Deletion of Stk11 and Fos in BLA projection neurons alters intrinsic excitability and impairs formation of long-term aversive memory

Abbreviated Title: The role of Stk11 in memory formation

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Abstract

Conditioned taste aversion (CTA) is a form of one-trial learning dependent on basolateral amygdala projection neurons (BLApn). Its underlying cellular and molecular mechanisms are poorly understood, however. We used RNAseq from BLApn to identify learning-related changes in Stk11, a kinase with well-studied roles in growth, metabolism and development, but not previously implicated in learning. Deletion of Stk11 restricted to BLApn completely blocks memory when occurring prior to training, but not following it, despite altering neither BLApn-dependent encoding of taste palatability in gustatory cortex, nor transcriptional activation of BLApn during training. Deletion of Stk11 in BLApn also increases their intrinsic excitability. Conversely, BLApn activated by CTA to express the immediate early gene Fos had reduced excitability. BLApn knockout of Fos also increased excitability and impaired learning. These data suggest that Stk11 and Fos expression play key roles in CTA long-term memory formation, perhaps by modulating the intrinsic excitability of BLApn. (149 words)

Introduction

Conditioned Taste Aversion (CTA) is a form of long-lasting aversive memory induced by a single pairing of exposure to an initially palatable taste with gastric malaise (Bures et al., 1998). Although multiple brain regions, including the brainstem, amygdala and the cortex, participate in various aspects of taste behavior (reviewed in Carleton et al., 2010), prior work suggests that the basolateral amygdala (BLA) plays a critical role in CTA memory. Disrupting neuronal activity within the BLA blocks the formation and retrieval of CTA memory (Yasoshima et al., 2000; Ferreira et al., 2005; Garcia-Delatorre et al., 2014; Molero-Chamizo, et al., 2017). This may reflect the fact that BLA projection neurons (BLApn) provide the principal output pathway from the amygdala to forebrain structures including the gustatory cortex and the central amygdala (Duvarci and Pare, 2014) enabling it to distribute taste valance information to these regions (Piette et al., 2012; Samuelsen et al., 2012). Consistent with this view, BLA neurons change their activity and their functional connectivity with their down-stream targets during CTA learning (Grossman et al., 2008). However, whether the BLA is a site of cellular and molecular plasticity during CTA learning, as opposed to merely gating plasticity in other structures, is not known.

Stages of memory formation are typically distinguished on the basis of duration and molecular mechanism. Short-term memory, lasting minutes to hours, requires only post-translational modification of preexisting proteins, whereas long-term memory, lasting days or longer, requires gene transcription and RNA translation, typically occurring in the hours following memory acquisition (Matthies, 1989; Alberini, 2009; Gal-Ben-Ari et al., 2012; Kandel, 2001). Production of new proteins is required to produce lasting changes in the efficacy of synaptic connections and in the intrinsic excitability of neurons, which are thought to be the cellular correlates of memory (Zhang and Linden, 2003; Mozzachiodi and Byrne, 2010; Takeuchi et al., 2014). The cellular correlates of CTA

learning are less completely understood than those of some other forms of learning, but the involvement of both synaptic plasticity (Li et al., 2016) and intrinsic plasticity (Yasoshima and Yamamoto, 1998; Zhou et al., 2009) have been demonstrated. CTA is known to require protein synthesis in the BLA (Josselyn et al., 2004) and to increase the expression of the activity dependent transcription factor Fos (Uematsu et al., 2015). In other behavioral paradigms, neurons increasing Fos protein undergo changes in synaptic strength and intrinsic excitability (Yassin et al., 2010; Ryan et al., 2015; Pignatelli et al., 2019) and are thought to be essential parts of the neuronal network underlying long-term memory (Tonegawa et al., 2015). However, the role of neurons expressing Fos in the BLA during CTA is unclear. Also unknown is whether CTA learning requires new transcription, and if so, the identities of the required transcripts and cellular processes they promote are not known.

In this study we found that new transcription in the BLA is required for CTA learning. Using RNA-seq from sorted neurons, we found that expression of the kinase Stk11, also known as LKB1, is altered following learning in BLA projection neurons (BLApn), but not in excitatory or inhibitory neurons within the GC. Stk11 is known to act as a master regulator of growth, metabolism, survival and polarity by phosphorylating 13 down-stream members of the AMP-related kinase family (Lizcano et al., 2004; Shackelford and Shaw, 2009). Recent work also suggests roles for Stk11 in the nervous system, where it controls axonal specification and dynamics during development (Barnes et al., 2007; Shelly et al., 2007) and synaptic remodeling during old age (Samuel et al., 2014). Stk11 can also regulate synaptic transmission in forebrain neurons (Kwon et al., 2016), but it is not known to play a role in learning or in the regulation of intrinsic neuronal excitability.

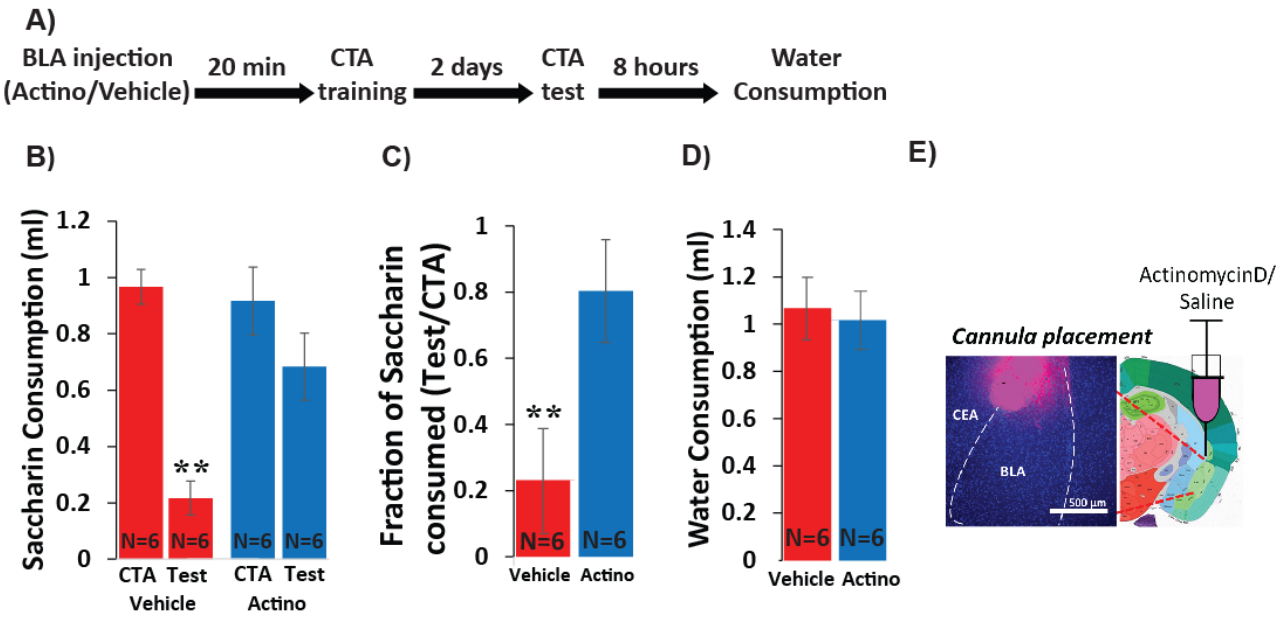
We find that Stk11 is required for CTA since conditional knockout from BLApn prior to training completely blocks learning. However, the same deletion performed two days after training—i.e., at a time when long-term memories have already been formed and stabilized—has no effect on subsequent memory retrieval. Deletion of Stk11 also increased the excitability of BLApn, but did not alter the ability of the BLA-GC circuit to become transcriptionally activated by training or to encode the palatability of gustatory stimuli. CTA training is associated with an opposing decrease in intrinsic excitability change in a sub-population of BLA projection neurons expressing the activity dependent gene Fos following learning. BLApn knockout of Fos also increased excitability and impaired learning. Together, these data suggest that Stk11 and Fos expression play key roles in CTA long-term memory formation, perhaps by modulating the intrinsic excitability of BLApn.

Results

CTA long-term memory requires BLA transcription

In order to determine whether CTA requires new RNA transcription within the BLA, we inhibited transcription by injecting Actinomycin-D (1 µl, 50 ng, bilaterally, Figure 1), a

widely used RNA polymerase 2 inhibitor (Alberini, 2009), into the BLA 20 min prior to CTA training, and tested memory 48 hours later. As a control, a separate group of mice received vehicle injection (1 µl of PBS, bilaterally). CTA training consisted of 30 min of access to 0.5% saccharin followed by an intraperitoneal injection of 0.15M LiCl, 2% body weight; (Figure 1-figure supplement 1). A two-way ANOVA comparing vehicle and



actinomycin-D injected mice before and after training revealed significant training and

Figure 1. Inhibiting BLA transcription impairs CTA Learning. (**A**) Protocol for injection (1 µl per hemisphere) of Actinomycin-D (50 ng) or vehicle (PBS with 0.02% DMSO). (**B**) Actinomycin D injection prior to CTA training impairs learning, expressed as a reduction in saccharin consumption between CTA and test sessions. Group and treatment effects were significant by two-way ANOVA (groups: $F(3,20)=13.05$; $p=6E-5$; treatment: $F(1,20)=4.83$; $p=0.04$; with a significant interaction: $F(1,20)=7.4$; $p=0.013$). Post hoc analysis (Bonferroni corrected) revealed significant reductions ($p=1E-4$) of saccharin consumption (training vs. test for vehicle treated, but not for actinomycin-D treated mice ($p=0.583$). (**C**) Fraction of saccharin consumed (Test/CTA) was significantly higher ($t(10)=-3.36$; $p=0.007$) following Actinomycin D treatment than vehicle, consistent with weaker memory. (**D**) Treatments did not differ in water consumption measured 8 hours later ($t(10)=0.279$; $p=0.794$) suggesting this does not account for differences in consumption during the test. **$p<0.01$. (**E**) Guide cannula was coated with fluorescent dye to assess placement (Left) relative to desired location in anterior BLA (Right; bregma -1.4 mm; Allen brain atlas). Note that the injection cannula extended 0.5 mm further into the BLA.

treatment effects and a significant interaction between the two (Figure 1B), and post-hoc analysis revealed significant reduction in the consumption of saccharin (CTA vs. Test) for the vehicle group, indicating impairment of learning for the actinomycin-D treated group compared to control mice. As a convergent measure, we also assessed the strength of CTA memory by calculating the relative consumption of saccharin during the test day to that consumed on the training day (Neseliler et al., 2011). The differences between the groups were large (23% in the vehicle group vs. 80 % for the actinomycin D group) and

significant. Meanwhile, actinomycin-treated mice were neither impaired in their ability to detect the palatability of saccharin, nor in their drinking behavior—consumption of saccharin during CTA training was similar for the two groups, as was consumption of water 8 hours after the test (Figure 1E), suggesting that these nonspecific effects cannot account for the memory impairment. Thus, BLA transcription is essential for CTA memory formation. These results extend prior work showing the importance of BLA protein synthesis for CTA memory (Josselyn et al., 2004) and together show that training induces both transcription and translation important for CTA in the BLA.

Stk11 expression is regulated in BLA projection neurons following CTA

In order to identify specific transcripts that might be necessary for CTA learning within the BLA, we used cell type-specific RNA-seq to profile transcriptional changes in sorted BLA projection neurons (BLApn). We manually isolated fluorescently labeled BLApn neurons from YFP-H mice (Feng et al., 2000) in which YFP is expressed under the Thy1 promoter in a large population of excitatory projection neurons located in the anterior part of the nucleus (Sugino et al., 2006; Jasnow et al., 2013; McCullough et al., 2016). RNA sequencing was performed separately on YFP^+ BLApn harvested 4 hours following training from mice undergoing CTA, and from taste-only controls (n=4/group) (Figure 2 and Table 1). Sequencing results (Figure 2A) also confirmed the purity and molecular identity of YFP-H neurons in the BLA, as transcripts known to be expressed in BLApn and other forebrain excitatory projection neurons were enriched and transcripts known to be expressed in inhibitory interneurons, glia cells and other neurons in the vicinity of the BLA (lateral amygdala or central amygdala) were virtually absent. Moreover, this observed pattern of expression was comparable to that reported in other studies profiling the same population (Sugino et al., 2006; McCullough et al., 2016).

CTA training changed expression of many genes. Table 1 lists the 20 transcripts showing differential expression between CTA and taste control group based on robust expression criteria: $2 \leq$ fold change ≤ 0.5, $p<0.01$ (unpaired t-test), transcripts-per-million (TPM) ≥ 30. Included among these transcripts is the activity dependent transcription factor, Fos which has previously been shown to be upregulated in BLApn following CTA and other learning paradigms (Zhang et al., 2002; Yasoshima et al., 2006; Mayford and Reijmers. 2015; Uematsu et al. 2015). Our screen for differentially expressed genes also identified Stk11 (also known as LKB1; Figure 2B) a kinase well studied in the context of cancer, cell growth and development, but not previously known to be involved in learning and neuronal plasticity (Bardeesy et al., 2002; Alessi et al., 2006; Barnes et al., 2007; Gurumurthy et al., 2010; Courchet et al., 2013). Changes in the expression of both Fos and Stk11 were validated by qPCR in separate experiments, which revealed a 4.6-fold increase in Fos mRNA ($t(6)=2.5$; $p=0.045$) and a 1.9-fold decrease in Stk11 ($t(6)=-2.5$; $p=0.046$) (Figure 2C). To further examine the significance of these transcriptional changes we also analyzed the levels of Fos and Stk11 protein in the BLA. Due to the availability of antibodies to Fos suitable for immunohistochemistry, we measured the

fraction of Fos-expressing YFP-H neurons in the BLA 4 hours following CTA and found a significant increase relative to lithium chloride-only and taste-only control groups (Figure 2-figure supplement 1). Lacking an antibody to Stk11 usable for immunostaining of brain sections, we examined Stk11 protein 4 hours following CTA and taste control conditions

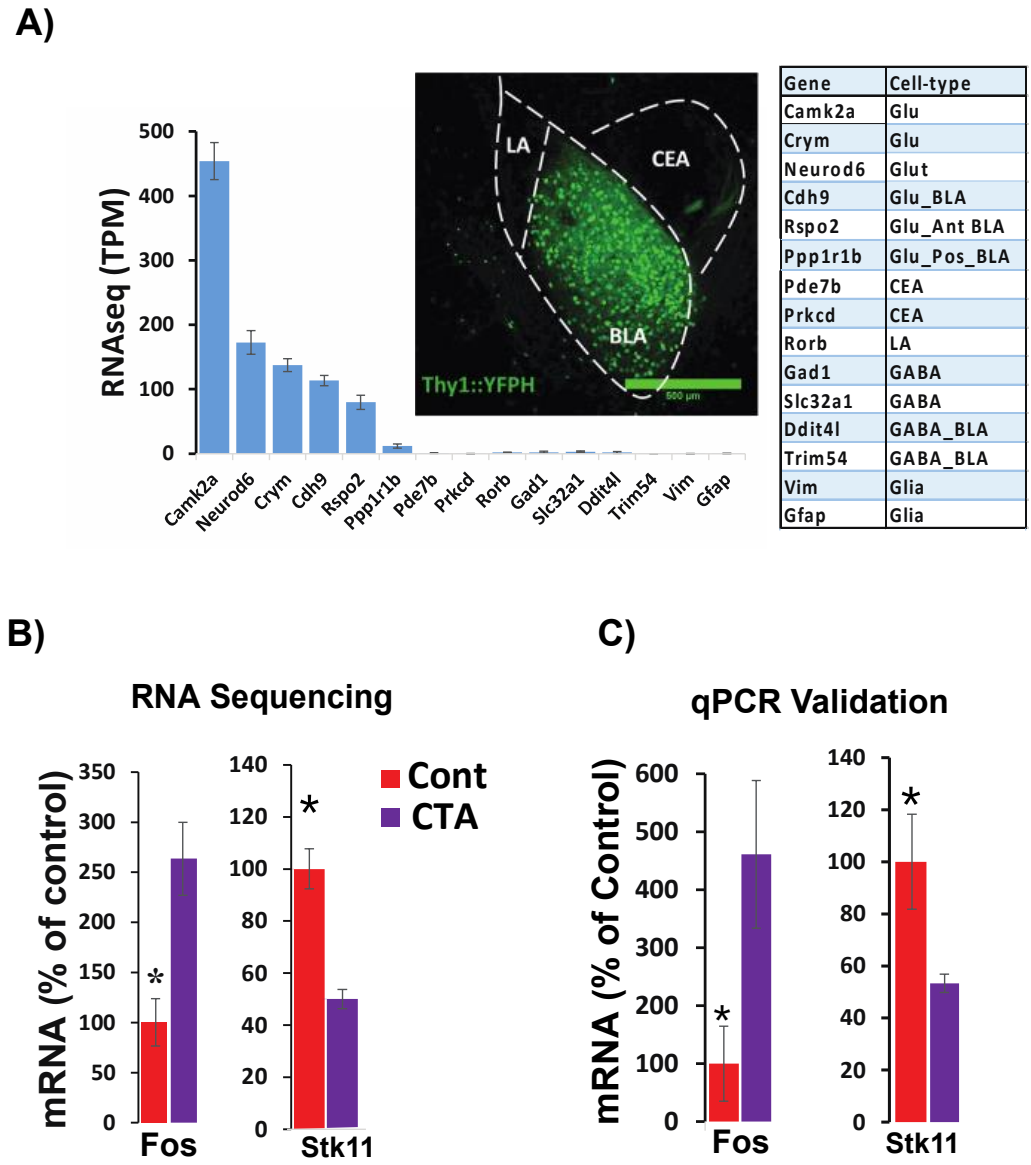


Figure 2. RNA sequencing from BLApn 4 hours following CTA training. (**A**) BLApn were isolated from YFP-H mice following CTA or taste control (N=4/group). Neurons (150-200) were manually sorted form coronal slices (LA-lateral amygdala; CEA-central amygdala). Abundant transcripts (histogram, averaged across both groups) are enriched for those expected in the population and depleted for those expressed in other nearby populations (table) including GABAergic interneurons, glia, neurons in LA or CEA; Sugino et al., 2006; Kim et al., 2016; Alan brain atlas). Glu- GABA-, glutamatergic, GABAergic neurons; AntBLA, PostBLA- Anterior and posterior portions of the BLA. TPM- transcript per million. (**B**) Among genes meeting robust criteria for differential expression (see table 1) Stk11 and Fos were selected for further analysis, including qPCR confirmation (**C)** in separate experiments (N=4/group; *p<0.05).

using immunoblotting of proteins isolated from the anterior BLA. Surprisingly, we found a 1.8- fold increase in Stk11 protein (Figure 2-figure supplement 2). This confirms the fact that Stk11 expression is altered following CTA, but suggests complexity in the dynamics of the expression and potential mismatch in the timing or magnitude of changes in transcript and protein.

Stk11 is at the apex of the AMP-related kinase pathway and mediates its effects by phosphorylating one or more of 13 different downstream kinases, all of which share some homology with AMP-kinase (Lizcano et al., 2004; Shackelford and Shaw. 2009). Among these, YFP^+ BLApn had moderate expression of Brsk2 (TPM=39.9) and Mark2/3 (TPM=59.3 and 73.7 respectively; Figure 2-figure supplement 3), which have known roles in establishing cell polarity during neuronal development (Barnes et al., 2007; Shackelford and Shaw. 2009). Expression of AMP kinase itself (Prkaa1/2), an important metabolic regulator in many cell types (Shackelford and Shaw. 2009) was lower (TPM=16.4 and 22.3 respectively). Comparing kinase expression between CTA and control groups revealed nearly two-fold less expression of Mark2 following CTA (fold-change=0.41; p=0.04, unpaired t-test; data not shown) but this was not significant after Bonferroni correction across the compared kinases.

We also analyzed the impact of CTA on the transcriptional profile of layer 5 pyramidal neurons labeled in strain YFP-H and Pvalb-expressing inhibitory interneurons in the GC, a brain region in which transcription is also known to be important for CTA learning (Imberg et al., 2016). RNA sequencing was performed on YFP^+ and Pvalb+ neurons in the GC harvested from mice 4 hours following CTA training, and from taste-only controls (n=3/4/group). Table 2 and 3 lists the transcripts showing the most differentially expressed genes using the same criteria used in the BLA: $2 \leq$ fold change ≤ 0.5, $p<0.01$ (unpaired t-test), transcripts-per-million (TPM) ≥ 30. While Pvalb+ neurons showed a robust transcriptional response, evident by 19 genes reaching the criteria, only one gene reached the same criteria in YFP+, suggesting a weaker CTA-driven transcriptional response in these neurons. Importantly, the expression of Fos and Stk11 in both YFP+ and Pvalb+ neurons, did not differ between CTA and control groups (Figure 2-figure supplement 4 and Table 2 and 3). This data suggest that the differential expression of Fos and Stk11 during CTA learning may be specific to a subset of cell-types within the circuit.

Fos and Stk11 expression in BLA projection neurons are necessary for memory formation

We next wished to determine whether any of the transcriptional changes in BLApns that correlate with learning are indeed necessary for learning to occur. Since both Fos and Stk11 protein increase following CTA, we pursued a loss of function (LOF) strategy. To restrict LOF to BLApns, we performed conditional deletion by injecting Cre recombinase into mice carrying alleles of Fos (Zhang et al., 2002) or Stk11(Nakada et al., 2010) in

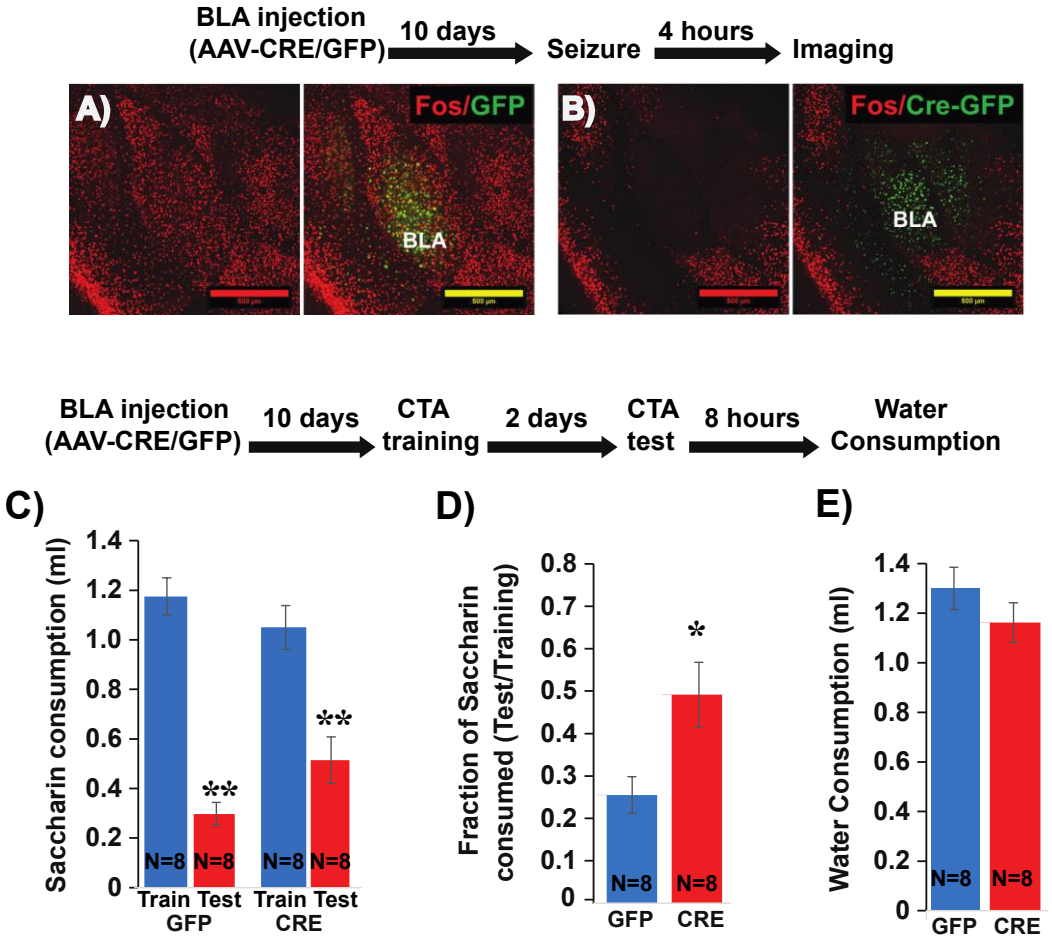


Figure 3. Deletion of Fos from BLApn reduces the strength of learning. (**A,B**) BLA of Fos$^{f/f}$ mice were infected with viruses expressing Cre-GFP **(B)** or GFP alone **(A)**. Fos induction was tested 10 days later, 4 hours after onset of seizures in response to kainic acid (20 mg/kg). Cre injected BLA's had reduced Fos expression confirming penetrance of the knock-out. (**C-E**) Fos deletion from BLApn attenuates CTA learning. Fos f/f mice received Cre and control viruses bilaterally and were trained for CTA 10 days later and then tested after an additional 48 hours. (**C**) Both groups exhibited significant memory reflected by reduced saccharin consumption between training and testing sessions. Two-way ANOVA revealed a significant effect of training ($F(1,28)=63.06$; $p=1.15E-8$), but not of genotype ($F(1,28)=0.261$; $p=0.614$), although there was a significant interaction ($F(1,28)=4.9$; $p=0.03$). Post hoc analysis (Bonferroni) revealed that both GFP (N=8) and Cre (N=8) group reductions following CTA (test vs. train) were significant (GFP: $p=7.17E-8$; Cre: $p=3.47E-4$) but differences between other groups were not. (**D**) Fos deletion from BLApn reduced memory strength measured as the fraction of saccharin consumed (test/training): 25% (GFP) versus 49% (Cre) and this difference in ratios was significant ($t(14)=-2.697$; $p=0.017$). (**E**) Reduced saccharin consumption cannot be attributed to overall inhibition of drinking as the amount of water drunk 8 hours later did not differ ($p=0.26$). *$p<0.05$; **$p<0.01$.

which key exons are flanked by lox-p sites. In both cases, recombination leads to a functionally null allele and analyses were carried out in homozygous ($^{f/f}$) animals. Cre was delivered by injecting AAV2/5-Camk2α::Cre-GFP into the BLA bilaterally. As expected,

this led to GFP expression in excitatory projection neurons, but not in BLA interneurons or adjacent GABAergic neurons in the Central Amygdala (CEA; Figure 3,5). Animals carrying the same genotypes but receiving AAV2/5-Camk2α::GFP served as controls. Injections were performed 10 days prior to analysis to allow time for LOF to occur.

To assess the efficacy of this approach for CTA learning, we first examined the necessity of Fos expression. Fos is known to contribute to multiple forms of learning and plasticity (Zhang et al., 2002; Tonegawa et al., 2015) and has previously been implicated in CTA (Lamprecht and Dudai. 1996; Yasoshima et al., 2006). To first confirm effective Cre-mediated recombination, we tested the ability of viral Cre to prevent widespread Fos expression in the BLA immediately following kainic acid-induced seizures. Fos staining performed four hours after seizures revealed strong induction of Fos protein throughout the BLA of control mice injected with the control virus, and diminished Fos expression in mice injected with Cre (Figure 3A,B). We then tested the effect of Fos deletion on CTA in separate animals. Cre-GFP and GFP control AAV's were injected into the BLA of $Fos^{f/f}$ mice, CTA training occurred 10 days later, and long-term memory was tested after an additional 2 days.
The results show that while both groups could form CTA memory, Cre injected mice showed significantly weaker memory (Figure 3C-E). This confirms our ability to manipulate memory by conditional knockout in BLApn neurons. The results refine those of a prior study using antisense injections and germline knockouts (Yasoshima et al., 2006).

Next, we used the same strategy to test the necessity of Stk11 expression in BLApn for CTA memory. BLA of $Stk11^{f/f}$ mice were injected bilaterally with AAV expressing Cre-GFP, or GFP alone. Ten days later both groups of mice were trained for CTA and tested after an additional two days. The results revealed a near complete loss of learning in the Stk11 KO mice (Figure 4A,B), which exhibited no significant reduction in the amount or ratio of saccharin consumed after training. Control mice exhibited significant reductions consistent with a similar degree of learning to that seen in previous control experiments. The differences in the consumption of saccharin during the test day were not attributable to overall reduced drinking, as both group of animals consumed comparable amounts of water 8 hours later (Figure 4C). Taken together, these results indicate that Stk11 expression in BLApn is essential for CTA memory.

Stk11 deletion prior to CTA training can potentially alter multiple memory stages including memory formation and retrieval (Levitan et al., 2016b). Because CTA memory is long-lasting after even a single training session, it is possible to distinguish an effect of Stk11 deletion on memory formation from an effect on retrieval by performing the deletion immediately after training and before testing. We performed the same knockout and control experiments as those described above, but altered our protocol so that Cre and control viruses were injected 2 days following CTA training. Testing occurred 10 days later, allowing the same period for Cre expression and recombination to occur. Since the memory was being tested after a longer period (twelve days vs. two days), we used a

stronger version of the CTA protocol (I.P injection of 2% LiCl instead of 1%; Figure 4-

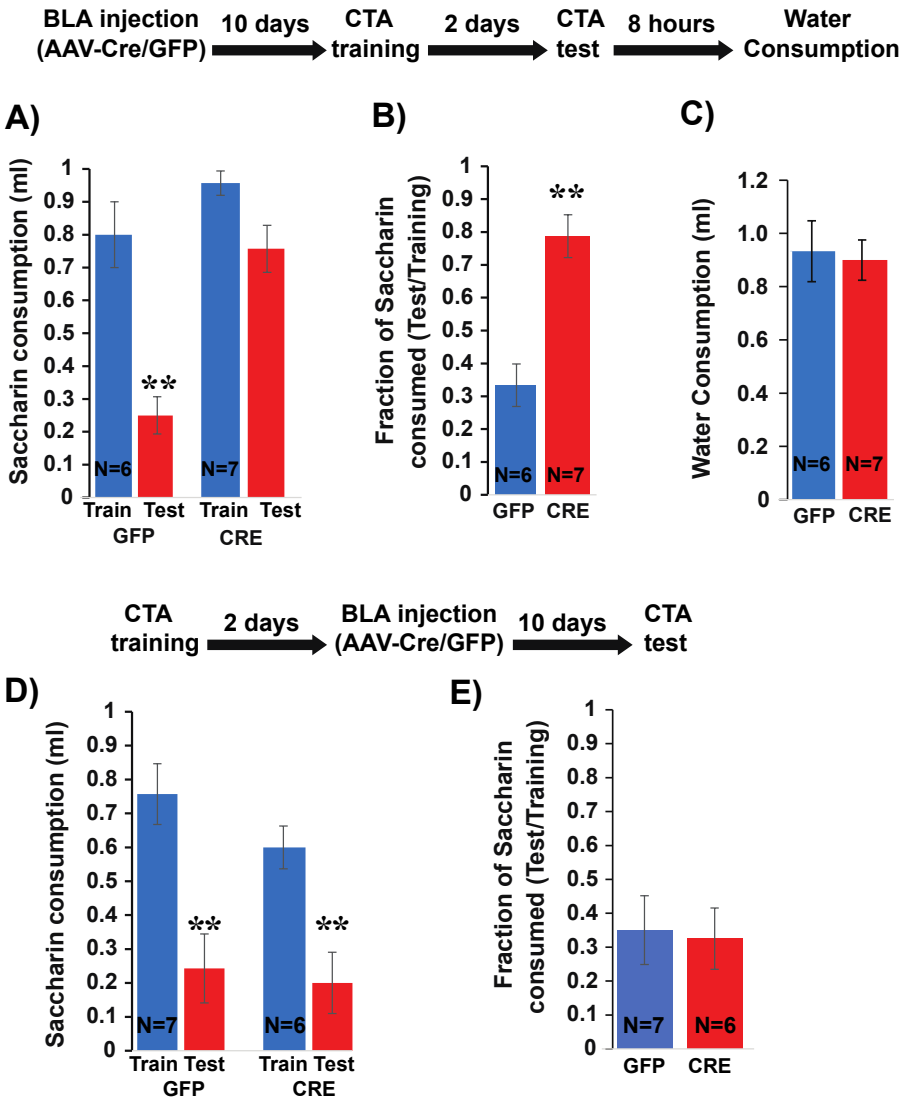


Figure 4. Deletion of Stk11 from BLApn impairs CTA learning. (A) Stk11$^{f/f}$ mice were infected with Cre or control viruses 10 days before CTA training and were tested 48 hours later. Despite significant reduction in saccharin consumption between testing and training sessions in control mice, Stk11 KO animals showed almost no learning. Two-way ANOVA revealed significant group and genotype effects (groups: F(3,22)=19.29; p=0.00002; genotype: F(1,22)=23.43; p=7E-5; as well as a significant interaction: F(1,22)=6.5; p=0.018). Post hoc analysis confirmed that GFP injected mice (N=6) developed strong CTA indicated by the significant reductions (p=0.0001) of saccharin consumption, but Cre injected mice (N=7) failed to significantly reduce their consumption (p=0.259). (**B**) GFP controls consumed only 33% during the test, relative to training, but KO mice consumed 78% and this difference was highly significant (t(11)=-4.91; p=4E-4). (**C**) Reduced saccharin consumption does not reflect overall reduction in drinking measured 8 hours later (t(11)=0.25; p=0.87). **p<0.01. (**D**) Stk11 deletion after long-term memory formation has no effect on CTA retension. Stk11 f/f mice received Cre and control viruses 2 days after CTA training and were tested 10 days later. Two-way ANOVA revealed a significant effect of training

(F(1,22)=21.06; p=8E-6) but no significant effect of genotype (F(1,22)=1.9, p=0.17) or interaction (F(1,22)=0.63, p=0.43). Post hoc analysis confirmed significant reductions in both groups following CTA (GFP: N=7, p=1E-4. Cre: N=6, p=0.006). (**E**) There was no significant difference in CTA intensity as measured by the fraction of saccharin consumed (t=-0.18, p=0.861). **p<0.01.

figure supplement 1).

Both Cre-GFP and GFP injected mice developed CTA (Figure 4D) – there was no significant between-group difference in the intensity of memory, assessed from the ratio of saccharin consumed during the test (Figure 4E). Since the same deletion produces a profound effect when occurring prior to training, this suggests that deletion of Stk11 from BLApn does not affect the retention and retrieval of CTA memory, provided memory was already formed prior to performing the knockout. This argues that Stk11 is required for CTA memory formation.

Stk11 deletion does not impact basal aspects of taste behavior

Since Stk11 deletion must occur prior to CTA training to have an effect on learning, we needed to rule out the possibility that its effect on memory came via disruption of either the responsiveness of BLA neurons to training stimuli, or the output of BLA neurons, which is known to be required for palatability coding within GC.

To assess the responsiveness of BLA neurons to training stimuli, we asked whether training would still activate Fos expression in BLApns after Stk11 deletion. As shown in (Figure 5- figure supplement 1), there is no significant difference between the number of Fos+ neurons following Cre injections and control GFP injections (N=2/group; p<0.05, t-test).

A more rigorous test of BLA function is to determine whether palatability coding in the GC is intact following knockout, since this is known to depend on intact output from the BLA (Piette et al., 2012; Samuelsen et al., 2012; Lin and Reilly. 2012; Lin et al., 2018). If Stk11 deletion disrupts gustatory activation of BLApn or their output to the GC, palatability coding recorded in the GC should be impaired. To test this, Stk11 deletion in BLApn was performed as before and 10 days later multi-channel *in vivo* recordings of GC taste responses were obtained. Recordings were targeted to the ventral part of GC, since BLA projects to these regions (Figure 5A; Haley et al., 2016; Levitan et al., 2019). Palatability coding was assessed with a battery of four tastes with hedonic values ranging from palatable (sucrose and sodium-chloride) to aversive (citric-acid and quinine; for details see Levitan et al., 2019).

Figure 5 shows the results of GC taste responses following Stk11 deletions in BLA. Figure 5B illustrates the peri-stimulus histogram of a representative GC neuron responding to the taste battery. As observed previously in rats and mice (Katz et al., 2001; Sadacca et al., 2013; Levitan et al., 2019), different aspects of taste processing are encoded in firing rates sequentially. In the first five hundred milliseconds or so post-taste delivery, neurons show different firing rates to different tastes (i.e., reflecting taste identity coding), while

later in the responses, differential response rates reflect the hedonic values of tastes (i.e., taste palatability coding). These properties were maintained in the mice studied here: as indicated by the dashed line, the magnitude of the correlation between the neuron's stimulus evoked firing rates and the behaviorally-determined palatability ranking rose significantly only after the first half a second following taste delivery. The averaged correlation magnitudes across neurons from Cre and GFP injected mice are shown in Figure 5C. Inspection of the figure suggests that BLA Stk11 deletion had little effects on GC taste palatability coding; the correlations in both GFP- and Cre-injected groups rise around half a second and peaks at about one second after taste delivery. An ANOVA found no significant group differences across each time bin, suggesting that Stk11 deletion in BLA has little detectable influence on GC taste processing.

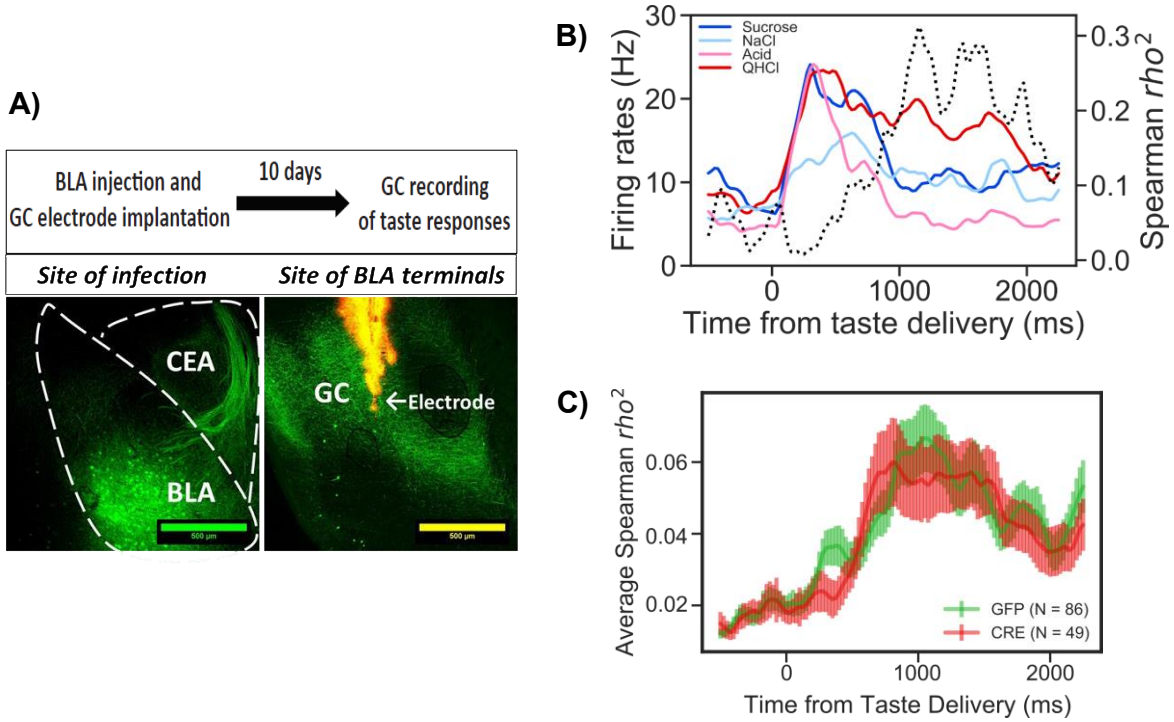


Figure 5. Stk11 deletion in BLApn does not affect taste palatability coding in the GC. (**A**) The BLA of Stk11$^{f/f}$ mice was infected bilaterally with Cre or control viruses. The ventral GC, where BLA projections terminate (Haley et al., 2016) was implanted with a multi-electrode array 10 days later to record GC taste responses to a battery of four tastes differing in their hedonic value, the palatable sucrose and sodium-chloride and the aversive critic acid and quinine (Levitan et al., 2019). Images show BLA injection site (left), and labeled BLA terminals in the ventral GC co-localized with the site of dye-labeled electrodes (right). (**B**) PSTHs (colored lines) from a representative GC neuron in a GFP-injected control mouse that responded significantly to all tastes. Dashed line represents the magnitude of correlation between firing rates and behaviorally measured palatability. (**C**) Correlation coefficients averaged across all recorded units in GFP(control) and Cre- injected mice. As revealed in a 2-way ANOVA, palatability correlations in both groups rise steeply between 800-1000 ms with no significant difference between genotypes ($F(1,133)=0.13$, $p = 0.72$) or interaction ($p =0.99$).

Taken together, our molecular and electrophysiological analyses suggest that the memory deficit observed after Stk11 deletion is unlikely to be due to a deficit in basic taste processing. Rather, Stk11 deletion likely impairs memory by affecting the process of memory formation.

Stk11 or Fos deletion and CTA produce opposing effects on BLA intrinsic excitability

What cellular mechanisms mediate the effects of Fos and Stk11 on memory formation? Obvious candidates abound: for instance, long-term memory is known to be accompanied by changes in both the intrinsic excitability of neurons (Zhang and Linden, 2003; Mozzachiodi and Byrne, 2010) and in the strength of their synaptic connections. Recent studies have shown that the intrinsic excitability of BLApn can be modulated bi-directionally during reinforcement learning, with positive reinforcement leading to increased and negative reinforcement to decreased intrinsic excitability (Motanis et al., 2014).

To determine whether Stk11 might influence memory formation by altering intrinsic excitability, we compared the excitability of BLApn recorded in ex-vivo slices from mutant animals receiving Cre-GFP (Figure 6). The results reveal a marked increase in the intrinsic excitability of BLApn following deletion of Stk11, relative to GFP-only controls. Cre infected neurons had higher firing rates than GFP

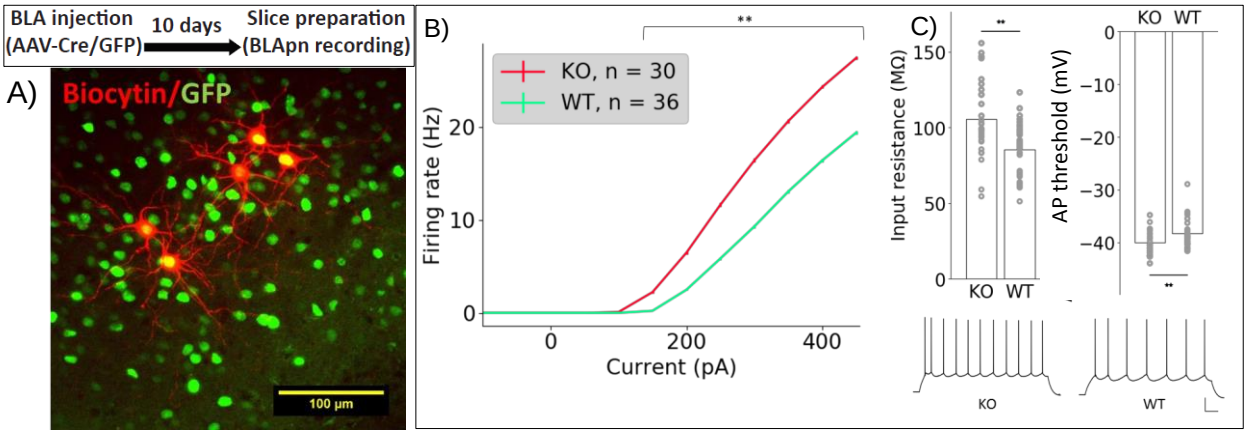


Figure 6. Stk11 deletion in BLApn increases intrinsic excitability. (**A**) Whole-cell recordings obtained from BLApn in *ex vivo* slices of Stk11$^{f/f}$ mice 10 days after injection of Cre or control virus. Stk11 neurons were targeted based on GFP expression and validated post hoc based on Biocytin fills. (**B**) Firing rates plotted against input current (F-I). Error bars (SEM) are too small to be seen for some points. Less current is needed to evoke firing in Stk11-KO neurons compared to Stk11-WT neurons (interpolated rheobase, F(1,64) = 10.63, p = 1.8e-3). The F-I slope is larger for Stk11 knockout neurons (F(1,64) = 23.47, p = 8.4e-6). (C**)** Stk11-KO neurons have increased input resistance (F = 14.41, p = 3.3e-4) and decreased threshold for generating action potential (F = 7.68, p = 7.3e-3). Traces are sample responses to 300 pA current steps. Scale bar: 100 ms, 20 mV.

neurons for any given amount of current input resulting in a steeper slope of the firing rate vs. current (F-I) curve. Threshold firing was initiated at a lower level of current injection

(i.e. the rheobase was lower; Figure 6C). This reflected a higher resting input resistance and a slightly lower voltage threshold. Some other electrophysiological properties also differed (see Table 4) including sag ratios, action potential amplitudes, and the medium and slow afterhyperpolarizations, while others, such as the degree of firing rate accommodation, spike widths and resting membrane potentials did not. Thus, Stk11 deletion from BLApn neurons increases overall intrinsic excitability, most likely by affecting multiple biophysical properties of these neurons.

Since Stk11 and Fos deletion both impair CTA learning, we wondered whether Fos deletion also increases the intrinsic excitability of BLApn. Analysis of a smaller sample of Fos KO neurons (n=11) revealed a similar increase in firing relative to control neurons (n=11; Figure 7A-B). Two-way ANOVA indicated a significant effect of genotype (in addition to the expected effect of current level). No significant differences in input resistance or action potential threshold were detected, reflecting either a difference in the underlying effect of Fos and Stk11 KO or the reduced power of the smaller sample tested.

Finally, we asked whether we could detect an effect of learning itself on neuronal excitability in the BLA. A difficulty with this experiment is that learning may have different effects on different populations of BLApn as evidenced by the fact that some neurons increase Fos expression following training, while others do not (Figure 2-figure supplement 2). In order to separately examine these two populations following training, we made use of the reporter system developed by Reijmers and colleagues, in which elements of the Fos promoter are used to drive the tet transactivator (tTA) which can then prolong and amplify expression of a reporter marking Fos-activated cells, when Doxycycline (Dox) is absent (Reijmers et al., 2007). In this case, we provided the tet-dependent reporter via an AAV (TRE::mCherry) injected into the BLA ten days prior to training. Animals were fed Dox until 24 hours prior to training to limit background activation (Figure 7C). Control experiments showed that CTA training resulted in a greater number of neurons expressing Fos 24 hours after training, compared to home cage controls (Figure 7D). Twenty-four hours following CTA training or taste-only control experiments, recordings were obtained from BLApn in acute slices. Two-way ANOVA revealed that BLApn in the CTA animals have lower input resistance than those in control animals and that Fos-activated neurons have lower input resistance than unlabeled neurons (Figure 7E). Notably, this change is in the opposite direction from that produced by deletions of Stk11 and Fos, two manipulations that impair learning. This suggests that these mutations may interfere with learning by impairing changes in intrinsic excitability that are required for learning to occur.

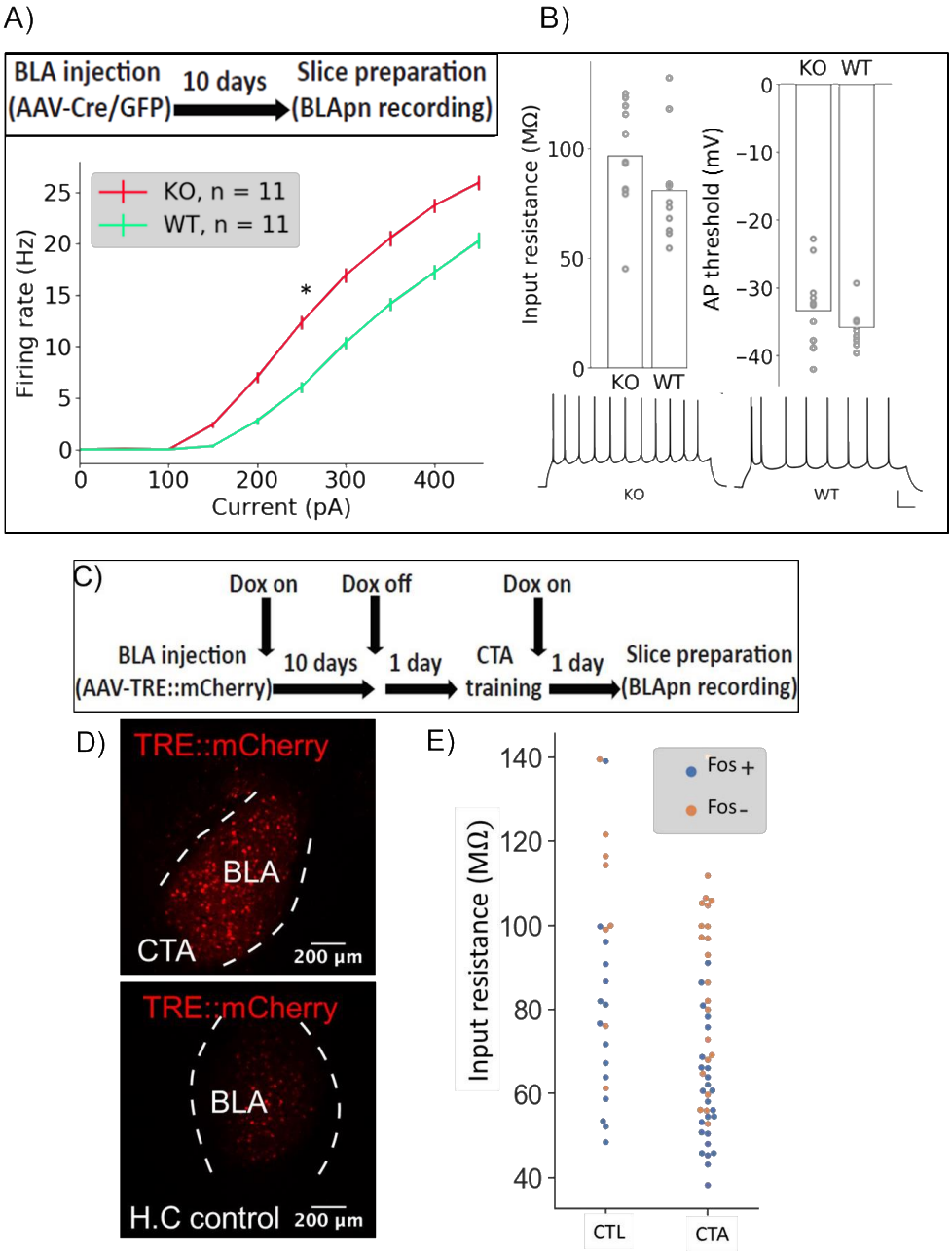


Figure 7. **Fos deletion and CTA have opposing effects on resting input resistance**. (**A,B**) Whole-cell patch-clamp recordings obtained from BLApn in *ex vivo* slices of Fos$^{f/f}$ mice 10 days after injection of Cre or control virus. Fos-KO neurons exhibit increased firing in response to current injection compared to Fos-WT neurons. **(A)** average frequency-current (FI) curves. Two-way ANOVA revealed a significant difference in firing F(1,20) = 22.6, p = 3.8e-6. (**B**) No significant differences in input resistance and threshold for generating action potential between Fos-KO and Fos WT neurons were detected. Sample responses to 250 pA current steps. Scale bar: 100 ms, 20 mV. (**C-E**) CTA training decreases intrinsic excitability. (**C**) Tet-dependent labeling of Fos expressing neurons (Reijmers et al., 2007) during CTA training. Fos::tTa mice were injected with AAV-TRE::mCherry and received food with 40 ppm Doxycycline (Dox) to suppress reporter expression. One day prior to CTA training, Dox was removed. Acute slices were prepared 24

hours following CTA (Saccharin+lithium) or control (Saccharin+saline) training. (**D**) Fos expressing neurons labeled through Tet-off system with mCherry fluorescent reporters in the BLA. (**E**) Input resistances of $Fos^{+/-}$ neurons in the BLA, following CTA training and taste-only control experiments. Two-way ANOVA reveals that neurons from CTA animals have lower input resistance than those from control animals ($F(1,65) = 10.26$, $p = 2.1e-3$) and that Fos^{+} neurons have lower input resistance than Fos^{-} neurons ($F(1,65) = 23.64$, $p = 7.5e-6$). Post-hoc tests show that among Fos^{+} neurons, neurons in CTA animals have lower input resistance ($p = 0.015$), while among Fos^{-} neurons, there was no significant difference between CTA and control animals ($p = 0.1$). Differences between Fos^{+} and Fos^{-} neurons did not reach post-hoc significance in either the CTA ($p = 0.25$) or control animals ($p = 0.12$) considered alone.

Discussion

This study is the first to identify a role for Stk11, a master kinase at the top of the AMP-related kinase pathway, in long-term memory. Using CTA as a behavioral paradigm, we first established a causal requirement for transcription in BLApn during establishment of long-term memory, and then using RNA sequencing, discovered that changes in Stk11 transcription and translation accompanies CTA learning. Cell-type specific conditional knock-out of Stk11 in BLApn revealed it was necessary for CTA memory formation but was dispensable for retrieval, once memories were established. Further investigations suggest that Stk11 may regulate CTA memory through the modulation of intrinsic excitability. Further support for the importance of excitability changes for memory comes from the observation that a second genetic manipulation affecting memory, deletion of the immediate early gene Fos in BLApn also alters excitability, while conversely activation of Fos during learning is associated with reduced excitability.

BLA projection neurons undergo transcription important for CTA learning

It is well established that BLA neurons play a necessary role in CTA learning. Multiple studies confirm that activity in the BLA is required for memory formation and retrieval (Yasoshima et al., 2000; Ferreira et al., 2005; Garcia-de la Torre et al., 2014; Molero-Chamizo, et al., 2017). It is also known, that CTA requires protein synthesis in the BLA (Josselyn et al., 2004), but whether new transcription is also required, and if so, the identities of the required transcripts and the cellular processes they promote were not previously known.

Here we show that BLA projection neurons (BLApn) undergo transcriptional changes important for CTA memory. Inhibiting transcription during CTA training impairs long-term memory tested 48 hours later. Using cell-type specific RNA sequencing, we then identified the transcripts that are altered in expression in YFP-H BLApn four hours after the onset of the unconditioned stimulus. For comparison, we also examined changes in transcript levels in two other populations: YFP-H pyramidal neurons and Parvalbumin-

positive interneurons in gustatory cortex. These profiling experiments provide a resource for future investigations of other molecules potentially involved in CTA in BLA and GC.

Perhaps the strongest case for the necessity of new transcription in BLApn can be made for the immediate early gene Fos. It is well known that Fos transcription and translation are activated by a variety of memory paradigms in the forebrain (Mayford and Reijmers. 2015), and more specifically by CTA in BLApn (Uematsu et al., 2015). The YFP-H neurons studied here, include the majority of BLApn in the anterior portion of the nucleus (Feng et al., 2000; Sugino et al., 2006; Jasnow et al., 2013; McCullough et al., 2016) and we found that many of these neurons express Fos protein (Figure 2-figure supplement 1) and that many project to GC (Figure 5A and Haley et al., 2016), suggesting that they are among the population of BLApn transcriptionally activated by training and participating in the BLA-GC circuit implicated in learning by prior studies (Grossman et al., 2008). Since Fos transcript and protein are short-lived (Spiegel et al., 2014; Chowdhury and Caroni. 2018) the most parsimonious explanation is that training induces new transcription and translation, and that it is these effects that are disrupted by the Fos KO in BLApn (Figure 3). Nevertheless, even for Fos, we cannot rule out the possibility that effects of the knockout preceding training, such as a change in neuronal excitability, are necessary for learning, but that new transcription and translation immediately following learning are not. Resolution of this issue will require new technologies like protein knockout (Clift et al., 2017) with temporal resolution measured in minutes rather than days.

The results of Fos KO selectively in BLApn clarify those obtained in earlier studies addressing the role of Fos in CTA learning. In these studies, Fos was manipulated with infusion of antisense oligonucleotides (Lamprecht et al., 1996; Yasoshima et al., 2006) or via global Fos knockout (which had no effect on CTA; Yasoshima et al., 2006), and loss of memory was attributed to inhibition of Fos in central amygdala (Lamprecht et al., 1996), or to loss in the amygdala as a whole, along with the GC (Yasoshima et al., 2006). In contrast, we show here that knockout restricted to BLApn is sufficient to impair memory.

The requirement for new transcription of Stk11 is less certain since this transcript is presumably less transient than that of immediate early genes. The issue is also unclear because we found that, at the time points measured, the transcript in profiled cells was decreased, while in anatomically sub-dissected portions of BLA, Stk11 protein was increased. Improved temporal and spatial mapping of transcript and protein levels will be required to understand the nature of the discrepancy. Despite this uncertainty, confirmed changes in transcription (Figure 2C) led us to examine the necessity of this gene in learning.

Necessity of Stk11 implicates the AMP-related kinase pathway in learning

Prior studies of CTA and other forms of aversive learning in the BLA have implicated the cAMP-dependent protein kinase, protein kinase C, and extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathways (Johansen et al., 2011; Adaikkan and Rosenblum, 2012), consonant with their established roles in other forms of

forebrain plasticity in the hippocampus and neocortex (Alberini, 2009). Here we raise the possible involvement of another kinase cascade, well studied in the contexts of cell growth, metabolism, cancer and polarity (Shackelford and Shaw. 2009) but hitherto unstudied in the context of learning and memory. That this pathway should have a role in learning is perhaps not shocking given its ubiquity and its previously demonstrated roles in axonal specification and dynamics during development (Barnes et al., 2007; Shelly et al., 2007), synaptic remodeling during aging (Samuel et al., 2014), and its ability to regulate axonal function and presynaptic neurotransmission (Kwon et al. 2016). In addition, Stk11 and downstream AMP-related kinases have been implicated in regulation of glucose metabolism, feeding and obesity through actions in multiple tissues including hypothalamus (Xi et al., 2018; Fei-Wang et al., 2012; Claret et al., 2011). Given the ability of hypothalamus to encode taste palatability and its connectivity with gustatory cortex (Li et al., 2013), it is tempting to speculate that the role of Stk11 signaling pathways in feeding may be functionally related to its role in gustatory learning. Further studies will be needed to distinguish whether the involvement of Stk11 in memory is specific to forms of learning regulating consumption, and whether its role is confined to the basolateral amygdala, or operates in the neural substrates of learning and memory in other structures as well.

Stk11 is a master kinase that regulates the kinase activity of 13 downstream AMP-related kinases by phosphorylating them (Lizcano et al., 2004). While the phosphorylation of Prkaa1/2 (also known as AMPK) by Stk11 is associated with metabolic regulation under cellular stress in response to alteration in nutrients and intracellular energy levels, the phosphorylation of BRSK and MARK are associated with cell polarity during development (Barnes et al., 2007; Shackelford and Shaw. 2009). We found (Figure 2-figure supplement 3) that, while AMPK transcripts (Prkaa1, Prkaa2) have low levels of expression in BLApn, BRSK and MARK transcripts showed higher levels of expression. Furthermore, MARK2 expression was found to be correlated with the expression of Stk11 during CTA learning (Figure 2-figure supplement 3). Recent studies showed that during development, the Stk11/BRSK/MARK cascade mediates the extracellular action of the neuropeptide BDNF and the intracellular actions of the second messengers, cAMP-PKA (Barnes et al., 2007; Shelly et al., 2007; Reviewed in Shelly and Poo. 2011), two well studied and highly conserved signaling pathways also known to be important in multiple memory paradigms, including CTA (Ma et al., 2011; Koh et al., 2002; Koh et al., 2003). Thus, Stk11 might exert its effect on CTA memory via activation of the BDNF and/or PKA signaling pathways in BLApn.

We found that deletion of Stk11 profoundly impaired memory when it occurred prior to training, but not when it occurred two days after training, at which time memory formation and consolidation have already occurred (Alberini, 2009; Gal-Ben-Ari et al., 2012; Bambha-Mukku et al., 2014; Levitan et al., 2016). This suggests that Stk11 expression in BLApn promotes memory formation, rather than memory maintenance or retrieval, but it remains difficult to entirely rule out a role for Stk11 prior to training, rather than during the training process itself. We found that after Stk11 deletion, activation of Fos by training in the BLA was not impaired, implying that at least the initial stages of transcriptional

activation leading to learning are intact. We also know that the ability of the BLA to convey palatability information to the GC remained intact after Stk11 knockout. Prior studies have shown that silencing of BLA neurons or of their axons within the GC impair palatability coding during the late phase of GC gustatory responses. We found that these responses were still present in GC following knockout, implying that this critical function of the BLA for CTA learning remained intact.

Intrinsic excitability of BLApn as a candidate mechanism for Fos and Stk11's effects on CTA memory.

Learning and memory paradigms leading to synaptic plasticity also frequently induce changes in neuronal excitability, and plasticity of intrinsic excitability has been known to accompany classical conditioning in the neocortex, olfactory cortex, hippocampus, amygdala and cerebellum for many decades (for reviews see Zhang and Linden, 2003; Frick and Johnston, 2005; Mozzachiodi and Byrne, 2010; Titley et al. 2017; Debanne et al. 2019).

Here we found that two genetic manipulations of BLApn that impair learning, deletion of Stk11 and Fos, also increase the intrinsic excitability of BLApn. Although there are likely multiple mechanisms involved, part of the increase in excitability reflects an increase in the resting input resistance and a corresponding decrease in the threshold current needed to evoke firing. Conversely, recordings from CTA-trained animals revealed an overall decrease in resting input resistance that was most marked in neurons transcriptionally activated by the training, identified by expression of a Fos promoter-dependent reporter. These results differ in sign from those in most prior studies of intrinsic plasticity, which usually report increased excitability with learning (Zhang and Linden, 2003; Mozzachiodi and Byrne, 2010; Pignatelli et al., 2019). However, they are similar in polarity to those found in BLApn following olfactory fear conditioning, another form of negative reinforcement learning (Motanis et al., 2014). Interestingly, Motanis et al. (2014) also found increases in BLA intrinsic excitability following positive reinforcement learning. Decreased excitability of Fos-activated neurons was also found using an earlier reporter of Fos promoter activation (Yassin et al., 2010). In both the prior studies and in the present study, it remains unclear whether or not the change in excitability is required for learning to occur. Studies in the lateral amygdala have suggested that activation of CREB, increases neuronal excitability (Zhou et al., 2009) and biases neurons for inclusion in subsequent synaptic plasticity and memory recall (Yiu et al., 2014; Rashid et al., 2016). In other circuits, increased excitability may also enhance memory retrieval (Piscatelli et al., 2019). In the present experiments, we found that mutations that cause enhanced excitability impair memory formation and at least for Stk11, the mutation does not affect subsequent memory retrieval provided that time for memory formation has elapsed. Conversely, at least for the portion of excitability tied to resting input resistance, training itself decreased excitability. Just as different forms and mechanisms of synaptic plasticity can accompany learning and sensory experience in synapses linking different parts of the

same circuit (Nelson and Turrigiano, 2008) plasticity of intrinsic excitability is unlikely to be identical in mechanism or function, even across the cell types involved in aversive learning.

In conclusion, we have demonstrated dual roles for the kinase Stk11 in BLApn. Conditional deletion increases their neuronal excitability and at the same time blocks acquisition of CTA memory without altering baseline contributions to taste coding or the ability to undergo the initial stages of transcriptional activation during training. Further work will be needed, first, to map out the intervening steps by which Stk11 affects downstream signaling partners leading to increased excitability and reduced learning, second, to better understand how these pathways intersect with transcriptional activation of immediate early genes, and third, to determine whether, and if so how, cellular changes in excitability and behavioral changes in learning are causally related.

Material and Methods

Subjects

Male and Female mice were used for behavior at age 60-80 days, or for electrophysiology at 25-35 days. Strains: wild-type; WT (C57BL/6J), YFP-H (B6.Cg-Tg(Thy1-YFP)HJrs/J), Stk11$^{f/f}$ (B6(Cg)-Stk11tm1.1Sjm/J, Lkb1fl), Fos$^{f/f}$ (B6;129-Fostm1Mxu/Mmjax), Fos-tTA (B6.Cg-Tg(Fos-tTA,Fos-EGFP*)1Mmay/J) all purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice were placed on a 12-hour light-dark cycle, and given *ad libitum* access to food and water except during training, at which time water access was restricted, while food remained available *ad libitum* (note that animals reliably consume less food when thirsty). All procedures were approved by the Brandeis University Institutional Animal Care and Use Committee (IACUC) in accordance with NIH guidelines.

Surgery

BLA cannulation for RNA synthesis inhibition experiments: WT Mice were anesthetized via ip injections of 100µg ketamine, 12.5 µg xylazine, 2.5 µg acepromazine per gram (KXA). Guide cannulae (23-gauge, 10 mm length) were implanted above the BLA (mm from bregma, AP= -1.4, DV= 4.2, ML= ±3.4) and stabilized using Vetbond and dental acrylic. Stainless steel stylets (30-gage, 10mm) were inserted into the guide cannula to ensure patency. Mice received postsurgical metacam (5 µg/g), penicillin (1500 Units/g) and saline (5 % body-weight) per day for three days and were allowed to recover a total of 7 days prior to training. Twenty minutes prior to CTA training, mice were infused with either 50 ng of actinomycin-D or vehicle control (PBS) bilaterally (in 1 µl over 2 minutes) via infusion cannulae extending 0.5 mm below the guide cannulae to reach the BLA. Each cannula was connected to a 10µl Hamilton syringe on a syringe pump (Harvard Apparatus, Massachusetts, MA, USA).

BLA viral infection: Stk11$^{f/f}$, Fos$^{f/f}$ and Fos-tTA mice were anesthetized with KXA. The skull was exposed, cleaned, and bilateral craniotomies were made at stereotactic

coordinates (AP= -1.4, ML= ±3.4). BLA were injected bilaterally with AAV2/5-Camk2α::Cre-GFP or AAV2/5-Camk2α::GFP (UNC, vector core) for Stk11$^{f/f}$ and Fos$^{f/f}$ mice and AAV2/5-TRE::mCherry for Fos-tTa mice, 10 days prior to CTA training using sterile glass micropipettes (10-20 µm diameter) attached to a partially automated microinjection device (Nanoject III Microinjector, Drummond Scientific). The micropipettes were lowered to 4.3 mm and 4.6 mm from the dura to reach the BLA. At each depth, virus (200 nl) was delivered via 10 pulses of 20 delivered every 10 sec, with 10 min between each injection. Mice were given 7 days to recover from surgery and received meloxicam (2 mg/kg), ip) and penicillin (0.1 ml), subcutaneously, for pain and infection management, respectively.

Conditioned Taste Aversion (CTA): Mice were housed individually with free access to food and maintained on a 23.0 h water deprivation schedule for the duration of training and experimentation. Three days prior to CTA training, water bottles were removed from the cages and water was given twice a day (10 am and 6 pm) for a duration of 30 min. On the day of CTA mice were given 30 min to consume 0.5 % saccharin, which was followed by intraperitoneal (I.P) injection of lithium-chloride (0.15M, 2% of body weight, unless indicated differently) 30 min later. Taste control groups received I.P injection of saline (0.9 % sodium chloride) instead of lithium and lithium control group received lithium injection alone 24 hours following Saccharin consumption. CTA testing: Mice were kept on watering schedule twice a day and 48 hours after CTA training mice received CTA testing which consisted of 30 min consumption of 0.5 % saccharin. 8 hours following testing mice were given 30 min water consumption.

Seizure induction: Fos$^{f/f}$ mice were housed individually with free access to water and food and received BLA viral infection with Cre and control viruses as described above. After 10 days, mice were injected I.P. with 20 mg/kg kainic acid in PBS. Four hours after injection, mice were perfused for Fos immunohistochemistry.

Immunohistochemistry: Mice were deeply anesthetized with an overdose of KXA and perfused transcardially with phosphate buffered solution (PBS) followed by 4% paraformaldehyde (PFA). Brains were post-fixed in PFA for 1-2 day, and coronal brain slices (60 µm) containing the BLA (-1mm to -2.5 mm anterior-poterior axis) were sectioned on a vibratome. Slices were rinsed with PBS and incubated in a blocking solution (PBS/.3%TritionX-100/5% Bovine serum albumin) for 12-24 hours at 4ºC. Blocking solution was removed and replaced with the primary antibody solution which consists of 1:100 c-Fos polyclonal rabbit IgG (SC-52G; Santa Cruz Biotechnology) for 24 hours at 4ºC. After incubation, slices were rinsed using a PBS/.3% Triton X-100 solution followed by the secondary antibody incubation of 1:500 c-Fos Alexa Flour 546 Goat-Anti-Rabbit IgG (H+L) (Life Technologies) and 5% natural goat serum for 12-24 hours at 4ºC. Sections were then rinsed 5-6 times over 90 mins (1XPBS/.3% Triton X-100), counterstained with DAPI, mounted with antifade mounting medium (Vectashield), and viewed by confocal fluorescence microscopy (Leica Sp5 Spectral confocal

microscope/Resonant Scanner). Imaging and quantification were performed blind to experimental group.

Fos quantification and analysis: To minimize systematic bias, Fos counts were performed blind and semi-automatically, using FiJi (University of Wisconsin-Madison; Schindelin et al. 2012). Eight-bit images were binarized and particles smaller than 10 μm^2 were rejected. Each cell count was from a separate animal and was the average of counts from six sections through the anterior, middle and posterior regions of the BLA of both hemispheres.

RNA sequencing experiment: RNA sequencing was performed on YFP^+ BLApn harvested from male YFP-H mouse line (Feng et al., 2000; Sugino et al., 2006; Jasnow et al., 2013; McCullough et al., 2016) which expresses YFP under the Thy1 promoter in the majority of excitatory projection neurons located in the anterior part of the nucleus. The mice underwent CTA training or taste-only controls (n=4/group) and 4 hours following training were subjected to manual cell-sorting, performed as previously described (Sugino et al, 2006; Hempel et al; 2007; Shima et al., 2016) by dissociating 150-200 fluorescently labeled neurons in 300µm thick brain slices and manually purifying them through multiple transfer dishes with the aid of a pipette viewed under a fluorescence dissection microscope. Total RNA was extracted from sorted cells using Pico-pure RNA isolation kit (Thermo fisher). Amplified cDNA libraries are prepared from isolated, fragmented RNA using the NuGen Ovation RNAseq V.2 kit (NuGEN, San Carlos, CA) and followed by purification using the Beckman coulter Genomic's Agencourt RNA Clean XP kit and Zymo DNA Clean & Concentrator. Sequencing adaptors are ligated per Illumina protocols and 50 bp single-ended reads are obtained from Illumina Hi-Seq machine. Libraries sequenced usually results in 25-30 million unique reads using 8-fold multiplexing.

Analysis: Reads are trimmed and then aligned to the mouse genome using TopHat (Trapnell et al. 2012). Sam files are converted to binary format using Samtools and visualized at the sequence level using IGV. Script written in python and R statistical package are used to convert unique reads to gene expression values and to filter genes by relative expression and statistical significance.

qPCR validation: The brains of a separate group of YFP-H mice receiving CTA (taste+lithium, N=4) or taste control (taste+saline, N=4) were harvested 4 hours following the end of the training and subjected to fax sorting. RNA was extracted using pico-pure kit and reverse transcribed to cDNA using iScript cDNA synthesis kit. qPCR was performed on Rotor-Gene qPCR machine using PCR master mix and transcript-specific sets of primers for Fos, Stk11 as target genes and Snap47 as loading control.

Acute slice electrophysiology: Ten days after virus injection, acute brain slices were prepared from P28-35 mice. Animals were deeply anesthetized with KXA and transcardially perfused with ice-cold oxygenated cutting solution containing (in mM): 10 N-methyl-D-glucamine (NMDG), 3 KCl, 1 NaH2PO4, 25 NaHCO3, 20 HEPES, 2 Thiourea, 3 Sodium Pyruvate, 12 N-acetyl-L-cystein, 6 MgCl2, 0.5 CaCl2, 5 Sodium

Ascorbate, 10 Glucose (pH: 7.25 – 7.4, adjusted using HCl). 300 µm coronal slices containing the BLA were cut on a vibratome (Leica), and then recovered for 15 min at 33 °C and for 15 min at room temperature in oxygenated recovery solution containing (in mM): 74 NaCl, 3 KCl, 1 NaH2PO4, 25 NaHCO3, 6 MgCl2, 0.5 CaCl2, 5 Sodium Ascorbate, 75 Sucrose, 10 Glucose, followed by at least another 1 hour at room temperature in oxygenated ACSF containing (in mM): 126 NaCl, 3 KCl, 1 NaH2PO4, 25 NaHCO3, 2 MgCl2, 2 CaCl2, 10 Glucose. During recordings, slices were perfused with oxygenated 34-35 °C ASCF. Target neurons in BLA were identified based on the presence of viral GFP reporter. ACSF included 35 µM d,l-2-amino-5-phosphonovaleric acid (APV) and 20 µM 6,7-dinitroquinoxaline-2,3-dione (DNQX) to block ionotropic glutamate receptors, and 50 µM picrotoxin to block ionotropic GABA receptors. Whole-cell recording pipettes (6 – 8 MΩ) were filled with internal solution containing (in mM): 100 K-gluconate, 20 KCl, 10 HEPES, 4 Mg-ATP, 0.3 Na-GTP, 10 Na-phosphocreatine, and 0.1% biocytin. Recordings were amplified (Multiclamp 700B, Molecular Devices) and digitized at 10 kHz using a National Instruments Board under control of IGOR Pro (WaveMetrics). Resting membrane potentials were adjusted to -70 mV and steady state series resistance was compensated. Series resistance and input resistance were calculated using -5 mV (voltage clamp) or 25 pA (current clamp) seal tests before each trial of recording. In current clamp, the calculated liquid junction potential (-10 mV) was compensated post hoc. Neurons with high series resistance (> 25 MΩ current clamp; >20 MΩ Voltage clamp) or membrane potentials that changed by > 5 mV were excluded. Hyperpolarization activated sag was measured from responses to -100 pA current steps. Action potential threshold, amplitude, afterhyperpolarization (AHP) and full width at half-height were averaged from the 5th-10th action potentials in trials with 10 to 20 Hz firing rates. Action potential threshold is the membrane potential at which the slope first exceeds 10 V/s. Amplitude was the difference between the peak value and the threshold value of the action potentials. Sag ratio is defined as the ratio between maximum voltage and steady-state voltage change with the negative current input. Medium AHP was measured as membrane potential hyperpolarization after the action potentials mentioned above compared to the action potential threshold. The slow AHP was measured from the peak hyperpolarization following positive current steps generating 10-20 Hz firing.

In-vivo recording of GC taste responses

Surgery: Stk11$^{f/f}$ mice were anesthetized and prepared for stereotaxic surgery and viral infection as above. Each mouse was also implanted bilaterally with multi-channel electrode bundles (16 formvar-coated, 25-µm diameter nichrome wires) in GC (1.2 mm Anterior and 0.4mm posterior to Bregma, 3 mm lateral) and a single intraoral cannula (IOC; flexible plastic tubing) was inserted into the cheek to allow controlled delivery of tastes. Electrode bundles were lowered to -2.25 mm and then further lowered by 0.25-0.5 mm to reach dorsal GC, and 0.75-1.00 mm to reach ventral GC (see Figure 5A).

IOC Fluid delivery protocol: Experiments began with three days of habituation to the recording setup and to receiving liquid through the IOC. Liquid consisted of 60 deliveries

of 15-µl aliquots (hereafter, “trials”) of water across 30 min. To ensure adequate hydration, mice were given two 30 min period of access to additional water.

On the following day, recording commenced and water trials were replaced with 4 different taste stimuli: sweet (0.2 M sucrose), salty (0.1 M sodium chloride), sour (0.02 M citric acid), and bitter (0.001 M quinine). A total of 15 trials were delivered for each taste in random order. These tastes and concentrations were chosen because they provided a broad range of hedonic values for palatability assessment. Fluid was delivered through a nitrogen-pressurized system of polyethylene tubes; flow was controlled by solenoid valves via a Raspberry Pi computer (construction details and code available on request from [https://github.com/narendramukherjee/blech_clust].

Taste palatability coding: To determine whether a neuron displays palatability activity, we performed a moving window analysis (window size: 250 ms; step size: 25 ms) to trace the dynamic of taste processing in GC. For each time window, we calculated a Spearman product-moment correlation between the ranked firing rates to each taste and the palatability rankings obtained previously in separate experiments (Levitan et al., 2019).

Statistical analysis: The results are expressed as means ± s.e.m unless otherwise stated. All effects were evaluated using either paired t test or one- or two-way ANOVA test with post hoc t-tests corrected (Bonferroni) for all possible pair-wise comparisons.

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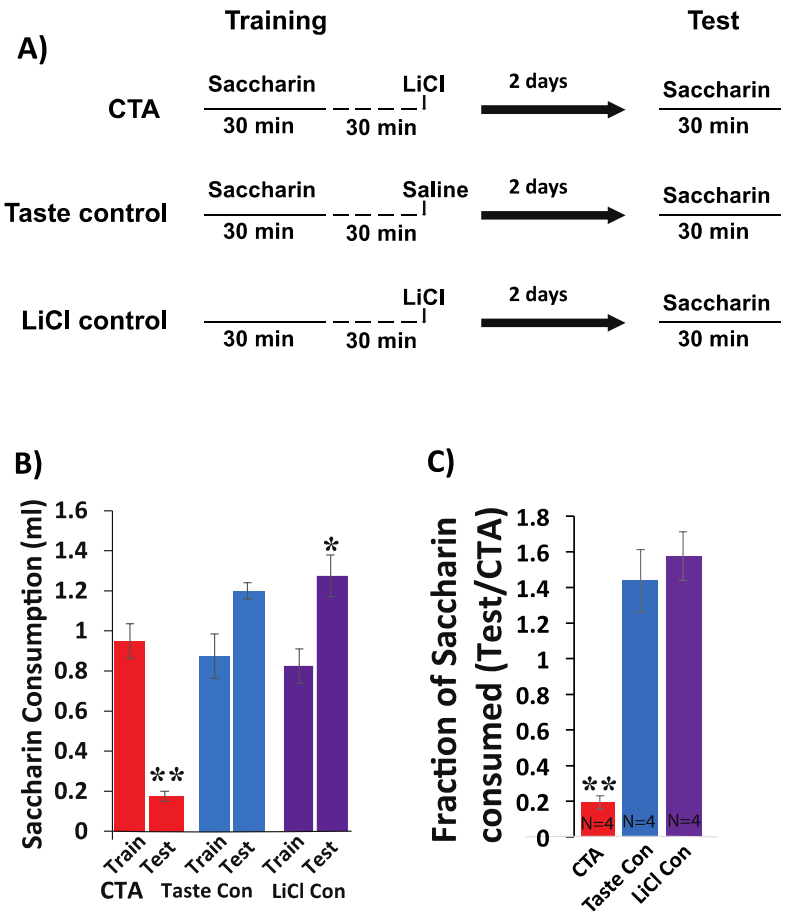

Figure 1-figure supplement 1. Testing CTA in mice. (**A**) Time line showing behavioral paradigm. CTA and Taste control receive 30 min of 0.5% saccharin consumption followed by intraperitoneal injection (I.P) injection of lithium-chloride (LiCl; 0.15 M, 2% body-weigh) or saline 30 min later. The lithium control group receives the same I.P injection of LiCl, but no saline during the training phase. All groups were tested for consumption of saccharin during 30 min exposure 48 hours later. (**B**) Saccharin consumption decreases following LiCl-induced malaise (N=4/group). There was main effect on drinking volume of saccharin between the groups across sessions: $f(4,18)=28.63$, $p=1E-7$ A subsequent pairwise comparison between training and test revealed $p=4E-5$ for CTA group, $p=0.016$ for LiCl group and $p=0.172$ for taste group. (**C**) Measuring CTA memory strength. The strength of CTA memory was determined by quantifying the fraction of saccharin consumption in the test day out of the consumption in the training day. Comparing CTA strength of all groups revealed main effect for difference between groups $f(2,9)=34.08$, $p=6E-5$; $p=2.5E-4$ for CTA vs taste control and $p=0.00011$ for CTA vs LiCl control and $p=1$ for taste control vs LiCl control (On way ANOVA with Bonferroni correction). *$p<0.05$; **$p<0.01$.labeled neurons from the BLA in the YFP-H mouse line (Feng et al., 2000; Sugino et al., 2006; Jasnow et al., 2013; McCullough et al., 2016) which expresses YFP under the Thy1 promoter in a large population of excitatory projection neurons located in the anterior part of the nucleus.

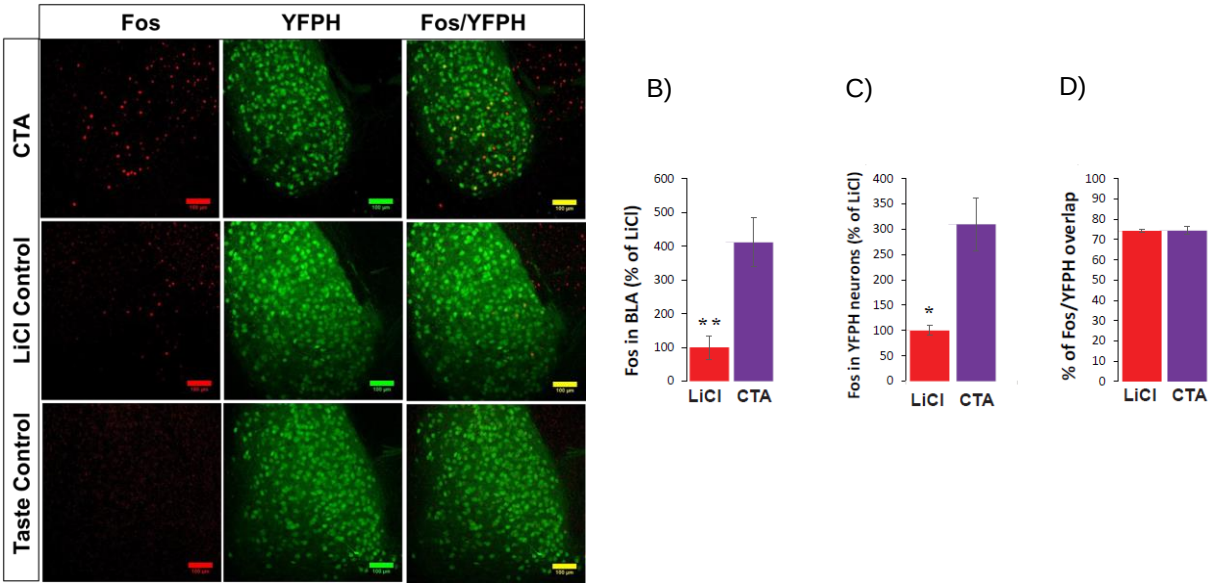

Figure 2-figure supplement 1. CTA increases Fos protein expression in BLApn including those in strain YFP-H. (**A**) Images of YFP$^+$ neurons in the BLA (green) and Fos protein (red) 4 hours following CTA training, LiCl and taste controls. Note that the taste control group has no Fos$^+$ signal and so is not shown in B-D. (**B**) CTA increased Fos expression in BLA relative to the LiCl and taste controls (F(2,11)=21.2; p=4E-4; Post hoc (Bonferroni corrected) difference between CTA and LiCl groups: p=0.003, and p=4E-4 between CTA and taste control groups, N=4/group). (**C**) As in (B), but only for Fos overlapping YFP expression (f(2,11)=23.5: p=0.008, post hoc: CTA and taste control groups: p=4E-4; N=3-4/group). *p<0.05; **p<0.01. (**D**) In both CTA and LiCl control conditions the expression of Fos protein was similarly localized to YFP$^+$ neurons (74.4% for CTA and 74.2% for LiCl control).

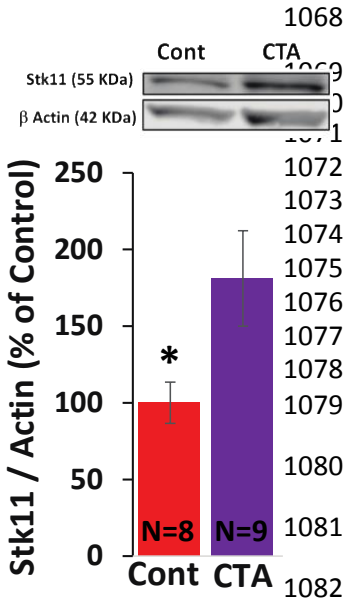

Figure 2-figure supplement 2. Stk11 protein expression following CTA training. YFP-H mice were trained for CTA or received a taste control and 4 hours later the anterior BLA (guided by YFP expression) was subdissected and used for immunoblotting with antibodies raised against Stk11 and actin (as loading control). CTA increased the expression of Stk11 (t(15)=2.28; p=0.037, N=8/9 per group). *p<0.05.

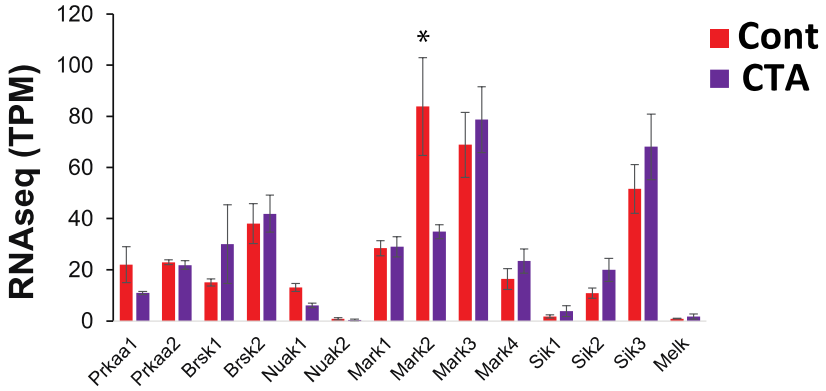

Figure 2-figure supplement 3. BLApn transcript levels of known downstream substrates of Stk11, the members of the AMP-related kinase family (Lizcano et al., 2004). RNA sequencing from BLApn 4 hours following CTA training. Note that Mark2 mRNA expression is reduced in taste control mice relative CTA trained mice (N=4/group; *p<0.05); TPM- transcripts per million.

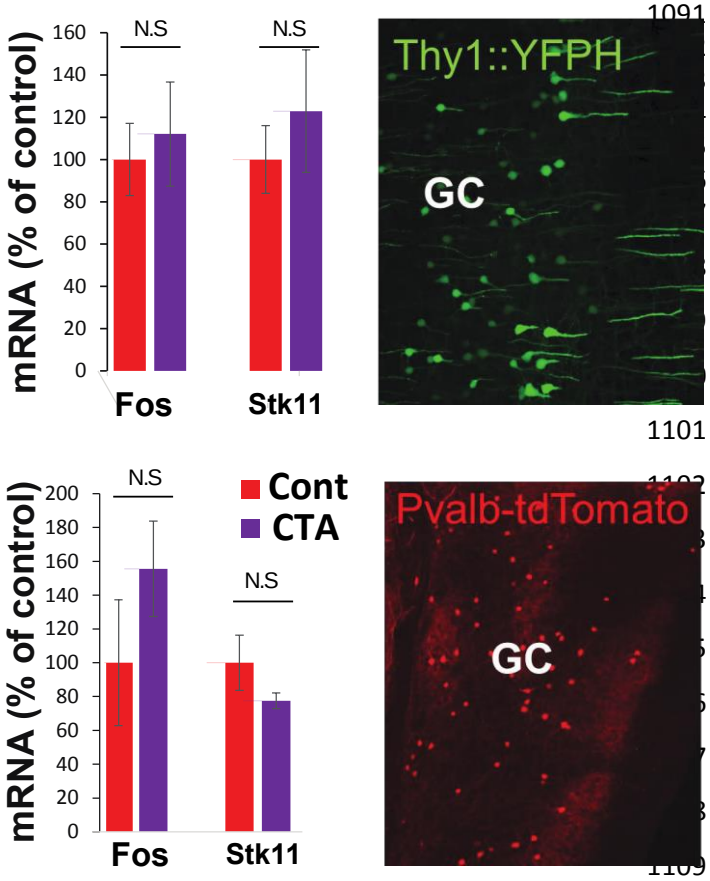

Figure 2-figure supplement 4. RNA sequencing from GC. Top. YFP-H L5 pyramidal neurons and Pvalb-tdTomato positive interneurons in the GC. Fos and Stk11 did not differ significantly between CTA and taste control groups.

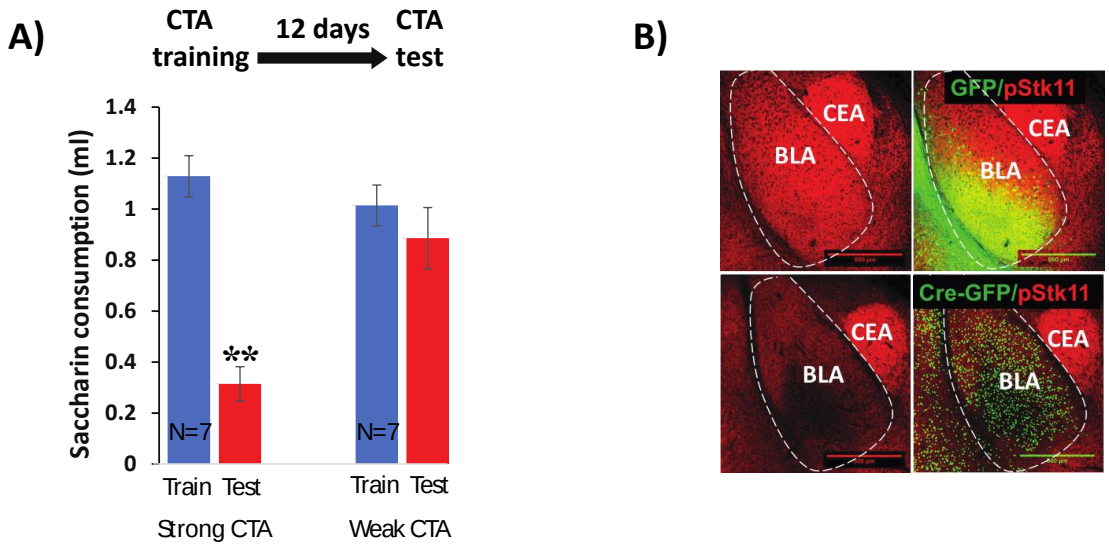

Figure 4-figure supplement 1. (**A**) CTA elicited by a dose of 0.15 M LiCl 1% body weight (Weak CTA) does not last 12 days following training (N=7, t(12)=0.89, p=0.391). On the other hand CTA elicited by a LiCl dose of 0.15M 2% body weight (Strong CTA) does (N=7, t(12)=7.757; p=5.15E-06). (**B**) Conditional knock-out of Stk11 reduces phospho-Stk11 protein expression. Immunostaining for Stk11 protein (phosphorylated at serine 431) was performed 10 days after BLA infection with Cre or control viruses. Scale bar: 500 µm.

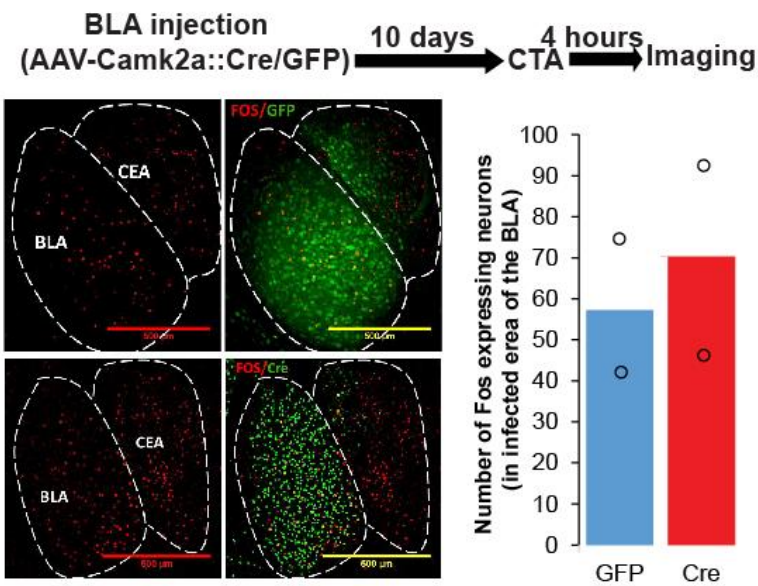

Figure 5- figure supplement 1. CTA induced Fos protein expression persists after Stk11 deletion in BLApn. Fos protein expression in the BLA was measured 4 hours following CTA training in Stk11$^{f/f}$ receiving Cre or control virus injection 10 days earlier.

Table 1. Transcripts in YFP$^+$ BLApn with significantly altered expression 4 hours following CTA. Criteria: 2 ≤ fold change ≤ 0.5, p<0.01, TPM > 30 (TPM=transcript per million).

Symbol	Fold-Change	P-Value	Gene name
Upregulated in CTA vs taste control			
Nptx1	2.02	1.57E-4	Neuronal pentraxin 1
Ric8	2.06	0.0017	RIC8 guanine nucleotide exchange factor A
Mmab	3.66	0.0019	methylmalonic aciduria (cobalamin deficiency) cblB type homolog
1110008F13Rik	2.08	0.0028	RAB5 interacting factor
Kbtbd4	2.09	0.0044	kelch repeat and BTB (POZ) domain containing 4(Kbtbd4)
Nudt21	2.14	0.0077	nudix (nucleoside diphosphate linked moiety X)-type motif 21
Fos	2.64	0.0094	FBJ osteosarcoma oncogene
Magoh	2.72	0.0095	mago homolog, exon junction complex core component
Downregulated in CTA vs taste control			
Surf2	0.40	5.42E-4	surfeit gene 2(Surf2)
Tmem136	0.39	8.69E-4	transmembrane protein 136
Stk11	0.50	0.0011	serine/threonine kinase 11(Stk11)
Kank3	0.28	0.0022	KN motif and ankyrin repeat domains 3
Lrrn1	0.49	0.0024	leucine rich repeat protein 1, neuronal
Trpc1	0.48	0.0032	transient receptor potential cation channel, subfamily C, memb. 1
Prpf6	0.49	0.0045	pre-mRNA splicing factor 6
Tctex1d2	0.49	0.0052	Tctex1 domain containing 2
Gpr108	0.37	0.0056	G protein-coupled receptor 108
Vkorc1	0.39	0.0069	vitamin K epoxide reductase complex, subunit 1
D10Wsu102e	0.50	0.0082	DNA segment, Chr 10, Wayne State University 102, expressed
Tmem107	0.28	0.0089	transmembrane protein 107

Table 2. Transcripts in YFP$^+$ L5 pyramidal neurons in the GC with significantly altered expression 4 hours following CTA. Criteria: 2 ≤ fold change ≤ 0.5, p<0.01, TPM > 30.

Gene Symbol	Fold-Change	P-Value	Gene name
			Transcript Down-regulated CTA vs taste control
Exosc1	0.43	0.008	exosome component 1

Table 3. Transcripts in Pvalb^{+} interneurons in the GC with significant altered expression 4 hours following CTA. Criteria: 2 ≤ fold change ≤ 0.5, p<0.01, TPM > 30.

Symbol	Fold-Change	P-Value	Gene name
Upregulated in CTA vs taste control			
Uprt	3.38	0.001	uracil phosphoribosyltransferase
Snca	2.66	0.001	synuclein, alpha
1810043H04Rik	2.57	0.004	NADH:ubiquinone oxidoreductase complex Assemb.Fact. 8
Dedd	2.47	0.001	death effector domain-containing
Fam149b	2.26	0.007	family with sequence similarity 149, member B
Jazf1	2.12	0.008	JAZF zinc finger 1
Nup54	2.09	0.007	nucleoporin 54
Downregulated in CTA vs taste control			
Dear1	0.006	0.0006	dual endothelin 1/angiotensin II receptor 1
Nt5c3b	0.20	0.002	5'-nucleotidase, cytosolic IIIB
Lrrc16b	0.26	0.010	capping protein regulator and myosin 1 linker 3
Enc1	0.31	0.005	ectodermal-neural cortex 1
Asap2	0.38	0.004	ArfGAP with SH3 domain, ankyrin repeat and PH domain 2
Pacs2	0.38	0.0002	phosphofurin acidic cluster sorting protein 2
Ncan	0.42	0.008	neurocan
uc008jhl.1	0.42	0.010	
Smarcd3	0.45	0.006	SWI/SNF Related, Matrix Assoc.Actin Dep.Reg. Chromatin, Subfamily D, Member 3
Tbce	0.46	0.002	tubulin-specific chaperone E
uc009mzt.1	0.47	0.008	
Anxa6	0.50	0.001	annexin A6

Table 4. Electrophysiological properties of BLApn: Stk11 knockout vs. GFP controls.

Group	Statistics	Resting membrane potential (mV)	Access resistance (mΩ)	Input Resistance (mΩ)	mAHP (mV)	sAHP (mV)	Action potential Amplitude (mV)	Action potential half width (ms)	Action potential threshold (mV)	Sag ratio
KO	mean	-68.23	19.68	105.33	7.44	0.92	74.41	0.80	-40.01	0.13
	S.D.	3.73	2.76	24.88	1.93	0.35	6.58	0.10	2.44	0.04
GFP	mean	-66.96	18.02	85.41	9.71	0.64	78.05	0.79	-38.30	0.15
	S.D.	2.66	3.47	17.65	1.64	0.26	4.39	0.07	2.55	0.03
	F	2.59	4.47	14.41	26.69	13.78	7.17	0.19	7.68	7.48
	p	0.112	0.0383	3.29E-4	2.54E-06	4.32E-4	0.0094	0.664	0.0073	0.008