the BLC and potentially break down the homeostatic regulatory system as a whole, leading to some degree of alterations in the mature protein expression levels of CaMKKß, LKB1, AMPK and CaMKIV in the BLC.

Western blot analysis

We performed Western blot analysis on whole tissue BLC from 4 non-stressed (NS) and 4 repeated restraint stressed (RRS) rats. To normalize the data and control for variability in sample loading, protein expression was determined relative to the expression of a housekeeping

peptide, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Shown in **Figure 13,** Western blot results revealed a significant increase in CaMKKß (n=4, p=0.015) and CaMKIV (n=4, p=0.035) protein levels, and a non-significant trend of a decrease in LKB1 protein levels (n=4, p=0.077), and increase in AMPKß1,2 (n=4, p=0.083) protein levels in BLC homogenates from RRS rats compared to the NS control group.

Immunoreactive intensity level analysis

We performed immunofluorescence staining on BLC slices from 4 non-stressed (NS) and 4 repeated restraint stressed (RRS) rats. One Z stack was taken from one brain section in each animal using Confocal spinning disk laser microscopy under the same level of exposure. Each Z stack had the same thickness and same number of counts. The means of the immunolabeling intensity level was collected using simple PCI program by developing maximum projection for each Z stack. Shown in **Figure 14**, Immunoreactive intensity level analysis revealed no significant changes in LKB1 (n=4, p=0.054) and AMPKα1,2 (p=n=4, 0.077) and a significant increase in CaMKIV (n=4, p=0.022) in immunolabeling intensity level.

Electrophysiology study: effect of AMPK activation on synaptic plasticity in BLC principal neurons from stressed animals .

Since our Western blot results revealed a selective increase in the level of CaMKKß, and no changes in AMPK expression in RRS animals, it raised the question of whether AMPK plays any functional role in the behavioral and electrophysiological changes observed following the RRS paradigm. Hence, we sought to directly examine the effects of AMPK activation by AICAR application on the reduction in LTP threshold normally observed in principal neurons recorded from stressed animals. As illustrated in **Figure 15**, our electrophysiological studies confirmed

previous observations that LTP could be induced in BLC principal neurons (165±12% of baseline, n=6) at a lowered threshold (2xHFS) in slices obtained from stressed animals that would not normally induce LTP in BLC principal neurons recorded from control animals. Significantly, the LTP induced by 2×HFS in stressed animals was significantly attenuated by intracellular application of AICAR (128±7% of baseline, n=6, $p<0.05$), indicating that activation of AMPK rescues the effect of stress on synaptic plasticity, and therefore plays a key functional role in regulating synaptic plasticity in BLC principal neurons.

Effect of food restriction on protein expression levels of AMPK and related cascades in the BLC.

Western Blot Analysis

Because stress-induced changes in behavior and synaptic plasticity were also associated with a net loss in weight in stressed animals, we wanted to examine the role of alterations in energy balance on intrinsic homeostatic processes in BLC principal neurons. Thus, we next performed Western blot analysis on another group of BLC tissue samples obtained from 4 rats under a food restriction protocol, and 4 rats allowed ad libitum food access. As described above, protein levels were determined relative to the expression levels of GAPDH. As shown in **Figure 16,** Western blot analysis revealed that the BLC from food restricted rats had significantly increased (n=4, p=0.033) CaMKKß protein levels compared to controls, but there were no significant changes (n=4, P>0.1) in LKB1, AMPKß1,2 and CaMKIV protein levels.

Electrophysiology study: effect of AMPK activation on synaptic plasticity in BLC principal neurons from food restricted animals.

Similar to the results obtained using the RRS paradigm, our Western blot results revealed an increased level of CaMKKß, and no change of AMPK protein levels in food restricted

animals, hence we continued to examine whether AMPK has a functional role under food restriction protocol by measuring synaptic plasticity directly using electrophysiology. As illustrated in **Table 5**, food restriction caused a 5mV shift in the hyperpolarizing direction for action potential generation threshold compared to principal neurons recorded from control animals fed ad libitum ($p<0.05$), indicating food restriction causes action potential threshold to lower, which is the reverse effect of previously reported electrophysiology data for AICAR and AMP inclusion in the patch recording pipette solution. Similarly, application of 2DG caused a hyperpolarizing shift of action potential generation threshold in BLC principal neurons from control animals ($n=12$, $p<0.01$). Significantly, no additional shift in the threshold for action potential generation was seen when 2-DG was applied to food restricted BLC principal neurons compared to control animals ($n=16$). These data suggest that food restriction/energy deprivation and application of 2-DG, which can induce hypoglycemia, share the same pathway to activate AMPK. Together these data also strongly suggest that AMPK has a functional role in BLC principal neurons whereby it acts to preserve an optimal firing rate in that face of alterations in energy balance.

Discussion

In summary, we have shown for the first time that mRNA and mature protein of the AMP-activated protein kinase (AMPK) as well as several of its upstream and downstream cascades, including CaMKKß, LKB1, and Kv2.1 are expressed in the BLC. In parallel, we have also shown that an additional downstream phosphorylation target of CaMKKß, CaMKIV, is also present in the BLC. Subsequently, using scRT-PCR and dual-immunofluorescence studies we have determined the expression levels of these cascades in specific neuronal populations in the BLC. Here, we report that CaMKKβ, LKB1, AMPK, and Kv2.1 as well as CaMKIV appear to be ubiquitously expressed in principal neurons of the BLC, whereas their expressions in GABAergic interneurons are more discrete. Significantly, data from our electrophysiological studies strongly suggest that AMPK is functionally active in BLC principal neurons where it acts to regulate the threshold for action potential generation, and by extrapolation regulate the threshold for LTP induction.

Previous studies from our lab had shown that stress caused a shift in the threshold for LTP induction whereby stimuli that would not normally induce LTP in control conditions (2xHFS) now induce robust LTP in BLC principal neurons; however the mechanism underlying this shift in threshold was unknown. Since stress was also associated with ~ 20% weight loss compared to control animals we reasoned that the change in LTP threshold may be related to changes in protein expression levels for AMPK signaling cascades. Furthermore, to control for the effects of stress versus weight loss we used a food restriction model to study the effects of weight loss alone on protein expression levels of AMPK signaling cascades. Moreover, since food restriction was expected to reduce available glucose levels in the CNS, we directly

examined the effects of *in vitro* application of 2-deoxyglucose (2-DG), which is known to activate hypothalamic AMPK by inducing hypoglycemia *in vitro* (Kawashima et al., 2012).

The presence of AMPK and related cascades in the control BLC.

Our initial whole tissue mRNA analysis in the BLC revealed the presence of PCR bands for CaMKKß, LKB1, AMPK, Kv2.1 and CaMKIV, and our Western blot analysis confirmed the presence of mature proteins for all these mRNA transcripts but Kv2.1, due to time limitations. However, although each of the Western blot bands were at the correct size for each protein in the cascade, the intensity level was low for CaMKKß, LKB1 and AMPK compared to that of CaMKIV. These data may suggest that CaMKIV may play a more significant role in regulating plasticity in the BLC, however it is hard to compare the intensity levels of western blot bands across the cascades as the actual amount of protein expressed in the BLC because the detection limits of our Chemiluminescence visualizing equipment can have an effect. Hence for future studies it might be helpful to extract and purify the proteins first to yield clearer results in Western blot analysis.

Differential expression of AMPK and related cascades in control BLC principal neurons and interneurons.

We elucidated the differential expression levels of AMPK and related cascades in specific subpopulations of BLC neurons, the principle neurons and local interneurons using scRT-PCR and dual-immunohistochemistry. scRT-PCR allowed us to investigate the AMPK pathway cascade expression levels specifically within BLC principal neurons, and immunohistochemistry allowed us to compare the expression pattern of these cascades in both principal and interneuron subpopulations using different antibody markers.

As noted above, scRT-PCR revealed that CaMKKß, LKB1, AMPK, Kv2.1 and CaMKIV were almost ubiquitously expressed in principal neurons, an observation that was confirmed by the finding of our dual-immunohistochemistry study using CaMKIIα as a marker for principal neurons. To compare this expression pattern with that of the GABAergic interneurons, we examined co-expression with parvalbumin-positive interneurons (PV), the second largest population of neurons within the BLC; calbindin interneurons (CB), the largest population of interneurons that show ~ 60% overlap with PV, calretinin interneurons (CR), a group of interneurons that are distinct from PV and CB interneurons; and neuropeptide Y interneurons (NPY) a distinct population of interneurons that express feeding related neuropeptide NPY. Although these four markers do not cover every subpopulation of BLC interneuron, they are sufficient to cover BLC interneurons with different electrophysiological characteristics (McDonald & Mascagni, 2006).

A limitation of our dual-immunofluorescence study was that some combinations of markers could not be tested because they were raised in the same host, and hence it would be impossible to stain the antibodies with different Alexa-fluor secondary antibodies. Therefore we could not perform dual-immunofluorescence studies on every cascade with every neuron-specific marker For example, the antibodies against CaMKKß, LKB1, AMPK and CaMKIV were raised in goat, rabbit, rabbit and mouse, respectively. Whereas, antibodies against CaMKIIα, PV, CB, CR and NPY were raised in mouse/rabbit, mouse/rabbit, mouse, goat and rabbit, respectively. Consequently, dual labeling was only possible with CaMKIIα and PV for all four AMPK pathway cascade proteins. An additional caveat to our study was that the studies on LKB1 were performed later than the CaMKKß, AMPK and CaMKIV studies and at the time the study was performed, the antibody against CR was picking up nonspecific epitopes and, hence, the dual-

immunofluorescence results on LKB1 were more limited than that for the other studies. Finally, due to time limitations we were unable to run dual-immunofluorescence staining for Kv2.1 and the neuron-specific marker antibodies, but observations from our immunofluorescence staining against Kv2.1 suggest that it is most likely ubiquitously expressed in all neuronal subpopulations.

For our dual-immunohistochemical experiments, we used confocal spinning disk laser microscopy to generate representative high-resolution photomicrographs (63× magnification) in Z stacks with which we could accurately quantify the percentage of specific neuronal subtypes that express the protein of interest by counting the number of cells that co-localize the marker and target antibodies. However, due to time limitations, on average, analysis was only conducted on 1-3 brain sections per dual-immunofluorescence staining protocol. However, every effort was made to ensure that similar amounts of total neurons were counted across groups. Therefore, our study only provides a general understanding of the full expression pattern in the BLC. For future directions, a larger sample population should be used to increase the total number of neurons detected in our co-localization studies so that we may more accurately quantify the results. Nevertheless, we believe that the differences seen in the expression pattern among the AMPK signaling cascades in our study indicate potential different functions of these cascades in the two distinct populations of neurons. However, without supportive electrophysiological studies or behavioral measurements after selective drug application, it is hard to interpret the functional roles of these cascades by just using immunohistochemistry.

In summary, we show evidence that CaMKKß, LKB1, AMPK, and CaMKIV are ubiquitously expressed in BLC principle neurons while a differential expression pattern was observed in the interneuron subpopulations. We also propose that Kv2.1 is expressed in both

BLC principal neurons and interneurons. For future studies, in order to elucidate and confirm our findings, we would need to investigate the localization of these cascades with antibodies that stain for specific intracellular structures, and use high resolution imaging techniques such as electron microscopy to localize the proteins within defined compartments of specific BLC neuronal subpopulations.

Examination of the functional role of AMPK in the BLC from control animals

Once the presence of AMPK and its related signaling cascades was established in the BLC, we next investigated the functional role of AMPK in brain slices obtained from control animals. By performing *in vitro* whole cell patch clamp electrophysiology studies we could directly examine the functional consequences of manipulating AMPK signaling cascades in BLC principal neurons. Here, we used intracellular application of the selective AMPK activator, AICAR, to artificially induce the activation of AMPK and then measure the drug effects on the active and passive membrane properties of BLC principal neurons. Most noticeable among the AICAR effects was a depolarizing shift of the threshold for action potential generation. This observation was consistent with that observed following inclusion of AMP in the recording pipette, and the reverse of the effects of including rolipram in the pipette. In normal conditions, AMPK works as a metabolic sensor and it is known to play a key role in energy conservation. Therefore it is reasonable that activation of AMPK by AICAR mimics a state of high intracellular AMP and acts to make the neuron less excitable and conserve energy by reducing the probability that incoming excitatory synaptic activity could drive the neuron to fire an action.

Effects of chronic stress on AMPK and related cascades.

Once we had confirmed both the presence and functional role of AMPK in the BLC in control conditions, we asked the question of whether, or not, the AMPK system may be broken under conditions of chronic stress. Chronic stress is known to disturb the balance of excitation and inhibition in the BLC, thus causing the amygdala to become hyper-excitable (Rainnie et al., 2004). We reasoned that excessive action potential firing in the BLC would have a direct impact on the AMPK signaling cascade. Therefore, we used a repeated restraint stress paradigm to examine the effects of chronic stress on protein expression level changes in stressed animals compared to controls. Unfortunately, for our immunohistochemical studies although the antibody against CaMKKß showed very clear cellular expression and we could quantify cell numbers, it also showed high non-specific background staining in the neuropil, and so we decided not to include quantitative intensity measurements for this antibody. Photomicrographs were obtained at the same magnification (40x), thickness (20um), and the same number of images (41) was obtained for each Z stack. In addition Z stacks were taken at the same exposure level across all sections collected for LKB1, AMPK and CaMKIV to ensure uniformity in data collection and estimates of intensity. When creating photomontages for each Z stack obtained, the same Simple PCI program intensity measurement tool was used. Therefore the immunoreactive intensity measurement provides a good estimate of the absolute protein expression levels in the BLC montages.

What is most noticeable from the results is that only CaMKKß showed an increase in protein expression level following RRS, whereas LKB1 and AMPK both showed no significant changes in protein levels. Similarly, our measurement of immunoreactivity intensity revealed no significant changes in LKB1 and AMPK levels following RRS. This suggests that the CaMKKβ

component of the signaling cascade is more sensitive to chronic stress manipulations. Normally, higher levels of CaMKKβ may be expected to facilitate activation of AMPK. However, following chronic stress the reduced threshold for LTP induction would suggest that AMPK activity has been attenuated. There are several possible explanations for this observation and we ran additional studies to confirm our interpretations.

The first possible explanation is that although no changes were observed in our Western blot analysis, AMPK may be functionally damaged following chronic stress, and might not operate normally. Therefore we performed electrophysiological studies to examine the functional role of AMPK in BLC principal neurons following repeated restraint stress. Consistent with previous observation, our results indicate that stress caused a reduction in the threshold for LTP induction, which is reasonable since stress is known to alter the excitation-inhibition balance in the BLC and, hence, may more easily allow LTP formation. However, the shift of LTP induction threshold was blocked by intracellular application of AICAR, which strongly suggests that the enzymatic activity of AMPK is not damaged in the BLC following chronic stress, and our first interpretation is not valid. Moreover, Western blot only detects protein expression levels and is not a direct measurement of enzymatic activity. Hence, increased CaMKKβ levels may not directly correlate with increased enzymatic activity. As is the case with AMPK, some enzymes function better following phosphorylation, without the necessity for elevated protein levels, and this would not be detected by Western blots. Hence, we cannot conclude from our Western blot data that the activity/function of AMPK signaling cascades remain constant following chronic stress. For future directions, an enzymatic assay for AMPK activity needs to be developed and performed to directly measure its functional role by detecting phosphorylation levels of AMPK substrates between control and RRS groups.

The second possible explanation is that following chronic stress AMPK has decreased sensitivity activation by its upstream kinases, e.g. signals from CaMKKß and LKB1. If this were the case, an increased level of either CaMKKß or LKB1 may be seen, because the neuron might try to compensate for an apparent reduction in AMPK activity by increasing the levels of the upstream kinase. As shown in our Western blot results, CaMKKß levels did indeed increase following chronic stress, however as previously mentioned these results do not directly address changes in functional activity. Therefore for future studies, it will be necessary to use electrophysiological studies to directly probe whether inhibiting/activating AMPK's upstream cascades can significantly alter the functional roles of these cascades.

Another theory that needs to be addressed is that differential activation of LKB1 might also play a role in homeostasis in the BLC. However, despite the fact that our Western blot analysis shows no change in LKB1 following repeated restraint stress, we cannot directly address whether, or not, LKB1 is required for AMPK activation. Intriguingly, previous studies have shown that LKB1 plays a role in axon differentiation during neuronal development (Shelly et al., 2007). Therefore it is possible that LKB1 is required for normal neuronal polarization during development of the BLC, and the signal that we detect in adult BLC neurons is a non-functional residual of this process. However whether LKB1 is required for AMPK activation in adulthood and what the upstream activators for LKB1 may be in neuronal systems still remains unknown and it will be interesting to see whether selective manipulation of LKB1 could mimic the effects of AICAR on AMPK activation in brain slices. Therefore future studies could use application of an LKB1 manipulator to reveal more information about the functional role of LKB1 in the AMPK signaling cascade.

Additionally, both Western blot analysis and immunoreactive intensity measurements revealed a significant increase of CaMKIV protein expression levels following RRS. This indicates the CaMKKβ and CaMKIV system could be an alternative route by which chronic stress may alter LTP induction threshold independently of a dysregulation the AMPK system. Further electrophysiological studies are needed to determine the functional role of CaMKIV in regulating homeostasis in the BLC.

Effects of food restriction on AMPK and related cascades

Since RRS animals display a ~20% weight loss during the stress paradigm, and AMPK is known to conserve energy by detecting AMP:ATP levels in the periphery, it was important to know whether the observed effects of RRS on the AMPK system were dependent on stress or chronic energy deprivation. Therefore we performed Western blot analysis on BLC tissues collected from food-restricted animals. Compared to RRS studies, food restriction did not cause any changes in CaMKIV levels. Hence, alterations in CaMKIV following RRS might be a stress-specific response rather than a response to energy deprivation. Nonetheless, results from our Western blot analysis following food restriction revealed a similar increase in CaMKKβ protein expression levels as observed following RRS suggesting that these two *in vivo* manipulations might share common mechanisms of action. Therefore we went on to test the effect of food restriction on functional role of AMPK in electrophysiological studies.

For these studies we first examined the effects of food restriction on action potential threshold in BLC principal neurons, and then compared these results with exogenous application of 2-deoxy-D-glucose (2DG), a drug that is known to induce hypoglycemia and activate AMPK in hypothalamic neurons. Our electrophysiological studies revealed that two week food

restriction resulted in a 5 mV decrease in the threshold for action potential generation, supporting our hypothesis that energy deprivation may affect neuronal homeostasis in the BLC by regulating the AMPK system. Significantly, the effect of food restriction on the threshold for action potential generation was mimicked by application of 2DG, but this effect was blocked in brain slices obtained from food restricted animals, indicating food restriction/energy sensing and 2DG activation might share the same pathway for conservation energy.

However paradoxically, the direction of action potential generation threshold change is opposite to the effects of AICAR and AMP application, where they directly activate AMPK as shown in our electrophysiology studies. We reasoned the AMPK system might function differently in a brain region specific manner. Previous studies have shown that sleep deprivation, another type of chronic stress caused elevated levels of PDE4 in the hippocampus (Vecsey et al., 2009), whereas our lab have shown lowered PDE4 levels following chronic stress. Hence stress could have markedly different effects on the AMPK signaling cascades depending on the brain region being studied. We reasoned regulation of the AMPK system might be dynamic and show differential regulation in the emotional circuits compared to previous studies done in feeding circuits (Kawashima et al., 2012).

In addition, previous studies on PDE4 activity have shown a differential response to acute and chronic stress, such that acute stress caused a reduction in PDE4 activity while chronic stress lead to increased activity of PDE4 in the frontal cortex and hippocampus of rats (Itoh et al., 2003). Taken together with our results, these observations suggest that sensors of AMP/ATP levels in the brain are dynamically regulated, and the AMPK system could be activated or attenuated depending on the time point as well as amount of stress received.

Finally, previous studies have shown that food deprivation/hunger results in increased firing rates in Agouti-related protein (AGRP) expressing neurons in the arcuate nucleus of the hypothalamus by upregulation of excitatory synaptic inputs onto these neurons (Yang et al., 2011). In addition, de Araujo and colleagues showed increased firing activity in the amygdala during hunger using in vivo electrophysiological recordings (De Araujo et al., 2006). Both studies support our observation of a lowered action potential generation threshold that increased the chances for an action potential to be fired. To some extent these data are consistent with the everyday observation that some people tend to get mood swings when they are hungry. The more food/energy deprived one gets, the easier it is for neurons in the amygdala to fire action potentials and elicit emotional responses. However, more studies would need to be conducted to test this hypothesis in the BLC.

Although our results from the food restriction studies were promising and support a potential role the AMPK signaling cascade in altering the threshold for action potential generation, before we can confirm the role of the AMPK system, we need to perform additional electrophysiological studies using AMPK activators and/or inhibitors to determine their effects on the 2DG induced shift in action potential threshold.

Metformin and AMPK

Interestingly AMPK can be activated by an anti-diabetic drug named Metformin, which is the first choice for treatment of type 2 diabetes. As reviewed by Hardie et al, Metformin is currently prescribed to more than 100 million people with type 2 diabetes worldwide (Hardie et al., 2012). Metformin is a very effective drug for treating diabetes due to its known role in activating AMPK in the liver and muscles, as well as increasing the activity of insulin. However,

results from our study suggest that activation of AMPK in the BLC could lead to increased neuronal excitability, which eventually could lead to the development of psychiatric disorders under prolonged exposure to metformin. However, it is unclear whether oral metformin administration can actively cross the blood-brain-barrier. In addition, recent studies also reported conflicting results on the mechanism of the action of metformin in the central nervous system. For example, Chau-Van and colleagues showed a decreased p-AMPK levels in primary cultures of hypothalamic cells in response to metformin, while Ropelle and colleagues showed increased p-AMPK levels in the hypothalamus under oral administration of metformin in tumor-bearing rats. However, the latter may be an indirect effect due to changes in circulating glucose levels (Chau-Van et al., 2007; Ropelle et al., 2007). Overall, the mechanism of metformin in the central nervous system is unknown and further clarification is necessary to elucidate the role of AMPK activation in different brain regions. One major concern for patients who take metformin as a treatment for type 2 diabetes is the potential to develop psychiatric side effects with prolonged treatment. However, this has not been reported to date, and metformin is relatively well tolerated.

Future Directions

We have taken the first step in examining the baseline expression of mRNA and protein for CaMKKß, LKB1, AMPK, Kv2.1 and CaMKIV in the BLC, and we have shown alterations in several of these protein expression levels following chronic stress or food restriction. However, our findings are far from complete.

Expression levels for many of these proteins appear to be low in the BLC, almost around the detection threshold for our Western blot and immunohistochemistry techniques. Therefore the biggest limiting factor for our study is the small sample size, particularly when trying to

determine the effects on in vivo manipulations on protein levels of specific members of the AMPK signaling cascade. Therefore additional studies should be performed to increase the sample number and strengthen the statistical power of our observations.

Additionally, samples used for our Western blot analysis were from homogenates of whole BLC tissue, including both BLC principal neurons and interneurons. Consequently, we are unable to determine if the change of protein levels in the AMPK system we see following a homeostatic challenge exists in a specific subpopulation of BLC neurons, or occurs across all cell types. Although the interneurons only represent ~20% of the total neuronal population specific alterations of AMPK signaling in these neurons would still have an effect on our measurement of protein level changes following stress/food restriction. For future studies it would be helpful if we can find a method to extract proteins from principal neurons only. In addition, Western blot does not provide a good understanding of the phosphorylation function/enzyme activity, so we would like to develop an enzyme assay for AMPK to directly measure its activity in control and treatment groups.

Additional electrophysiology studies can be performed using a wider variety type of drugs that affect the signaling cascade, for example manipulators of CaMKKß, LKB1, AMPK and Kv2.1 to study in greater depth the functional roles of these members of the AMPK cascade.

Finally, we propose that AMPK acts as a homeostatic regulatory sensor that might play a major role in controlling the overall BLC excitability and therefore dysfunction of this system may be associated with certain psychiatric disorder, e.g. PTSD and schizophrenia. More work remains to be done on understanding the role of the AMPK pathway in regulating neuronal

excitability in the BLC before we can propose this pathway as a potential therapeutic target for homeostasis imbalance related psychiatric disorders.

Figure 1

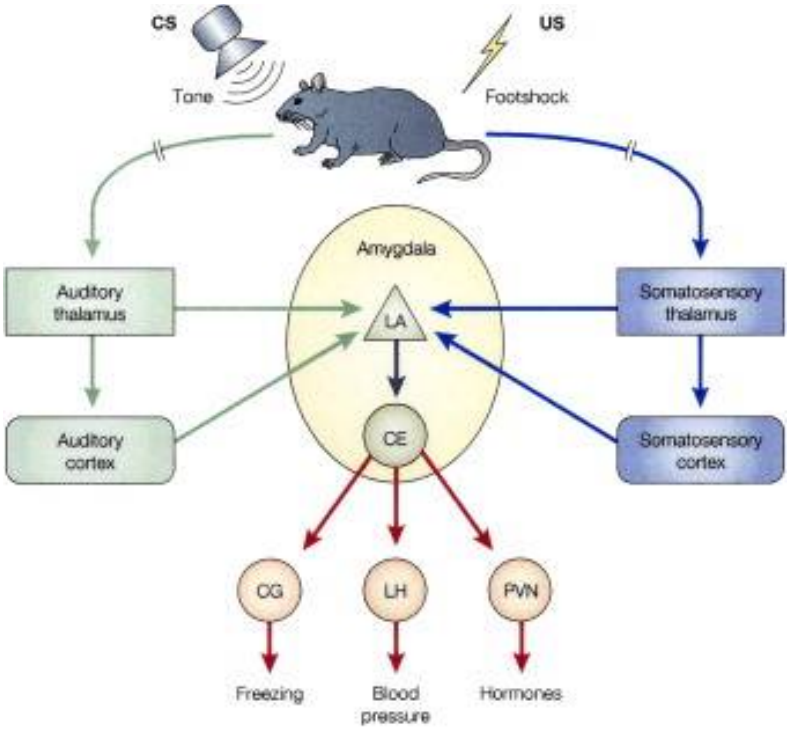

Figure 1: A schematic of the neural circuits underlying auditory fear conditioning adapted from LeDoux, 2007.

Abbreviations: LA, Lateral nucleus of the amygdala; CE, central nucleus of the amygdala; CG, central grey; LH, lateral hypothalamus; PVN, paraventricular hypothalamus.

Figure 2a

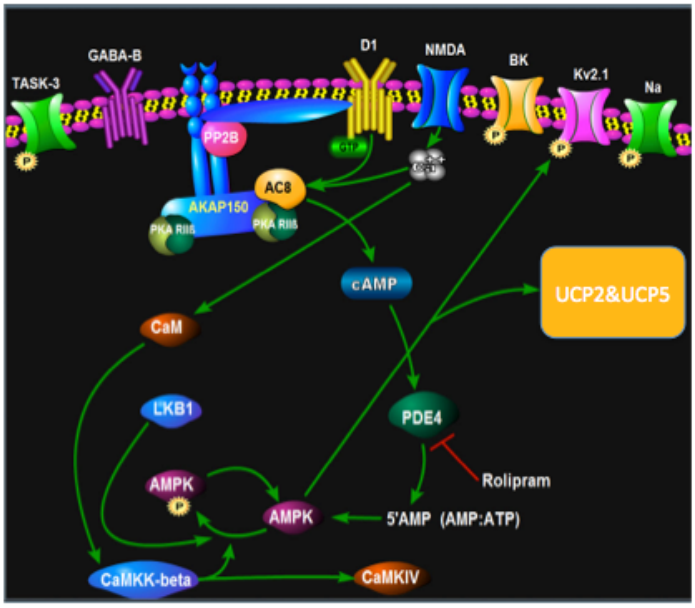

Figure 2a: A summary of the cAMP/PKA second messenger cascade and AMPK system in the BLC. Dopamine actives D1 receptors, which activates adenylate-cyclase (AC).AC catalyzes the transformation of ATP into cAMP, which then activates protein kinase A (PKA) and downstream targets, including the cAMP response element binding protein (CREB). cAMP levels are returned to baseline via the phosphodiesterases, including PDE4. PDE4 is inhibited by rolipram.

Figure 2b

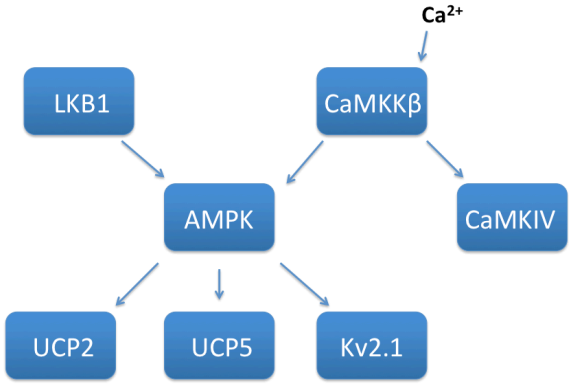

Figure 2b: A summary of proposed AMP-activated protein kinase (AMPK) system in the BLC. AMPK is activated upon detecting AMP:ATP levels in the BLC, which can also be activated via calcium/calmodulin second messenger cascades, specifically CaMKKß. In addition CaMKKß can also phosphorylate downstream cascade CaMIV. Another potential upstream cascade for AMPK activation is Liver Kinase B1 (LKB1). Downstream targets of AMPK include Kv2.1, UCP2 and UCP5.

Figure 3a

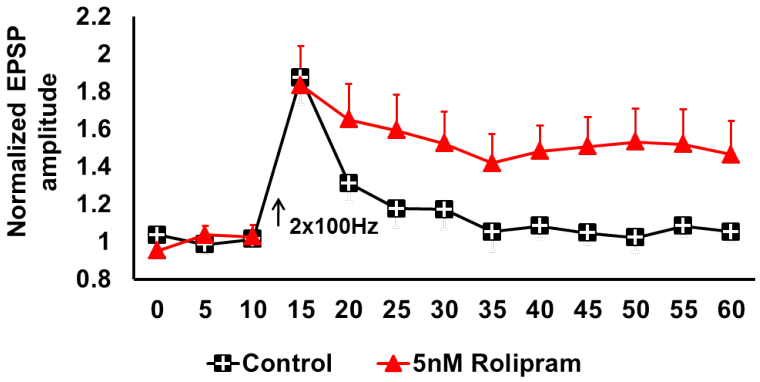

Figure 3a: Rolipram caused down shift of LTP induction threshold. Administration of rolipram, a PDE4 inhibitor, reduced the threshold for LTP induction in the BLC. In control conditions, a 5x 100Hz stimulus is required for LTP induction. Following administration of rolipram, a 2x 100 Hz stimulus successfully induced LTP.

Figure 3b

	AP threshold before Rolipram application	AP threshold after Rolipram application
Control BLC principal neurons	-41.2±1.9mV	-46.2±3.6mV

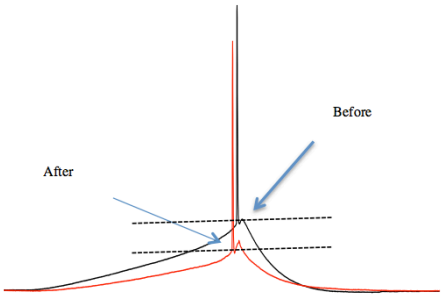

Figure 3b: Rolipram caused down shift of action potential threshold. Here it shows the example trace of action potential generation threshold change after application of rolipram. A 5 mV shift in the hyperpolarizing direction was detected.

Figure 4

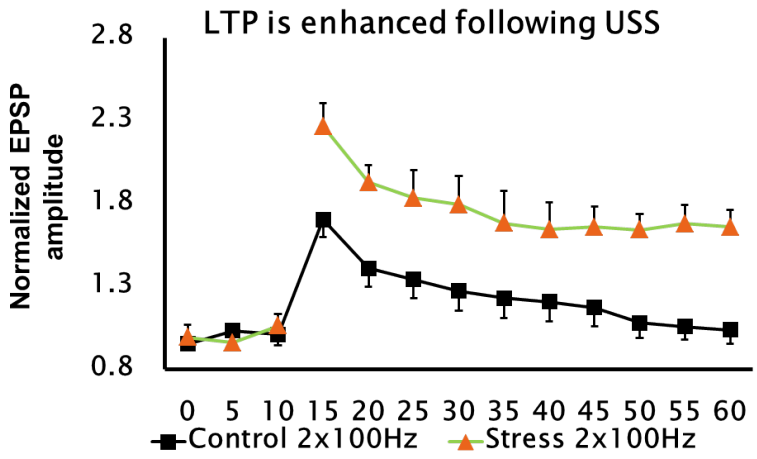

Figure 4: Stress caused down shift of LTP induction threshold. Following stress, a 2x 100 Hz stimulus successfully induced LTP, indicating a reduction of the threshold for LTP induction. 2x 100 Hz stimulus did not enhance LTP induction in control animals.

Figure 3 & 4 are courtesy of Dr. Chen-Chen Li.

Table 1

Gene Name	Accession No	Primer Sequence	PCR produce size (bp)
CaMKKβ	NM_031338.1	F: 5'-GCTCCCCACAGTCCTCTCCCC-3' R: 5'-CCTTGAACTCATTGCTCACGC-3'	600
LKB1	NM_001108069.1	F: 5'-CGGACCGTCGCCTCCGTCCC-3' R: 5'-ACCTTGCCGTACGAGCCCTC-3'	592
AMPK	NM_023991.1	F: 5'-ATGAATGGTTTAAACAGGATTTGC-3' R: 5'-TGTGCTGGAATCGACACTTGACCGA-3'	510
CaMKIV	BC128706.1	F: 5'-GCTCAAAGTCACGGTGCCCTCC-3' R: 5'-TTCTCTGGTTTGAGGTCGCGAT-3'	470
Kv2.1	NM_013186	F:5'GAAGGAGCAGATGAACGAGGAG-3' R:5'GTTGAGTGACAGAGCGATGGTG-3'	208

The PCR primer sequences used in the study and the resulting PCR product size

Figure 5

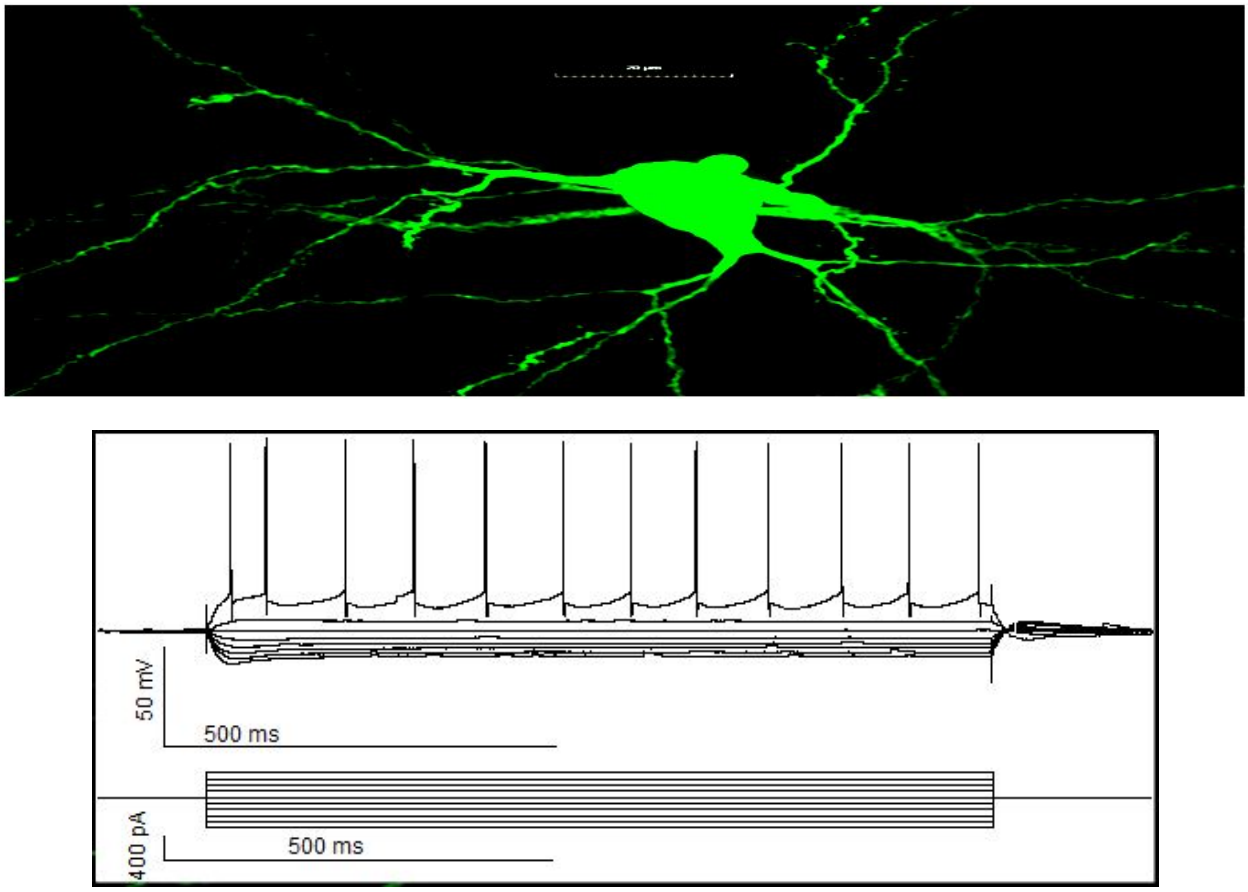

Figure 5: Here is an example of a principle BLC neuron along with a sample of its electrophysiological profile. Principal neurons can be identified by their input resistance, spike train properties, and expression of voltage-dependent currents such as Ih and It. Here, the neuron was filled with biocytin and stained with streptavidin, then processed for immunohistochemistry. Image is courtesy of Steven J. Ryan, B.S., Ph.D candidate.

Figure 6

Figure 6 shows the mRNA expression of AMPK, CAMKKβ, LKB1,CaMKIV and Kv2.1 in control whole BLC tissue.

Table 2

	LKB1	CaMKKβ	AMPK	Kv2.1	CaMKIV
Neuron 2	+	+	+	+	+
Neuron 3	+	+	-	+	+
Neuron 5	+	+	+	+	+
Neuron 6	-	+	+	+	+
Neuron 7	+	-	-	-	+
Neuron 9	+	-	-	-	+
Neuron 10	-	-	+	+	+

Table 2 shows the neurons out of 7 BLC principal neurons that express AMPK signaling cascade. Significantly, 2 out of 7 BLC principal neurons expressed all five cascades.

Figure 7

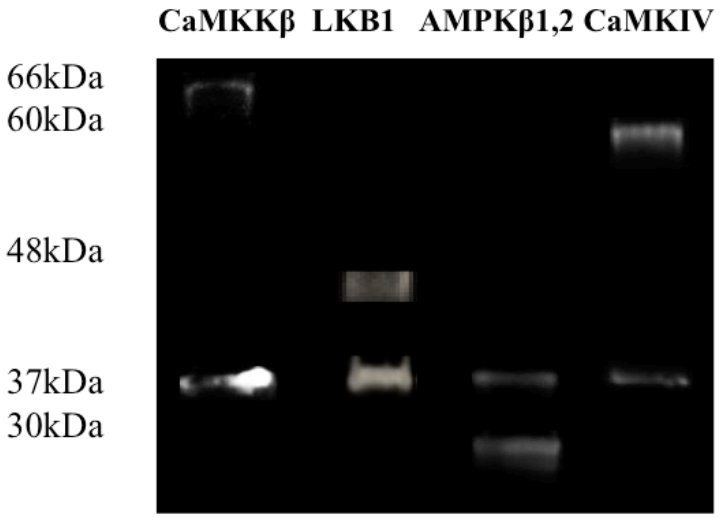

Figure 7 shows the protein expression of AMPK, CAMKKβ, LKB1 and CaMKIV in control whole BLC tissue. Protein bands at 37kDa represent housekeeping protein GAPDH.

Figure 8

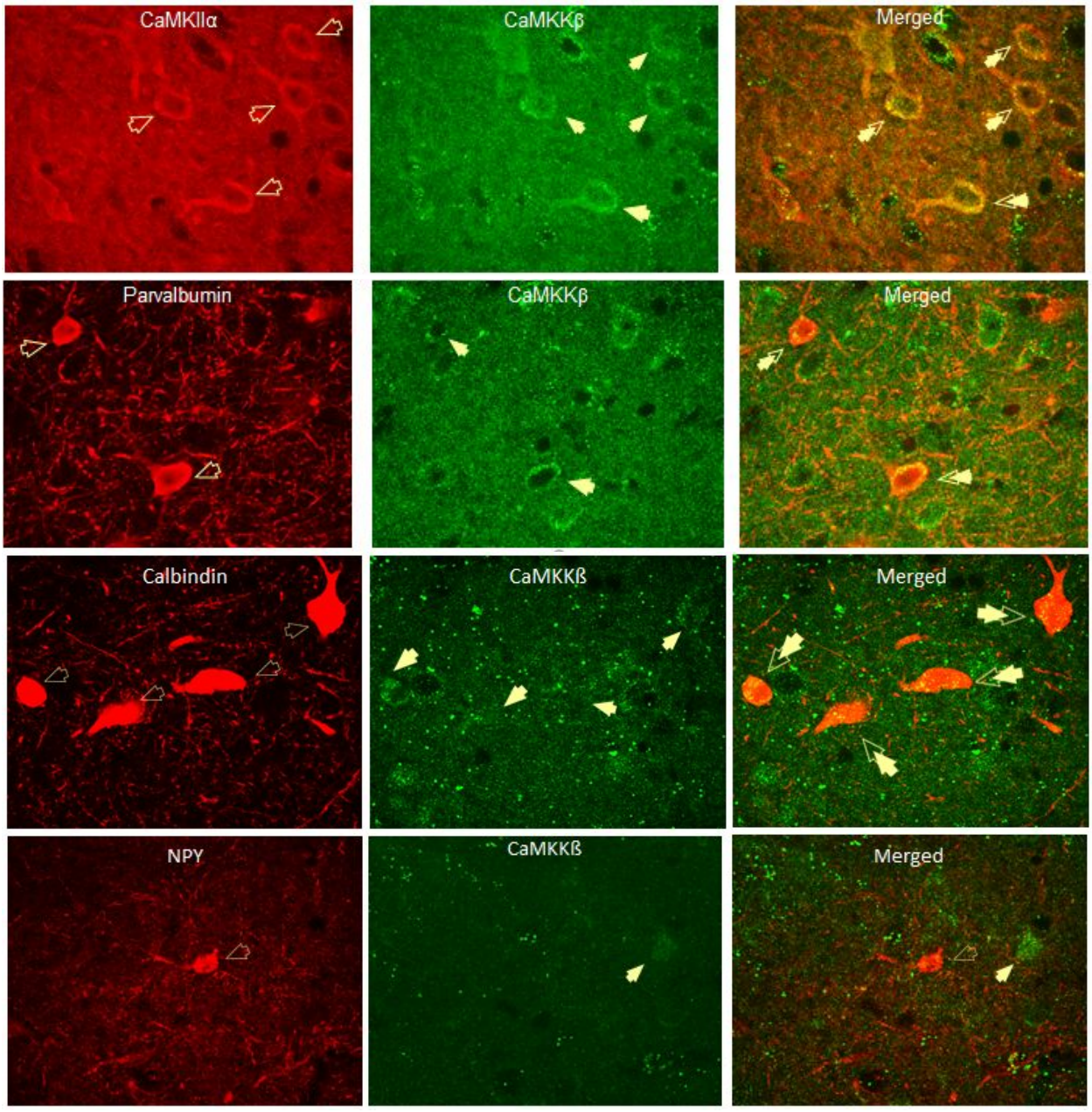

Figure 8 shows dual-immunofluorescence studies on CaMKKβ and specific neuronal markers, including CaMKIIα, Parvalbumin, Calbindin, and NPY.

Figure 9

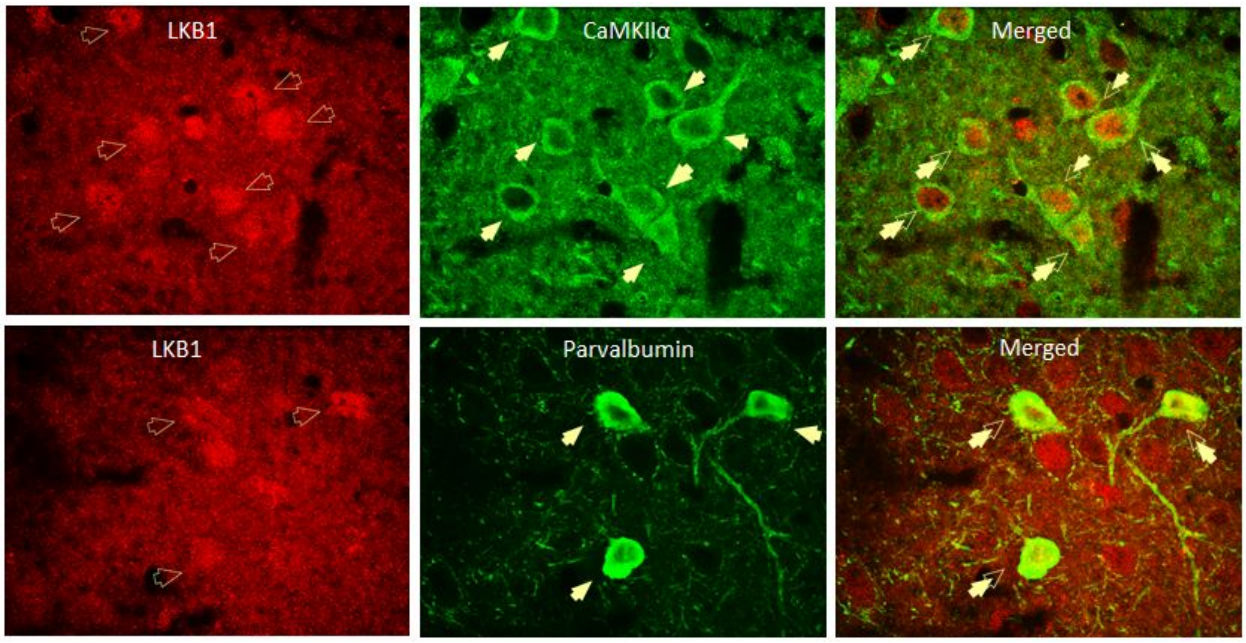

Figure 9 shows dual-immunofluorescence studies on LKB1 and specific neuronal markers, including CaMKIIα and Parvalbumin.

Figure 10

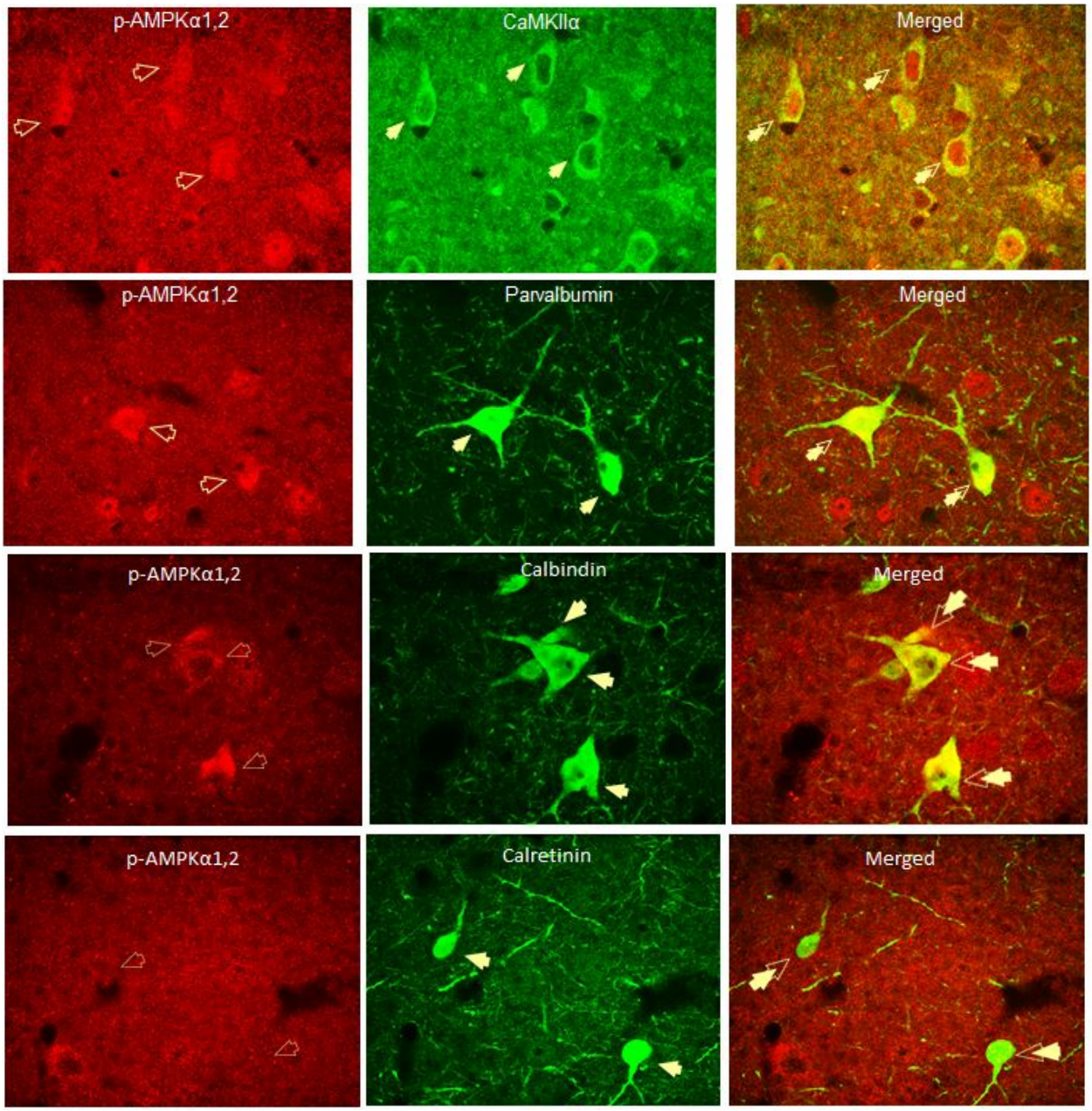

Figure 10 shows dual-immunofluorescence studies on p-AMPKα1,2 and specific neuronal markers, including CaMKIIα, Parvalbumin, Calbindin and Calretinin.

Figure 11

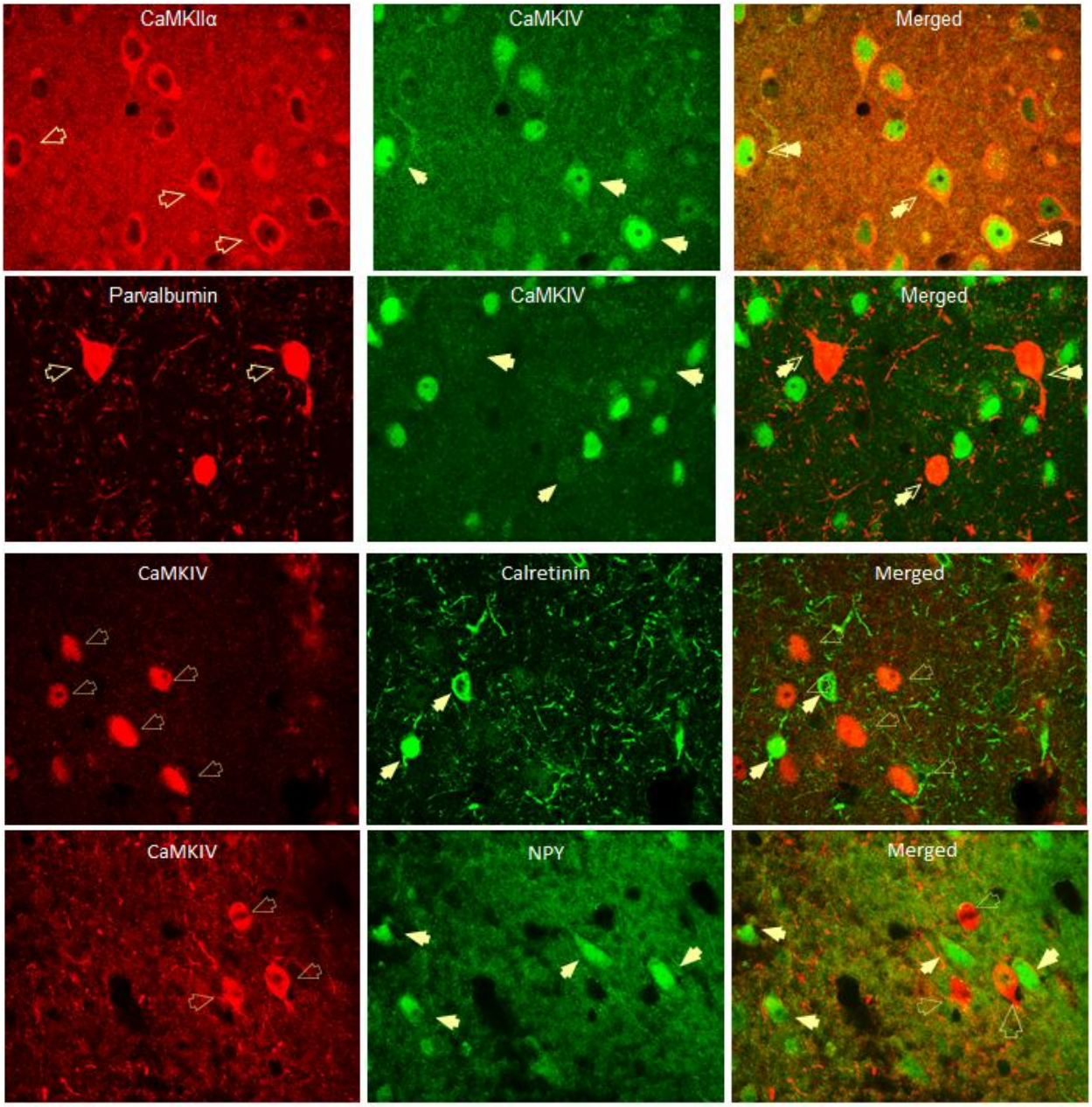

Figure 11 shows dual-immunofluorescence studies on CaMKIV and specific neuronal markers, including CaMKIIα, Parvalbumin, Calretinin and NPY.

Figure 12

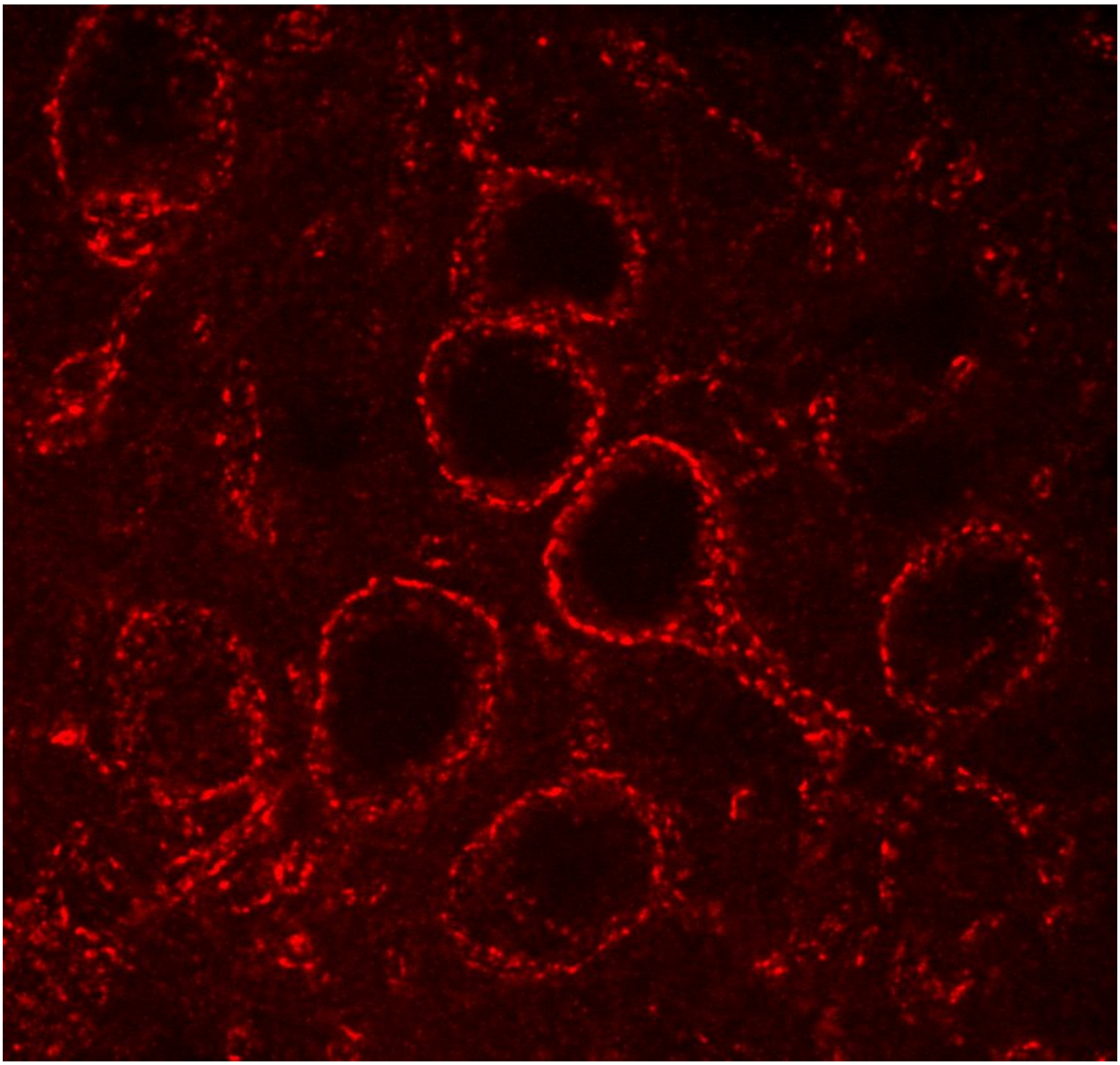

Figure 12 shows immunofluorescence staining against Kv2.1.

Table 3

	% marker cells co-expressing CaMKKß	% marker cells co-expressing LKB1	% marker cells co-expressing p-AMPKα1,2	% marker cells co-expressing CaMKIV
CaMKIIα	87.8%(65/74)	100% (78/78)	100%(27/27)	100% (26/26)
Parvalbumin	50%(3/6)	100%(9/9)	100%(10/10)	75%(9/12)
Calbindin	100% (4/4)	-	100%(14/14)	-
Calretinin	-	-	42.9%(3/7)	36.4%(4/11)
NPY	50%(1/2)	-	-	0%(0/4)

Table 3 shows co-localization of AMPK pathway cascades with markers for defined subpopulations of BLC neurons in the rat BLC.

Average number of immunoreactive cells counted per section is indicated in brackets. Due to some marker antibodies have the same host as target AMPK pathway cascade antibodies do, we cannot analyze the same group of markers for every cascade.

Table 4

	AP threshold before AICAR application	AP threshold after AICAR application
Control BLC principal neurons	-40.6±3.5mV	-32.6±5.0mV

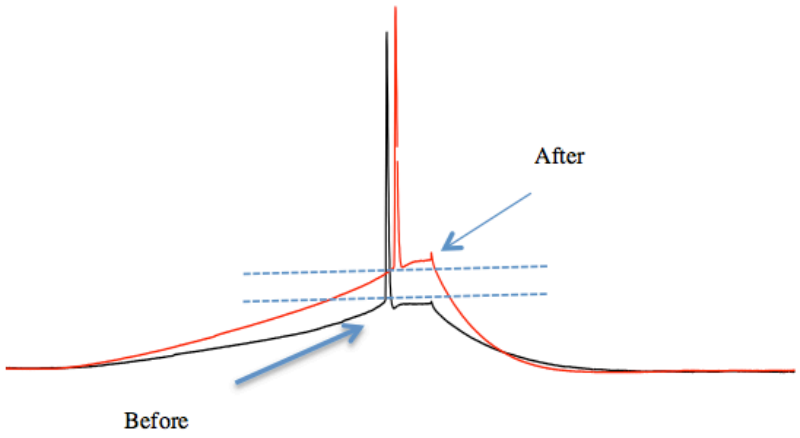

Table 4 shows action potential generation threshold shift in the depolarizing direction after the application of AICAR in patch recording solution in control BLC principal neurons ($n=8$, $p<0.05$) and the example trace.

Figure 13

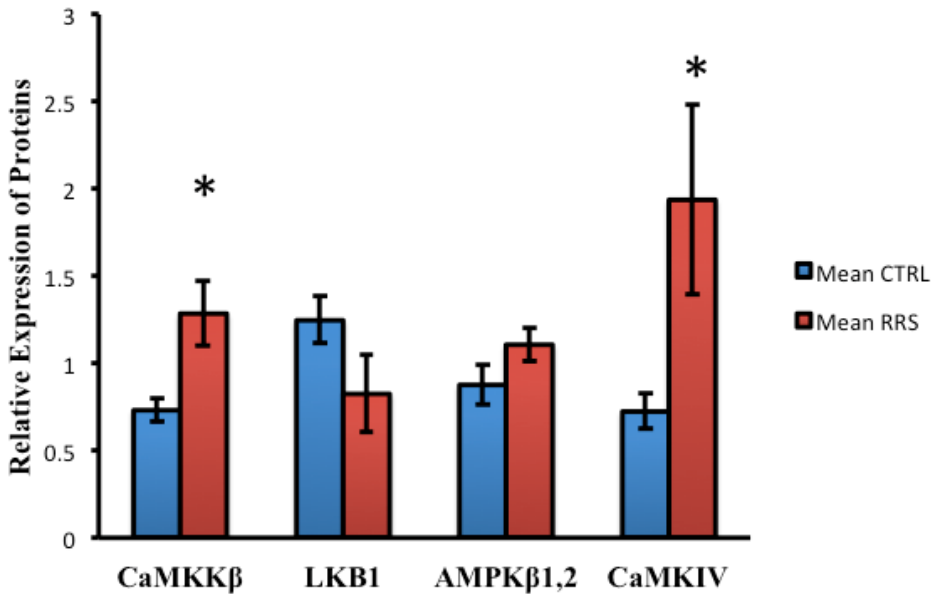

Figure 13 represents relative expression of specific proteins as calculated for GAPDH (35kDa) from Western blot data. Repeated restraint stress (RRS) significantly increased expression levels of CaMKKβ (66kDa, p=0.015,n=4) and CaMKIV (60 kDa, p=0.035, n=4). RRS also caused a non-significant trend of decrease in the expression level of LKB1(48kDa, p=0.077, n=4) and increase in the expression level of AMPKβ1,2 (30kDa, p=0.082,n=4) in the BLC at day 10 compared to control animals. Error bars show standard error of the mean.

Figure 14a

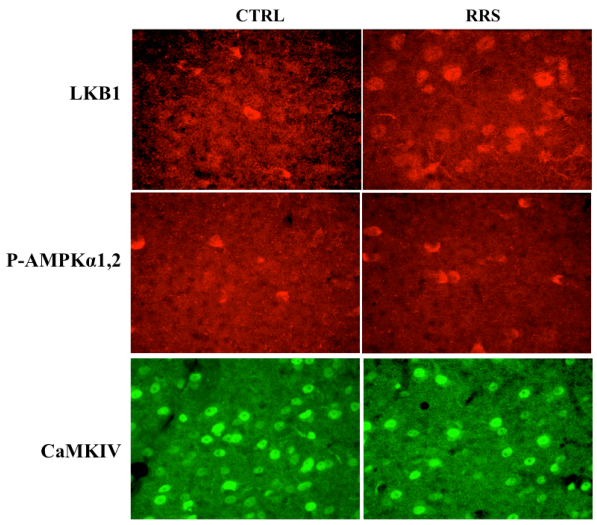

Figure 14b

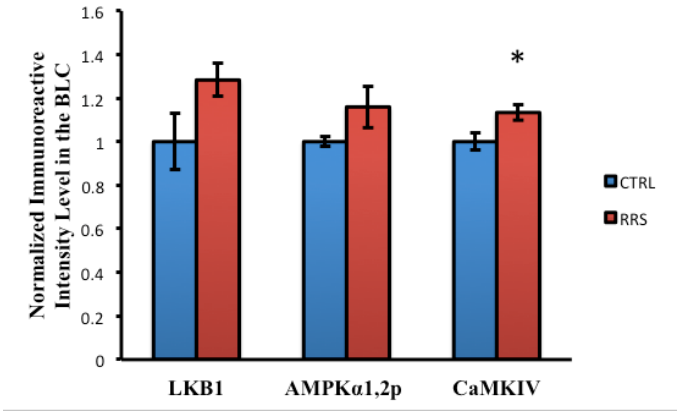

Figure 14a shows representative immunofluorescence staining pictures from control and repeated restraint stress for CaMKKβ, LKB1 and CaMKIV.

Figure 14b shows CaMKIV (n=4, p=0.022) immunoreactive level is significantly increased, while LKB1 (n=4, p=0.054) and AMPKα1,2 (n=4, p=0.077) levels show a non-significant trend of increase in RRS animals compared to controls. Error bars show standard error of the mean.

Figure 15

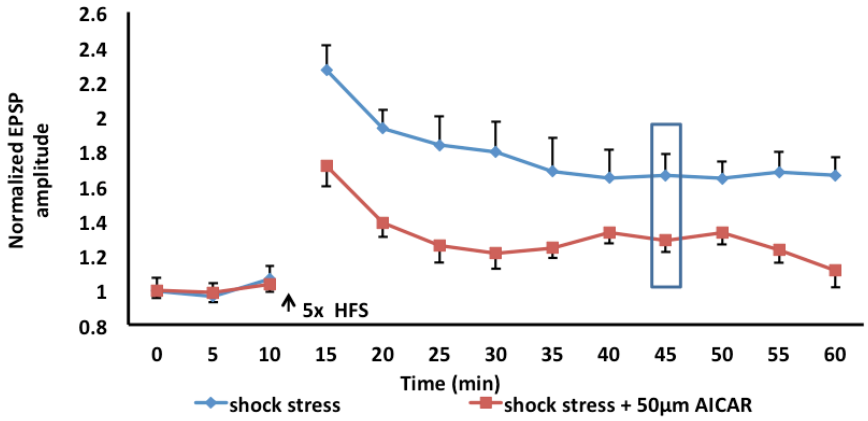

Figure 15 shows LTP could be induced in BLC principal neurons (165±12% of baseline, n=6) at a lowered threshold (2xHFS) in slices obtained from stressed animals that would not normally induce LTP in BLC principal neurons recorded from control animals. The LTP induced by 2×HFS in stressed animals was significantly attenuated by intracellular application of AICAR (128±7% of baseline, n=6, $p<0.05$).

Figure 16

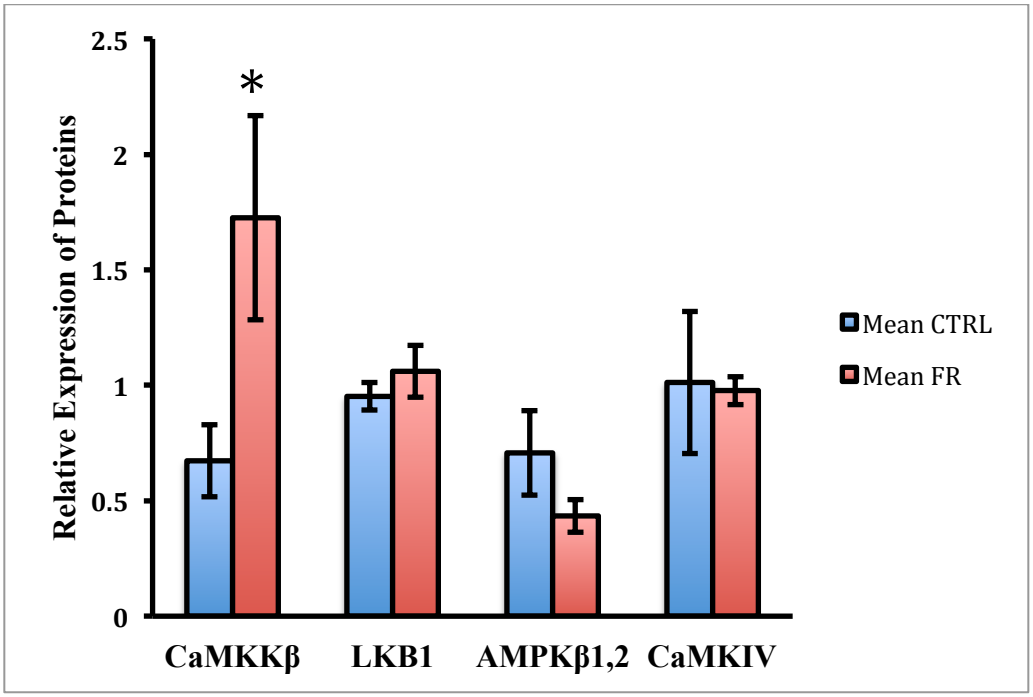

Figure 16 represents relative expression of specific proteins as calculated for GAPDH (35kDa) from Western blot data. Food restriction significantly increased the expression level of CaMKKβ (66kDa, p=0.033,n=4) and caused a non-significant trend of increase in expression level of LKB1 (48kDa, p=0.213,n=4) and decrease in expression levels of AMPKβ1,2 (30kDa, p=0.107,n=4) and CaMKIV (60 kDa, p=0.458, n=4) in the BLC of 7.6 week-old rats compared to control animals. Error bars show standard error of the mean.

Table 5

	AP threshold before 2DG application	AP threshold after 2DG application
Control BLC principal neurons	-36.90±1.41mV	-41.04±1.16mV
Food restricted BLC principal neurons	-40.48±0.93mV	-40.79±1.13mV

Table 5 shows a shift in the hyperpolarizing direction of action potential generation threshold following food restriction compared to controls ($p<0.05$), as well as a hyperpolarizing shift of action potential generation threshold following 2DG application ($n=12$, $p<0.01$) in controls and no additional action potential generation threshold shift after application of 2DG in patch recording solution in BLC principal neurons collected from food restricted animals ($n=16$).

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