Amygdala circuits underlying valence-specific behaviors

by

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Abstract

Threatening and rewarding stimuli evoke a set of distinct stereotyped behaviors, which can be categorized as negative and positive valence-related behaviors, respectively. The stereotypic nature of negative and positive valence-related behaviors suggests that threatening and rewarding stimuli engage evolutionarily predetermined neural circuits in the brain. The amygdala is an important mammalian brain region that is activated by negative and positive stimuli and mediates negative and positive valence-related behaviors. The current prevailing circuit model of the amygdala mainly considers negative behaviors and only recently has cell-type specific models have been proposed. Hence, the substrates, genetically distinct neuronal populations, for negative and positive behaviors are not known. The work presented here describes a genetically-defined amygdala circuit model for negative and positive behaviors. Development of a genetic-based circuit model of the amygdala revealed anatomical and genetic circuit motifs that underlie that amygdala circuits that mediate valence-specific behaviors.

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Michele Pignatelli: Performed the patch clamp recordings for the BLA
Sangyu Xu: Performed the operant behaviors for the BLA
Shruti Muralidhar: Performed the patch clamp recordings for the CeA
Xiangyu Zhang: Contributed to performing in situ hybridization and behavioral work for the CeA
Sarah A. LeBlanc: Helped genotype mice and organize colony for CeA study

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PREFACE. PHILOSOPHICAL CONSIDERATIONS

Modern study of the brain mainly originates from two distinct schools of thought, the psychological tradition and the modern biological or neurobiological tradition. In recent days, these two distinct philosophical frameworks for understanding the brain has been hybridized into a field called, neuroscience. Hence, in order to avoid confusion and provide clarity, it is important to distinguish between these two ways of understanding and to articulate the philosophical framework underlying the work presented here.

The psychological tradition posits the mind, the mind being of the subjective and metaphysical in nature. For instance, psychology deals with metaphysical concepts such as emotions, memory, attention, awareness, thought, cognition, consciousness, to name a few. In contrast, the modern biological approach focuses on the objective and material. For instance, modern biology attempts to understand physically definable processes based on a mechanistic description of matter, interaction, localization, and spatiotemporal action.

These two philosophical approaches in brain research are not absolutely independent nor is the validity of such a descriptive framework or superiority of a particular philosophy being necessarily argued for. However, the clear distinction between metaphysical versus material statements should be noted in regards to scientific research on brain and behavior. Thus, the work presented here, although in a topic area highly pursued in the field of psychology, will solely deal with materialistic/biological claims and, though inevitable in terminology, does not take seriously any metaphysical/psychological claims or implications.

I. Genetic ontology of the study of neural circuits and behavior

For this work, the functional units for the study of neural circuits and behavior are genetically-defined neuronal populations. Genetically-defined neuronal populations are
populations of neurons defined by at least one genetic marker within a brain region. Pertaining to this work, the nomenclature will be structured by abbreviation of the brain region followed by the abbreviation of the gene in italics. BLA Rpso2 refers to Rpso2 (gene known as R-spondin 2) expressing neurons in the basolateral amygdala (brain region, BLA). A single genetically-defined neuronal population generally consists of anywhere from $10^1$-$10^6$ neurons in the brain of a mouse.

In regards to ontological basis of function (behavior), psychological or ethological terminology will be adapted for their operational uses, but not their metaphysical implications. Listed are terms and their definitions.

- **valence**: pertaining to negative and positive valence
- **negative valence-related**: a category of behaviors that include defensive behavior, and avoidance.
- **positive valence-related**: a category of behaviors that include self-stimulation, eating, drinking, approach.
- **threatening/negative stimuli**: stimuli that evoke negative valence-relate behaviors
- **rewarding/positive stimuli**: stimuli that evoke positive valence-relate behaviors

II. Epistemology of the study of neural circuits and behavior

What can this genetic-based ontology of neuronal populations tell us about function (behavior)? This ontological framework does not presume that all genetically-defined neuronal populations will necessarily have homogenous function(s) and or neuroanatomical structure(s) nor does it suggest that there must be a genetically defined neuronal population for an arbitrarily definable function (i.e. a unique genetically defined population of neurons that responds to Jennifer Aniston). Rather, this ontological framework presumes or hypothesizes that genetically-defined neuronal populations may have homogenous function(s). In other
words, a genetically-defined neuronal population can serve as the limiting feature to reflect the basic fundamental units and hierarchical organization (categorization) of function (behavior).
Chapter 1. CURRENT PREVAILING AMYGDALA MODEL

I. Introduction

The amygdala is an almond-shaped mammalian brain region that consists of a several divisions—basolateral amygdala (BLA), central amygdala (CeA), lateral amygdala (LA), medial amygdala (MeA), basomedial amygdala (BMA), cortical amygdala (CoA), interacted cells (ITC)^1^3. Early lesion studies have implicated the amygdala in valence-related behaviors and have attempted to attribute distinct roles to distinct amygdala regions^4^-^6^. Although early lesions studies have alluded to a causal role of the amygdala in positive valence-related behaviors, the vast majority of studies have focused on negative valence-related behaviors and a circuit model for negative-valence related behaviors was developed.

II. Current prevailing amygdala circuit model

The circuit model of the amygdala has been developed mainly around the behavioral paradigms known as tone fear conditioning (TFC) and contextual fear conditioning (CFC). In TFC paradigms in mice, the pairing of a conditioned stimulus (CS), a neutral tone, with a negative unconditioned stimuli (US), footshocks, leads to the association of the CS with the unconditioned response (UR), the UR being defensive behavior, which is characterized a mouse in a stereotypic immobile freezing state. Learning is reflected by the conditioned response (CR), which is measured by freezing levels in response to presentation of the tone CS in a neutral context^7^8.

The prevailing amygdala circuit model for TFC proposes that CS and US converge onto the principle cells of the lateral amygdala (LA). The convergence of CS and US onto LA neurons leads coincidental activation of LA neurons, which potentiates the CS inputs onto LA
neurons. As a result, subsequent CS presentation leads to the activation of LA neurons and ultimately leads to the CR. LA neurons are thought to ultimately reach effectors of defensive behavior in the periaqueductal gray (PAG) via the basolateral amygdala (BLA) and central amygdala (CeA)\(^7,8\).

CFC is similar to TFC except that CFC involves presentation of footshocks without any pairing to a tone in a distinct context. The context serves as a CS. The CR is tested in the context where the footshocks occurred. In the amygdala circuit model for CFC, the CS (context) and US converges onto the BLA. BLA neurons are thought to reach the PAG via the CeA\(^7,9\).

This amygdala circuit model describes the circuit underlying CS- and US-induced defensive behavior. Discussions and characterizations of this model in previous works do not describe well or articulate the circuit distinctions between CS- and US-induced defensive behaviors\(^7-10\). However, regardless of the distinctions between CS- and US-induced defensive behaviors, it is believed that such structure, the LA, BLA, CeA, and PAG and the circuit through these structures mediate defensive behaviors.

Recently, a working circuit model for negative and positive behaviors in the amygdala was proposed\(^11,12\). This model utilizes a projection-based definition for populations in the BLA. This model proposes that CeA-projecting and nucleus accumbens (NAc)-projecting BLA neurons are mediators of negative and positive behaviors, respectively. This model is highly consistent with the current prevailing amygdala circuit model and simply builds off an early observation that NAc-projecting BLA neurons mediate positive behaviors\(^7,8,13\).

III. Problems with the prevailing amygdala circuit model

Structurally, the circuit model of the amygdala suggest circuit that from the LA to BLA to CeA to the PAG or LA directly to CeA to PAG mediate defensive behavior\(^7,9\). However, early
findings on the anatomical connectivity between the LA, BLA, CeA do not fully support such model of defensive behavior. For instance, early lesions studies have implicated the anterior BLA, but less so, the posterior BLA in defensive behaviors. Anatomically, the anterior BLA does not directly project to the CeA subdivisions shown to project to the PAG; rather, the posterior BLA mainly projects to the CeA subdivisions shown to project to the PAG. In addition, the LA to BLA or LA to CeA connection has not been convincingly shown to exist; rather the LA appears to mainly send projections to the basomedial amygdala (BMA) and amygdala striatal transition zone (Astr). Hence, such anatomical evidence does not fully falsify such circuit model. Rather, such anatomical inconsistencies, previous to the work presented here, suggested at least some level of structural incompleteness of the amygdala circuit model.

Functionally, the role of LA, BLA, and CeA in defensive behaviors in TFC and CFC paradigms and the contributing factors of such structures has been controversial. Many of the contradictory studies utilized lesions or pharmacological manipulation, which suffer from specificity. Thus, it is not unexpected that such controversies should arise. To solve such technical limitations of earlier perturbation methods, a genetic-based approach offers a method to provide more specificity and clarity on the role of distinct amygdala populations in defensive behaviors. Only recently has such genetic-based approaches been implemented, and as a consequence, has brought more clarity to the amygdala circuit (further discussion in Chapter 3). Thus, in order to better develop an amygdala circuit model for negative and positive behaviors, it is pertinent to implement a genetically-defined framework.

IV. Aims of this work

The aims of the work presented here involve:
1. Identifying and classifying genetically distinct populations in the amygdala
2. Phenotyping genetically distinct populations in regard to valence-related behaviors
3. Deriving a circuit model for negative and positive valence-related behaviors
4. Examine organizing principles underlying distinct amygdala circuits

The main findings of these works have been published and can be read in manuscript form, (Kim et al, 2016) (Kim, Zhang, et al, 2017). In addition to the content based on these two published works, further preliminary works will be described and discussed and a more speculative model will be proposed and broader topics will be discussed.

Chapter 2. GENETICALLY-DEFINED AMYDALA CIRCUIT MODEL

I. Basolateral amygdala
   A. Activity-dependent transcriptional profiling and screening of BLA neurons

   The BLA is a cortical-like brain structure consisting of two-types of nonlaminally organized excitatory pyramidal, magnocellular and parvocellular neurons segregated in the anterior and posterior BLA (aBLA, pBLA), intermingled with populations of genetically-defined interneurons. Two cytoarchitecturally distinct regions have been previously identified, the magnocellular and parvocellular cells. The magnocellular and parvocellular cells are spatially segregated, corresponding to the anterior and posterior BLA (aBLA, pBLA).

   Previous correlative studies have demonstrated that the BLA is activated by negative and positive stimuli, and perturbation studies have demonstrated the necessary role of the BLA in negative and positive behaviors. In more recent studies utilizing optogenetic and genetic approaches, it has been showed that activation of BLA neurons result in the expression of negative and positive behaviors. Therefore, previous evidence suggests
that BLA may be a key site for the regulation of negative and positive behaviors. Yet, despite the critical role of the BLA in valence-related behaviors, it is not established whether the BLA pyramidal neurons that contributes to negative and positive behaviors (negative neurons and positive neurons) are structurally distinct, let alone, genetically distinguishable.

Recent studies demonstrated that BLA neurons, which express the activity-dependent gene, c-Fos, during a negative or positive stimulus, were capable of driving a behavioral response consistent with the valence of the experience\textsuperscript{32}. Therefore, we reasoned that by utilizing a c-Fos-based genetic expression system, molecular profiles of the putative negative and positive neurons within the BLA can be obtained. Activity-dependent molecular profiles of BLA neurons may reveal genetic markers unique to negative and positive neurons.

Genetics-based RNA profiling strategies in mammalian models have involved ectopically expressing epitope-tagged RNA associated proteins or exploiting molecular modifications of RNA-associated substrates\textsuperscript{37-40}. In order to obtain transcriptional profiles, we implemented a strategy involving ectopically expressing an epitope-tagged RNA binding protein, poly(A) binding protein with a c-terminus FLAG tag (PABP-FLAG)\textsuperscript{41}. Two AAV\textsubscript{9} constructs were used, one containing the tetracyclinc-based transcription factor, tTA, under the control of the activity-dependent promoter of c-Fos (AAV\textsubscript{9}-c-Fos-tTA), and the other containing Pabp-flag under the control of the tetracycline response element TRE (AAV\textsubscript{9}-TRE-Pabp-flag). Activation of the c-Fos promoter drives the expression of tTA. In the absence of doxycycline (Dox), tTA binds TRE to induce the expression of PABP-FLAG. PABP-FLAG competes with endogenous PABP and bind the polyA tails of mRNA, which then can be isolated via immunoprecipitation using an anti-flag antibody and A/G coated magnetic beads (Figure 1a).

The putative negative and positive neurons were targeted by exposing male mice to footshocks and a female mouse, respectively. AAV\textsubscript{9}-c-Fos-tTA and AAV\textsubscript{9}-TRE-Pabp-flag were
introduced into the BLA in mice kept on a Dox diet. Once placed off a Dox diet for 2 days, mice were exposed to footshocks or female mouse, then immediately placed back on a Dox diet for 2 days prior to sacrifice. A similar number of BLA neurons were FLAG+ in the shock and female groups, but were greater than mice that were kept in their home cages or kept on a Dox diet (Figure 1b,c,d,e,g-j). In contrast, a greater number of BLA neurons were FLAG+ in the mice that underwent kainic acid-induced seizures compared to the shock or female group (Figure 1b,f-j). This affirms the activity-dependency of the genetic system. Therefore, RNA immunoprecipitation using antibodies against FLAG was performed from the shock and female group. Isolated RNA was reverse-transcribed to cDNA and underwent microarray analysis using Affymetric Mouse 430A chip. After RMA or MAS5 normalization (see Methods), differential gene expression profiles were compared between the shock and female group and were used as the basis of the screen for identifying genetic markers for the putative negative and positive neurons of the BLA (Figure 1k, Figure 8).

Based on previous observations32,34, we hypothesized that the putative negative and positive BLA neurons would be non-overlapping; therefore, we sought to select from our potential list of candidate genetic markers, a single gene candidate, one for each, putative negative and positive neurons. As a corollary, this posits that each of the gene markers would label a subpopulation (<100%) of BLA principle neurons. First, independent of statistical significance, hundreds of genes that were the most enriched in the shock and female groups were individually screened on Allen Mouse Brain Atlas42. 37 genes were selected for single label fluorescent in situ hybridization, of which, 16 probes yielded a quantifiable signals in the -1.0 to -1.6 anterior-posterior (AP) plane of the BLA (Figure 9). Quantification of gene expression in the BLA revealed that the majority of candidate genes were expressed in virtually all BLA neurons (Figure 1l, Figure 9). Several genes were expressed in the majority of
BLA neurons, while, only one gene, *Pp1r1b*, labeled less than 50% of neurons. *Rpso2* was very regionally specific for labelling the BLA as well as had very low variability as a genetic marker. Therefore, Rspontin-2 (*Rpso2*) and Protein phosphatase 1 regulatory subunit 1B (*Pp1r1b*) (encodes for DARPP-32) were selected for further characterization.

B. Characterization of *Rpso2*+ and *Pp1r1b*+ BLA neurons

Double label single molecule fluorescent in situ hybridization (smFISH) and quantification across the anterior-posterior (AP) axis of the BLA (-0.8 to -2.8mm from bregma) revealed that *Rpso2* and *Pp1r1b* labeled spatially segregated population of neurons (Figure 2a-c). Less than 1% of BLA neurons were *Rpso2*+*Pp1r1b*+ (Table 1). *Rpso2*+ and *Pp1r1b*+ BLA neurons are co-labelled with the pyramidal neurons marker, *Camk2a*, and non-overlapping with the inhibitory neuron marker, *Gadi* (Figure 2d-g, Table 1). *Rpso2*+ neurons correspond to magnocellular pyramidal neurons in the anterior BLA (aBLA). In contrast, *Pp1r1b*+ neurons correspond to the parvocellular pyramidal neurons or posterior BLA (pBLA). Double smFISH with a *Camk2a* probe and the combined probes of both the *Rpso2* and *Pp1r1b* showed that virtually all *Camk2a*+ BLA neurons express either *Rpso2* or *Pp1r1b* (Table 1, Figure 10). Therefore, *Rpso2*+ and *Pp1r1b*+ neurons collectively define the entirety of BLA pyramidal neurons.

The electrophysiological and morphological properties of *Rpso2*+ and *Pp1r1b*+ neurons were examined using patch clamp recordings. *Rpso2*+ and *Pp1r1b*+ were targeted by patching magnocellular and parvocellular BLA neurons (Figure 2h). To ascertain genetic identity, *Rpso2*+ and *Pp1r1b*+ neurons were identified by the use of single-cell quantitative polymerase chain reaction (qPCR) from cytoplasmic harvest of patch clamped recorded BLA neurons. Of 37 magnocellular neurons, single cell qPCR yielded 10 *Rpso2*+ and 0 *Pp1r1b*+ neurons; of 38 parvocellular neurons, single cell qPCR yielded 0 *Rpso2*+ and 11 *Pp1r1b*+
neurons (Figure 2i). Soma diameter was larger in Rspo2+ neurons than Ppp1r1b+ neurons; membrane resistance was smaller in Rspo2+ neurons than Ppp1r1b+ neurons; membrane capacitance was larger in Rspo2+ neurons than Ppp1r1b+ neurons (Figure 2j,k). qPCR-confirmed Rspo2+ and Ppp1r1b+ neurons were not significantly different from unconfirmed magnocellular and parvocellular neurons, respectively (Table 1). Taken together, Rspo2+ and Ppp1r1b+ BLA neurons defined spatially segregated, genetically, morphologically, and electrophysiological distinct cell-types.

If Rspo2+ and Ppp1r1b+ neurons represent negative and positive neurons of the BLA, respectively, then stimuli that elicit valence-specific behaviors may differentially activate the aBLA and pBLA. Mice were exposed to the stimuli used to identify BLA gene markers—shocks or female mice—and were sacrificed 90 minutes later. c-Fos+ neurons were quantified separately in the aBLA and pBLA (defined by cytoarchitectural boundaries) by measuring the total number of c-Fos+ neurons per section at intervals across the AP axis (Figure 3a-c, Figure 11). The relative c-Fos expression, measured by the number of c-Fos+ neurons in the aBLA or pBLA as a percentage of total c-Fos+ BLA neurons, was significantly greater in the aBLA in response to footshocks compared to exposure to a female mice or control mice, which received no stimulus in a context (Figure 3d). Conversely, relative c-Fos expression was significantly greater in the pBLA in response to female mice compared to exposure shock or control, which were exposed to a neutral context (Figure 3d). In response to valence-specific olfactory stimuli—2,3,5-Trimethyl-3-thiazoline (TMT), or peanut oil—relative c-Fos expression was significantly greater in the aBLA in response to TMT compared to exposure to a neutral odor benzaldehyde (BA) or peanut oil, while relative c-Fos expression was significantly greater in the pBLA in response to peanut oil compared to exposure to a BA or TMT (Figure 3e). In response to valence-specific gustatory stimuli—quinine (bitter), water, sucrose (sweet)—
relative c-Fos expression was significantly greater in the pBLA in response to water and sucrose water compared to mice that received no water or quinine water (Figure 3f). In contrast, no significant difference was observed in relative c-Fos expression between exposure to quinine water (which did not elicit much water drinking) compared to no water, as well as between sucrose water and water (Figure 3f). Overall, the aBLA is recruited by stimuli that elicits negative behaviors (shocks, TMT), while the pBLA is recruited by stimuli that elicits positive behaviors (female, water, sucrose, peanut oil).

Double smFISH was performed to directly assess the expression of c-Fos in Rspo2o or Ppp1r1b+ BLA neurons in response to valence-specific stimuli (stimuli that will be used in subsequent behavioral experiments). Shocks significantly increases c-Fos expression in Rspo2o (Figure 3g,k), but not in Ppp1r1b+ neurons (Figure 3h,l), compared to context (Figure 3g, h, m, n). In contrast, administration of water significantly increases c-Fos expression in Ppp1r1b+ (Figure 3j,p), but not in Rspo2o neurons (Figure 3i,o), compared no water (Figure 3i, j, q, r, Figure 12c,d). These data suggest that negative and positive information is represented by genetically-defined populations of neurons in the BLA that are spatially segregated; Rspo2o neurons, which define the aBLA, represent negative valence, while Ppp1r1b+ neurons, which define the pBLA, represent positive valence.

Valence-specific activation of Rspo2o and Ppp1r1b+ neurons posits that these populations may be necessary for valence-specific behaviors; therefore, the effects of inhibiting these BLA populations were performed in a fear and reward conditioning paradigm. Rspo2o and Ppp1r1b+ neurons were genetically targeted using Rspo2-Cre and Cartpt-Cre mice, respectively. Ppp1r1b+ BLA neurons are accessible by Carpt-Cre mice (Figure 12), and hereafter, virus-injected Cartpt-Cre mice will be referred to using “Ppplrlb”. Light-activated inhibitory ion channel, eArch3.0, was expressed in Rspo2o (Rspo2-Arch) and Ppp1r1b+
(Ppp1r1b-Arch) BLA neurons using a Cre-dependent viral vector (AAV5-EF1α-DIO-eArch3.0-eYFP) bilaterally targeted to the BLA of Rspo2-Cre and Cartpt-Cre mice, respectively. Control mice (Rspo2-eYFP, Ppp1r1b-eYFP) received a viral vector lacking eArch3.0, (AAV5-EF1α-DIO-eYFP) (Figure 4a,1,m).

On day 1 of contextual fear conditioning, mice received green light, bilaterally targeted to the BLA, during footshocks (Figure 4b). Rspo2-Arch mice displayed reduced levels of freezing in response to footshocks compared with Rspo2-eYFP mice. Ppp1r1b-Arch mice displayed similar levels of freezing compared to Ppp1r1b-eYFP mice. On day 2, mice were tested in the context without shock or light stimulation. Reduction of freezing was observed in Rspo2-Arch mice compared to Rspo2-GFP mice, while, similar levels of freezing was observed in Ppp1r1b-Arch mice compared to Ppp1r1b-eYFP mice (Figure 4c). Thus, Rspo2+, but not Ppp1r1bp+, BLA neuronal activity is critical for freezing to shock stimuli and for the association of a context to freezing behavior.

Reward conditioning took place in an operant conditioning chamber, where water was dispensed contingent on a nose poke following an external light cue (Figure 4d). Green light was bilaterally delivered into the BLA simultaneously with the presentation of water. Rspo2-Arch and Rspo2-eYFP mice displayed similar levels of nose pokes and cue-reward association (z-score of time spent in the reward port during cue period, see Methods). In contrast, Ppp1r1b-Arch mice displayed reduced levels of nose pokes and cue-reward association compared to Ppp1r1b-eYFP mice (Figure 4e). Thus, Ppp1r1b+, but not Rspo2*, BLA neuronal activity is critical for reward-seeking behavior and for the association of a conditioned stimulus to appetitive behavior.

Next, the effects of activating these BLA neurons were assessed. Light-activated excitatory ion channel, ChR2, was expressed in Rspo2+ (Rspo2-ChR2) and Ppp1r1b+
(Ppp1r1b-ChR2) BLA neurons using a Cre-dependent viral vector (AAV5-EF1α-DIO-ChR2-eYFP) unilaterally targeted to the BLA of Rspo2-Cre and Cartpt-Cre mice, respectively. Control mice (Rspo2-eYFP, Ppp1r1b-eYFP) received a viral vector lacking ChR2, (AAV5-EF1α-DIO-eYFP) (Figure 4a,n,o).

On day 1 of the optogenetic freezing test, mice were placed in a neutral context while receiving blue light stimulation (Figure 4f, see methods). Rspo2-ChR2 mice displayed greater levels of freezing compared to Rspo2-eYFP mice, while Ppp1r1b-ChR2 and Ppp1r1b-eYFP mice displayed similar levels of freezing (Figure 4g). On day 2, mice were returned to the context and freezing was measured without shock. Rspo2-ChR2 mice displayed greater levels of freezing compared to Rspo2-eYFP mice, while Ppp1r1b-ChR2 and Ppp1r1b-eYFP mice displayed similar levels of freezing (Figure 4g). Thus, Rspo2*, but not Ppp1r1b*, BLA neurons are capable of eliciting freezing, which can be conditioned to a neutral context.

On day 1 of the optogenetic self-stimulation test, mice were placed in an operant conditioning chamber in which blue light stimulation was administered when poking into a nose port (Figure 4h). Ppp1r1b-ChR2 mice displayed greater number of pokes compared to Ppp1r1b-eYFP mice, while Rspo2-ChR2 and Rpso2-eYFP mice displayed similar number of pokes. On day 2, mice were returned to the operant condition chamber in which no light stimulation was delivered. Ppp1r1b-ChR2 mice displayed greater number of pokes compared to Ppp1r1b-eYFP mice, while Rspo2-ChR2 and Rspo2-eYFP mice displayed similar number of pokes (Figure 4i). Thus, Ppp1r1b+, but not Rspo2*, BLA neurons are capable of eliciting self-stimulation and support reward conditioning.

In real-time optogenetic place preference test (Figure 4j), Rspo2-ChR2 mice spent less time in the light-stimulated side compared to corresponding controls, while Ppp1r1b-ChR2 mice spent more time in the light-stimulated side compared to corresponding controls (Figure
Therefore, *Rspo2* BLA neurons are sufficient to elicit place aversion while *Ppp1r1b* BLA neurons are capable of eliciting place preference.

C. Antagonism between *Rpso2* and *Ppp1r1b* BLA neurons

*Rpso2* and *Ppp1r1b* neurons drive opposing behaviors; therefore, we examined whether these two types of neurons contribute to the antagonistic control of emotional behaviors and memories. For this purpose, we examined the behavioral effects of optogenetically activating *Rpso2* or *Ppp1r1b* neurons during the presence of valence-specific stimuli. On day 1 of contextual fear conditioning, ChR2-expressing mice received bilateral blue light stimulation in the BLA during footshocks (Figure 5a). *Rspo2*-ChR2 and *Rspo2*-eYFP mice displayed similar levels of freezing in response to footshocks, while *Ppp1r1b*-ChR2 mice displayed lower levels of freezing than *Ppp1r1b*-eYFP mice (Figure 5b,c). On day 2, conditioned freezing was assessed by returning mice to the context without footshock or light stimulation. Similar to day 1, no difference in freezing was observed between *Rspo2*-ChR2 and *Rspo2*-eYFP mice, while less freezing was observed in *Ppp1r1b*-ChR2 mice compared to *Ppp1r1b*-eYFP mice (Figure 5b,c). Thus, activation of *Ppp1r1b* BLA neurons is capable of disrupting freezing to footshocks and the association of a conditioned contextual stimulus with footshocks.

In reward conditioning, ChR2-expressing mice received blue light stimulation during reward delivery (Figure 5d). *Rspo2*-ChR2 displayed reduced levels of nose pokes and cue-reward association compared to *Rspo2*-eYFP mice, while *Ppp1r1b*-ChR2 and *Ppp1r1bp*-eYFP mice displayed similar levels of nose pokes and cue-reward association (Figure 5e,f). Thus, activation of *Rspo2* BLA neurons is capable of disrupting reward-seeking behaviors and the association of a conditioned stimulus with a reward.
Although *Rspo2* and *Ppp1r1b* neurons antagonize behaviors elicited by stimuli of the opposing valence, behavioral antagonism may be result of interference by downstream circuits rather than by direct interactions between these two neuronal populations. Therefore, the effect of optogenetic activation of one of the two neuronal populations on the activation of the other in response to valence-specific stimuli was measured using *c-Fos*. In *Ppp1r1b-ChR2* mice, which received blue light stimulation in the presence of footshocks, *c-Fos* was increased in *Ppp1r1b* neurons and decreased in *Rspo2* neurons compared to *Ppp1r1b-eYFP* mice (Figure 5g,h,i). In water-deprived *Rspo2-ChR2* mice, which received blue light stimulation during the consumption of water, *c-Fos* was increased in *Rspo2* neurons and decreased in *Ppp1r1b* neurons compared to *Rspo2-eYFP* mice (Figure 5j,k,l). Thus, *Ppp1r1b* and *Rspo2* neurons are capable of reducing the activity elicited by valence-specific stimuli in the opposite neuronal population.

Antagonism observed at the behavioral and *c-Fos* activation level of valence-specific BLA neurons was further examined at the microcircuit level by combining patch clamp recording with optogenetic stimulation of valence-specific neurons. The functional relationship between *Rspo2* and *Ppp1r1b* neurons were examined by combining patch clamp recordings with optogenetic stimulation of cell type-specific axons (Figure 6a-d). Patch clamp recordings of *Rspo2* and *Ppp1r1b* neurons revealed distinct intrinsic physiological properties (Table 1). Therefore, the postsynaptic cell target was recognized based on a combination of anatomical position, soma size, and intrinsic electrophysiological properties (Figure 6m,n).

Electrophysiological recordings of *Rspo2* neurons in response to optogenetic stimulation of *Ppp1r1b-ChR2* fibers and recordings of *Ppp1r1b* neurons in response to stimulation of *Rspo2-ChR2* fibers resulted in inhibitory post-synaptic potentials (IPSPs) (Figure 6e-h,k,l). The probability of connections of *Rspo2* to *Ppp1r1b* and vice versa were 100% and 100%.
inhibitory, respectively (Figure 6i,j), of which 25% of connections of \textit{Ppp1r1b} to \textit{Rspo2} BLA neurons and 17% of connections of \textit{Rspo2} to \textit{Ppp1r1b} were both inhibitory and excitatory (Figure 6i,j). These data suggest that these two populations interact predominantly through mutual inhibition.

D. \textit{Rspo2} and \textit{Ppp1r1b} BLA neuron projections

The distinct projection targets of the \textit{Rspo2} and \textit{Ppp1r1b} neurons may reveal divergent brain structures that mediate negative and positive behaviors. Therefore, retrograde tracing from putative projection targets was examined using cholera toxin subunit b (CTB). CTB targeted to the capsular nucleus of the central amygdala (CeC), revealed CTB$^+$ neurons primarily in the aBLA (Figure 7a,c,d). CTB targeted to the lateral/medial nucleus of the central amygdala (CeL/CeM), resulted in CTB$^+$ neurons distributed along the lateral side of the pBLA (Figure 7a,e,f). CTB targeted to the nucleus accumbens (NAc), resulted in CTB$^+$ neurons distributed along the medial side of the BLA, spanning the posterior end of the aBLA to the posterior end of the pBLA (Figure 7a,g,h). Dual-labelled CTB targeted to the prelimbic (PL) and inframlimbic (IL) cortex resulted in spatially segregated distribution of CTB$^+$ neurons in the BLA—PL-CTB$^+$ neurons primarily in the aBLA, IL-CTB$^+$ neurons primarily in the pBLA (Figure 7b,i,j). smFISH of \textit{Rspo2} or \textit{Ppp1r1b} probe in CTB injected mice, revealed that CeC-CTB$^+$ BLA neurons are 96% \textit{Rspo2} and 4% \textit{Ppp1r1b}; CeL/CeM-CTB$^+$ neurons are 6% \textit{Rspo2} and 94% \textit{Ppp1r1b}; NAc CTB$^+$ neurons are 30% \textit{Rspo2} and 70% \textit{Ppp1r1b} (Figure 7k-n, Table 1).

For anterograde characterization, ChR2-eYFP$^+$ fibers in \textit{Rspo2-ChR2} and \textit{Ppp1r1b-ChR2} mice was examined. In \textit{Rspo2-ChR2} mice, dense fibers were found in the CeC, NAc, PL, but not in the CeL, CeM, or IL (Figure 7o). In \textit{Ppp1r1b-ChR2} mice, dense fibers were found in the CeL, CeM, NAc, and IL but not in the CeC or PL (Figure 7p). Together, CTB retrograde tracing and anterograde characterization of projection fibers suggest that \textit{Rspo2} distinctly
project to the CeC and PL, \textit{Ppp1r1b}+ neurons distinctly project to the CeL, CeM, and IL, while both \textit{Rspo2}+ and \textit{Ppp1r1b}+ BLA neurons both project to the NAc.

II. Central amygdala

The central amygdala (CeA), like the BLA, is involved in the control of valence-related behaviors\textsuperscript{3,44}. As previous described (I. Basolateral amygdala), the BLA contains two spatially segregated, genetically distinct populations of cortical-like excitatory pyramidal neurons—Protein phosphatase 1 regulatory subunit 1B+ (\textit{Ppp1r1b}+ also known as DARPP-32) parvocellular neurons and R-spondin 2+ (\textit{Rspo2}+) magnocellular neurons\textsuperscript{1-3,25,29,43}. BLA \textit{Ppp1r1b}+ neurons elicit appetitive behaviors, inhibit defensive behaviors, and send projections to the lateral (CeL) and medial (CeM) nucleus of the CeA. BLA \textit{Rspo2}+ neurons elicit defensive behaviors, inhibit appetitive behaviors, and send projections to the capsular (CeC) nucleus of the CeA\textsuperscript{25}. The CeA consists of GABAergic striatal medium spiny-like neurons, and similar to the BLA, is critical for appetitive and defensive behaviors\textsuperscript{3,44,45}.

The CeA has been mainly studied on the basis of its role in innate and learned fear-related behaviors\textsuperscript{4,6,8,46-50}. Cell-type specific studies have shown evidence for the involvement of several genetically defined CeA neurons in aversive behaviors such as defensive responses and anxiogenesis\textsuperscript{51-58}. However, despite early evidence suggesting the involvement of the CeA in appetitive behaviors\textsuperscript{59,62} and more recent activation studies demonstrating a modulatory role of the CeA in appetitive behaviors\textsuperscript{63-65}, how appetitive behavior integrates into a structural and functional model of amygdala has yet to be established. For instance, a genetically defined population of CeA neurons that are positive mediators of appetitive behavior has not been identified. Given the strong projections from BLA \textit{Ppp1r1b}+ parvocellular neurons to the CeL and CeM\textsuperscript{25}, we hypothesized that the CeA may contain neurons that are positive mediators of...
appetitive behavior. Therefore, we first examined the role of genetically distinct CeA populations in both appetitive and defensive behaviors. Furthermore, although the BLA and CeA are both important for appetitive and defensive behaviors, it is not known how BLA $Ppp1r1b^+$ parvocellular and BLA $Rspo2^+$ magnocellular neurons are connected to genetically defined CeA neurons. Therefore, we also examined the connectivity from genetically defined neurons from the BLA to CeA. Lastly, cytoarchitectural studies suggest that the BLA and CeA are structurally similar to the cortex and striatum, respectively. For this reason, we explored the expression pattern of striatal markers in the CeA to examine if there exists, an organizing principle in the BLA to CeA circuit that is common with the cortex and striatum.

A. Identification of genetically distinct CeA neurons

Gene expression studies have shown a wide range of genetic diversity within the CeA. To identify genetically distinct populations in the CeA, we performed single molecule in situ hybridization (smFISH) (ACDBio RNAscope) of genes that are expressed in the CeA—calcitonin receptor-like ($Calcr$), corticotropin-releasing hormone ($Crh$), serotonin receptor 2a ($Htr2a$), neurotensin ($Nts$), protein kinase C-δ ($Prkcd$), somatostatin ($Sst$), and tachykinin 2 ($Tac2$). It should be noted that for the CeC only the anterior region was quantified due to the ambiguity of the CeC in the posterior CeA (Figure 24). $Calcr$ and $Prkcd$ were expressed in the CeC; $Crh$, $Htr2a$, $Nts$, $Prkcd$, $Sst$, and $Tac2$ were expressed in the CeL; $Htr2a$, $Nts$, $Sst$, and $Tac2$ were expressed in the CeM (Figure 24A and 24B). Overlap of the expression of $Calcr$, $Crh$, $Nts$, $Prkcd$, $Sst$, and $Tac2$ was examined in the CeA. In the CeC, $Calcr$ labeled 89% of $Prkcd$ neurons, while $Prkcd$ labeled 56% of $Calcr^+$ neurons (Figure 17A and 17D). $Calcr^+$ neurons were non-overlapping with $Sst$ (which delineates the CeL) in the CeA (Figure 17I), further indicating that $Calcr^+$ neurons reside in the CeC rather than the CeL. In the CeL, several of the genes had significantly high levels of overlap (>50%) (Figure 17B, 17J-1O).
Using the levels of overlap among pairs of genes, hierarchical clustering revealed 3 major clusters. The first containing Prkcd, the second containing, Sst, and third containing Crh, Tac2, and Nts (Figure 17B). In the CeM, Crh, Nts, Sst, and Tac2 were minimally (<15%) overlapping (Figure 17C). Htr2a expression was weak and difficult to quantify with a high degree of confidence in the CeA. However, based upon a few double label smFISH, several Htr2a+ neurons were found to coexpress Crh, Prkcd, Nts, Sst, and Tac2 in the CeL and Nts, Sst, Tac2 in the CeM (Figure 24D). Therefore, Htr2a may be less specific for labelling a distinct population compared to the other genes. Collectively, characterization using these sets of genes revealed as many as 8 or 9 genetically and regionally distinguishable populations of neurons in the CeA.

For this study, we have chosen to study 7 major neuronal populations of the CeA, distinguishable based on gene expression and region—CeC Prkcd+, CeL Prkcd+, CeL Crh+Nts*Tac2i, CeL Sst+, CeM Nts*, CeM Sst+, CeM Tac2* (Figure 17P). To determined what proportion of neurons CeL Prkcd+, CeL Crh+Nts*Tac2i, and CeL Sst+ neurons constitute in the CeL and what proportion of neurons CeM Nts*, CeM Sst+, and CeM Tac2* neurons constitute in the CeM, the expression of Nts, Prkcd, Sst, and Tac2 with glutamate decarboxylase 1 (Gad1), a marker of inhibitory neurons, was examined. Prkcd, Sst, and Tac2 collectively labeled 96% of Gad1+ neurons in the CeL, whereas Prkcd+, Sst+, and Tac2+ neurons were 100% Gad1+ (Figure 24C). Nts, Sst, and Tac2 collectively labeled 95% of Gad1+ neurons in the CeM, whereas Nts+, Sst+, and Tac2+ were 100% Gad1+ (Figure 24C). This suggests that CeL Prkcd+, CeL Crh+Nts*Tac2i, and CeL Sst+ neurons constitute the majority of GABAergic neurons in the CeL and that CeM Nts+, CeM Sst+, and CeM Tac2+ neurons constitute the majority of GABAergic neurons in the CeM.

B. Characterization of genetically distinct CeA neurons
The CeA has been shown to participate in defensive behaviors and appetitive behaviors\(^4,6,47,59,61\). Therefore, each of the 7 CeA neuronal populations was subjected to optogenetic stimulation experiments to assess these functions. CeC \textit{Prkcd}\textsuperscript{+} and CeL \textit{Prkcd}\textsuperscript{+} neurons were targeted using a Cre-dependent channelrhodopsin (ChR2) virus injected into the CeC and CeL, respectively, of the \textit{Prkcd-Cre} mice; CeL \textit{Sst}\textsuperscript{+} and CeM \textit{Sst}\textsuperscript{+} using injections into the CeL and CeM, respectively, of the \textit{Sst-Cre} mice; CeL \textit{Crh\textsuperscript{+}Nts\textsuperscript{+}Tac2\textsuperscript{+}} neurons using injections into the CeL of \textit{Crh-Cre} mice; CeM \textit{Nts}\textsuperscript{+} neurons using injections into the CeM of \textit{Nts-Cre} mice; CeM \textit{Tac2\textsuperscript{+}} neurons using injections into the CeM of the \textit{Tac2-Cre} mice (Figure 25A). Control mice were Cre\textsuperscript{-} mice that underwent identical surgical procedures in the CeC (\textit{Prkcd-Cre}\textsuperscript{-} mice), CeL (\textit{Sst-Cre}\textsuperscript{-} mice), and CeM (\textit{Tac2-Cre}\textsuperscript{-} mice). In the optogenetic freezing test, mice were placed into a neutral conditioning chamber, where no light stimulation occurred during the 0- to 3-min period (OFF) followed by 20-Hz blue light stimulation during the 3- to 6-min period (ON). Stimulation of CeC \textit{Prkcd}\textsuperscript{+} neurons elicited freezing (Figure 18A), measured by an increase in freezing during the ON period compared to the OFF period, while stimulation of CeL \textit{Prkcd}\textsuperscript{+}, CeL \textit{Crh\textsuperscript{+}Nts\textsuperscript{+}Tac2\textsuperscript{+}}, CeL \textit{Sst}\textsuperscript{+}, CeM \textit{Nts}\textsuperscript{+}, CeM \textit{Sst}\textsuperscript{+}, and CeM \textit{Tac2\textsuperscript{+}} neurons did not elicit freezing (Figure 18B-18G). It should be noted, that stimulation of CeM \textit{Tac2\textsuperscript{+}} neurons elicited immobility-like behaviors, but did not reflect stereotypical freezing. Rather, this immobility-like behavior due to activation of CeM \textit{Tac2\textsuperscript{+}} neurons coincided with biting behavior in 5 out of 8 mice. Cre\textsuperscript{-} control mice did not demonstrate light-induced freezing (Figure 25B). Following the freezing test, mice were tested for self-stimulation. In the optogenetic self-stimulation test, mice were freely allowed to poke into two nose ports for a single session of 60 min where only one port delivered 20-Hz blue light stimulation. Activation of CeL \textit{Sst}\textsuperscript{+}, CeL \textit{Crh\textsuperscript{+}Nts\textsuperscript{+}Tac2\textsuperscript{+}}, CeM \textit{Nts}\textsuperscript{+}, CeM \textit{Sst}\textsuperscript{+}, and CeM \textit{Tac2\textsuperscript{+}} neurons resulted in self-stimulation, based on an increased in nose pokes in the light stimulated port (ON) compared to the
unstimulated port (OFF) (Figure 18C-18G), while CeC Prkcd+ and CeL Prkcd+ neurons did not result in self-stimulation (Figure 18A-18B). Cre- control mice did not demonstrate light induced self-stimulation (Figure 25B). These optogenetic stimulation experiments demonstrate that CeL Sst+, CeL Crrh*Nts*Tac2+, CeM Nts+, CeM Sst*, and CeM Tac2* neurons are capable of driving appetitive behaviors, CeC Prkcd+ neurons are capable of driving defensive behaviors, while CeL Prkcd+ neurons are not capable of driving either appetitive or defensive behaviors.

Distinct CeA neurons are capable of eliciting defensive behaviors and appetitive behaviors. Therefore, to assess how these populations are activated by external stimuli, the expression of the activity-dependent gene, Fos, was measured in each of the 7 CeA neuronal populations in response to five different conditions associated with defensive or appetitive behaviors. Wild-type mice were exposed to either footshocks or no footshocks; contextual fear extinction recall or contextual fear recall without fear extinction; ad libitum food or no food in food-deprived mice; ad libitum water, quinine water, or no water in water-deprived mice; injection of cholecystokinin (CCK, an agent that induces satiety) or saline in mice 30 min prior to sacrifice (see Methods). The percentages of Fos labelling within each of the CeA populations were measured. Fos expression was increased in CeC Prkcd+ neurons in response to footshocks compared to corresponding control (Figure 19A) and contextual fear recall compared to contextual fear extinction (Figure 19B). Fos expression was also measured in CeC Calcrt+ neurons in response to footshocks and was found to be significantly increased in CeC Calcrt+ neurons (Figure 26B). This is consistent with the observation that CeC Prkcd labels a subpopulation of CeC Calcrt+ neurons (Figure 17A). Fos expression was increased in CeL Prkcd+ neurons in response to contextual fear extinction recall compared contextual fear recall (Figure 19B). Fos expression was increased in CeL Prkcd+, CeL Sst*, CeL Crrh*Nts*Tac2+, CeM Nts*, CeM Sst*, and CeM Tac2* neurons in response to ad libitum food.
and ad libitum water compared corresponding controls (Figure 19C-19D). Fos expression was increased in CeL Prkcd+ in response to CCK (Figure 19E). Fos expression was increased in CeC Prkcd+ in response to quinine in water-deprived mice (Figure 19D). These results suggest that CeC Prkcd+ neurons are activated by threatening stimuli and aversive tastes. CeL Prkcd+ neurons are activated by states of suppression of defensive behaviors and stimuli that suppress appetitive behaviors. CeL Sst+, CeL Crh+Nts+Tac2+, CeM Nts+, CeM Sst+, and CeM Tac2+ neurons are activated by stimuli that elicit appetitive behaviors.

Differential Fos expression in the CeA in response to food, water, and footshock suggests there may be differential involvement of these 7 CeA populations in feeding, drinking, and freezing behaviors. Therefore, to dissociate the contribution of distinct CeA populations in these behaviors, each of the 7 CeA neuronal populations was subjected to a series of optogenetic inhibition experiments (see Methods). For targeting these populations, a Cre-dependent archaerhodopsin (Arch) virus was injected in the same fashion as in the previous optogenetic stimulation experiments, while littermate Cre-mice that underwent identical procedures were used as controls. When presenting ad libitum food in food-deprived mice during a 10-min trial, inhibition of each of the CeA populations—CeC Prkcd+, CeL Prkcd+, CeL Sst+, CeL Crh+Nts+Tac2+, CeM Nts+, CeM Sst+, and CeM Tac2+—did not result in any significant changes in the duration of feeding behavior compared to corresponding controls (Figure 20A-G). Subsequently, when presenting ad libitum water in water-deprived mice during a 5-min trial, inhibition of CeL Prkcd+ neurons resulted in an increase in drinking behavior (Figure 20B), inhibition of CeL Sst+ and CeL Crh+Nts+Tac2+ resulted in a decrease in drinking behavior (Figure 20C and 20D), and inhibition of CeC Prkcd+, CeM Nts+, CeM Sst+, and CeM Tac2+ did not result in changes to drinking compared to corresponding controls (Figure 20A, 20E-G). Finally, mice underwent contextual fear conditioning in which CeA neurons were
inhibited during the presentation of 3 footshocks in a contextual fear conditioning protocol (Day 1) and were subsequent reexposed to the conditioning chamber 24 hr later with no light inhibition (Day 2). Inhibition of CeC Prkd+ neurons resulted in a minor, but statistically significant, reduction in freezing on Day 1 and subsequently reduced levels of freezing on Day 2 compared to corresponding controls (Figure 20A). Although there is a trend for increased freezing levels from inhibition of CeL Prkd+ neurons as previously reported, inhibition of any of the CeA populations did not affect freezing on Day 1 or 2 compared to corresponding controls (Figure 20B-G). Together, these data suggest that CeL Prkd+ and CeL Prkd (CeL Sst and CeL Crt*Nt*Tac2+) neurons have opposing roles on drinking, CeC Prkd+ neurons are required for defensive behaviors, while inhibition of any one of these populations does not affect feeding behavior in food-deprived mice.

Inhibition of CeM Nts+, CeM Sst+, or CeM Tac2+ neurons did not affect feeding, drinking or freezing behaviors. Therefore, the effects of collectively inhibiting all three CeM populations were assessed. CeM Nts+, CeM Sst+, and CeM Tac2+ neurons collectively constitute almost all CeM Drd1+ neurons (Figure 27E). Thus, a Cre-dependent Arch virus was injected into the CeM of Drd1-Cre mice and underwent the same procedures as the previous inhibition experiments (Figure 20). Inhibition of CeM Drd1+ neurons resulted in reduction of feeding behavior in food-deprived mice, reduction of drinking in water-deprived mice, and showed no differences in freezing in response to footshocks compared to corresponding controls (Figure 27). Therefore, CeM Drd1+ neurons are critical for both feeding and drinking behavior and suggest that CeM Nts+, CeM Sst+, and CeM Tac2+ neurons may collectively function to mediate feeding and drinking.

C. CeA projections to the midbrain
The CeA is one of the output structures of the amygdala and is known to project to several brain regions including the periaqueductal gray (PAG) and it has been widely hypothesized that PAG-projecting CeA neurons mediate freezing. Therefore, the relationship between PAG-projecting CeA neurons and genetically distinct CeA populations was examined. The retrograde tracer, cholera toxin subunit B (CTB), was injected into the PAG. This resulted in CTB+ neurons in the CeL and CeM that were Sst+, Tac2+, and Nts+ (Figure 28A-C). In contrast, CTB retrograde tracing from the PAG did not label Prkcd+ neurons in the CeL (Figure 28A-C) which is consistent with previous reports. Using ChR2 mice (Figure 18), anterograde tracing fibers were found in the PAG of CeL-Sst-ChR2, CeL-Crh-ChR2, CeM-Nts-ChR2, CeM-Sst-ChR2, and CeM-Tac2-ChR2 mice, but not CeC-Prkcd-ChR2 and CeL-Prkcd-ChR2 mice (Figure 28D). These data suggest that CeL Sst+, CeL Crh+Nts+Tac2+, CeM Nts+, CeM Sst+, and CeM Tac2+ neurons project to the PAG, while CeC Prkcd+ and CeL Prkcd+ neurons do not project the PAG. These findings suggest that, independent of whether or not appetitive functions are necessarily mediated by transmission from the CeA to PAG, PAG projections may not be a unique structural feature of CeA neurons that mediate defensive function.

D. Cell-type specific BLA-to-CeA connectivity

Genetically defined BLA pyramidal neurons that are capable of driving defensive and appetitive behaviors send projections to the CeA. Therefore, the anatomical relationship between the BLA and CeA was examined using cell-type specific monosynaptic rabies tracing in the 7 CeA neuronal populations. To target these populations, AAV helper virus, AAV-synP-FLEX-sTpEpB, (construct containing Cre-dependent TVA, Rabies G-protein, eGFP) was injected in the same fashion as in the optogenetic stimulation experiments, incubated for 3 weeks prior to injection of the G-deleted rabies mCherry virus, then sacrificed 1 week later.
Tissues were labeled using antibodies against PPP1R1B to determine the BLA cell-type—
$Ppp1r1b^+$ or $Ppp1r1b^-$ (as a measure of $Rspo2^+$ neurons because $Rspo2^+$ and $Ppp1r1b^+$
constitute virtually all BLA excitatory neurons $^{25}$) (Figure 21D-J). Monosynaptic tracing from
CeL $Prkcd$, CeL $Sst^+$, CeL $Crh^+Nts^+Tac2^+$, CeM $Nts^+$, and CeM $Sst^+$, and CeM $Tac2^+$ neurons
resulted in retrograde labeled neurons in the BLA that are predominantly PPP1R1B$^+$ (Figure
21A). In contrast, monosynaptic tracing from CeC $Prkcd^+$ neurons resulted in retrograde
labeled neurons in the BLA that were PPP1R1B$^-$ but also PPP1R1B$^+$ (Figure 21A). These results
suggest that BLA $Rspo2^+$ neurons directly project to CeC $Prkcd^+$ neurons, while BLA $Ppp1r1b^+$
neurons directly projects to all CeA neurons that were examined. It should be noted that the
connectivity from BLA $Ppp1r1b^+$ neurons to CeC neurons was not observed in our previous
study $^{25}$. This apparent contradiction may be explained by the fact that we previously targeted
the dorsal portion of the CeC for retrograde labelling, whereas CeC $Prkcd^+$ neurons reside
more ventrally in the CeC (Figure 24B).

Monosynaptic tracing experiments were further analyzed using an antibody against
PKC-$\delta$ for determining the retrograde labeled CeA cell-type ($Prkcd^+$ or $Prckd^+$). Monosynaptic
tracing from CeL $Prkcd$, CeL $Sst^+$, CeL $Crh^+Nts^+Tac2^+$, CeM $Nts^+$ and CeM $Tac2^+$ neurons
resulted in several (>5 neurons/section) retrograde labeled neurons in the CeC that were PKC-$\delta^+$
and PKC-$\delta^-$ (Figure 5B). Monosynaptic tracing from CeC $Prkcd$, CeL $Sst^+$, CeL
$Crh^+Nts^+Tac2^+$, CeM $Nts^+$ and CeM $Tac2^+$ neurons resulted in several (>5 neurons/section)
retrograde labeled neurons in the CeL that were PKC-$\delta^+$ neurons (Figure 21C). Monosynaptic
tracing from CeM $Nts^+$ neurons resulted in several (>5 neurons/section) retrograde labeled
neurons in the CeL that were PKC-$\delta^-$ neurons (Figure 21C). Although no leaky viral expression
was found (Figure 28E), due to possibilities of differential tropism across mouse lines,
nonspecific targeting (Figure 28F), and unmeasurable leakiness, there may be more or less
connectivity than demonstrated by these rabies experiments. Nevertheless, using an arbitrary threshold for retrograde labelling (>2.5 neurons/section in the BLA and >5 neurons/section in the CeA), a model of the BLA to CeA connectivity was generated (Figure 31A). Overall, these results demonstrate monosynaptic connections from BLA Ppp1r1b+ neurons to the CeA neurons that mediate appetitive behaviors and a monosynaptic connection from BLA Rspo2+ neurons primarily to CeC neurons that inhibit several of the CeA neurons that are capable of eliciting appetitive behavior.

The BLA to CeA connectivity was further assessed using slice patch clamp recordings in conjunction with cell-type specific optogenetic stimulation of the BLA. Cre-dependent ChR2 virus was injected into the BLA of Rspo2-Cre and Cartpt-Cre mice for targeting BLA Rspo2+ (Rspo2-ChR2) and BLA Ppp1r1b+ (Ppp1r1b-ChR2) neurons, respectively. Patch clamped CeA neurons were recorded in response to ChR2 stimulation and were genetically identified using biocytin filling followed by immunohistochemistry against PKC-δ in the CeL or cytosolic harvesting followed by quantitative polymerase chain reaction (qPCR) in the CeC and CeM. The electrical properties were also measured of these neurons (Figure 29A-D). Blue-light stimulation in Ppp1r1b-ChR2 slices resulted in monosynaptic excitation, determined by latency (Figure 29F-29I), in 100% of neurons in the CeC, 100% of neurons in the CeL, and 97% of neurons in the CeM. Among these neurons, disynaptic inhibition was also observed following monosynaptic excitation in 57% of neurons in the CeC, 53% of neurons in the CeL, and 60% of neurons in the CeM (Figure 22A). Connections with only disynaptic inhibition were observed in 3% of neurons in the CeM. Based on genetic marker-based confirmation (Figure 29E and 29J), monosynaptic excitatory connections were observed in CeC Prkcd+, CeL Prkcd+, CeM Nts+, CeM Sst+, and CeM Tac2+ neurons (Figure 22C and 29J). Blue light stimulation in Rspo2-ChR2 slices resulted in monosynaptic excitation in 52% of neurons in the CeC, 11% of neurons in the
CeL, and 0% of neurons in the CeM. Among these neurons, disynaptic inhibition following monosynaptic excitation was observed in 58% of neurons in the CeC and 50% of neurons in the CeL. Connections with only disynaptic inhibition were observed in 11% of CeL neurons (Figure 22B). Based on genetic marker-based confirmation (Figure 29E and 29K), monosynaptic excitatory connections were observed in CeC Prkcd+ neurons as well as a CeL Prkcd neuron (Figure 22D and 29K). These results show that BLA Rspo2+ neurons mainly innervate CeC Prkcd+ neurons, while making minimal connections to the CeL and CeM. In contrast, BLA Ppp1r1b+ neurons innervate CeC Prkcd+, CeL Prkcd+, CeM Nts+, CeM Sst+, and CeM Tac2+ neurons. In addition, 100% connectivity from BLA Ppp1r1b+ neurons to CeL neurons suggests that BLA Ppp1r1b+ neurons also innervate CeL Sst+ and CeL Crrh*Nts*Tac2+.

Although the identity of the neurons that mediates the polysynaptic inhibitory responses cannot be identified from these experiments, these functional experiments confirm the results of the rabies tracing experiments (Figure 21) and connectivity model of the BLA to CeA connectivity (Figure 31A).

E. Striatal marker expression in the CeA

The BLA and CeA are cytoarchitecturally similar to the cortex and striatum, respectively. In the cortex, the direct pathway promotes movement and is characterized by intratelencephalic-type (IT-type) cortical neurons innervating dopamine receptor 1+ (Drd1+), dynorphin+ (Pdyn+), and substance P+ (Tac1+) striatonigral medium spiny neurons. The indirect pathway inhibits movement and is characterized by pyramidal tract-type (PT-type) neurons innervating dopamine receptor 2+ (Drd2+) and enkephalin+ (Penk+) striatopallidal medium spiny neurons. Therefore, the expression of Drd1, Drd2, Pdyn, Penk, and Tac1 was examined in the CeA to examine how the BLA to CeA circuit is organized compared to the direct and indirect pathway of the cortex and striatum. Drd2 and Penk were expressed in the CeC. Pdyn
and Penk were expressed in the CeL. Drd1, Drd2, Pdyn, Penk, and Tac1 were expressed in the CeM (Figure 30A and 30B). Overlap of the expression of these striatal markers was examined in the CeA (Figure 23A-B, 30C). In the CeC, Penk and Drd2 were highly (>90%) overlapping (Figure 23A). In the CeL, Penk labeled 78% of Pdyn neurons, while Pdyn labeled 35% of Penk neurons (Figure 23B). In the CeM, hierarchical clustering using overlaps of the genes showed 2 major clusters. The first was Drd2, which was minimally overlapping (<15%) with the other markers. The second contained, Drd1, Pdyn, and Penk, which all moderately overlap with one another (~30-60%) except for Pdyn neurons, with which most or all (>90%) coexpressed Drd1 (Figure 23C). Using these sets of striatal markers, 7 or 8 genetically and regionally distinct populations can be identified. It should be noted that Drd2 expression in the CeM is questionable. Though Drd2 is expressed within the bounds of the CeM, its sparseness and expression pattern may reflect expression in the ventromedial extent of the CeC rather than the CeM. Further assessment using CTB retrograde tracing from the PAG resulted in no detectable CTB+ neurons that were Drd2* (data not shown). Thus, like CeC neurons, Drd2* neurons likely do not project to the PAG and therefore these CeM Drd2* neurons may reflect CeC Drd2* neurons.

The relationship between striatal markers and the CeA markers that were behaviorally and functionally characterized was examined using smFISH (Figure 23D-H). In the CeC, Drd2 and Penk were coexpressed in the majority (>80%) of Prkcd+ neurons (Figure 23D), while Prkcd was coexpressed in a subset (~40%) of Drd2 and Penk neurons (Figure 23E). In the CeL, Penk labeled the vast majority (>90%) of Prkcd+ neurons and a subpopulation of Sst+ neurons. Pdyn labeled virtually all (97%) of Sst+ neurons and the majority (70-80%) of Nts*, Tac2*, and Crh+ neurons (Figure 23D). In the CeL, hierarchical clustering using overlaps between all the genes revealed 3 major clusters, the first containing Penk and Prkcd, the
second containing $P_{dyn}$ and $Sst$, and the third containing $Crh$, $Nts$, and $Tac2$ (Figure 23F). In the CeM, $Drd1$ was coexpressed in the majority (>85%) of $Nts^*$, $Sst^*$, and $Tac2^*$ neurons. $P_{dyn}$ and $Penk$ were expressed in a subpopulation of $Nts^*$, $Sst^*$, and $Tac2^*$ neurons, with slightly more expression of $P_{dyn}$ in $Sst^*$ neurons and more expression of $Penk$ in $Tac2^*$ neurons (Figure 23D). In the CeM, heirarchical clustering using overlaps between all the genes revealed 2 majors clusters, the first containing $Drd2$, the second containing $Drd1$, which can be further clustered into 3 groups—$Penk$ and $Tac2$, $Drd1$ and $Nts$; $P_{dyn}$, $Sst$ and $Tac1$ (Figure 23G). Results of gene expression in the CeA were summarized in a model (Figure 31B). These results show that CeC $Prkcd^*$ neurons express the striatal markers for the corticostriatal indirect pathway, $Drd2^*$ and $Penk^*$, while CeM $Nts^*$, CeM $Sst^*$, and CeM $Tac2^*$ neurons mainly express the striatal markers for the corticostriatal direct pathway, $Drd1$, $P_{dyn}$ and $Tac1^{75,76}$.

III. Summary of Findings

A. Summary of behavioral and anatomical findings

Several genetically distinct populations were characterized in the BLA and CeA for negative and positive valence-related behaviors. Neurons that positively participate in negative valence-related behaviors included:

1. BLA $Rspo2^*$
2. CeC $Prkcd^*$

Neurons that positively participate in positive valence-related behaviors included:

1. BLA $Ppp1r1b^*$
2. CeL $Sst^*$
3. CeL $Crh^*Nts^*Tac2^*$
4. CeM $Nts^*$
5. CeM Sst^+ 
6. CeM Tac2^+

Neurons that do not positively promote negative or positive valence-related behaviors, but suppress negative or positive valence-related behaviors.

1. CeL Prkcd^+

B. BLA-to-CeA amygdala circuit model for valence-specific behaviors

A circuit from the BLA to CeA is summarized in Figure 31A. BLA Rspo2^+ neurons primarily send projections to:

1. CeC Prkcd^+

BLA Ppp1r1b^+ neurons primarily send projections to:

1. CeC Prkcd^+
2. CeL Prkcd^+
3. CeL Sst^+
4. CeL Crh^+ Nts^+ Tac2^+
5. CeM Nts^+
6. CeM Sst^+
7. CeM Tac2^+

Chapter 3. DISCUSSION

I. Basolateral amygdala

Here, we employed a forward genetic strategy in order to transcriptionally profile active neurons in BLA. This approach revealed genetic markers for distinct populations of BLA neurons and was predictive of neuronal function. Rspo2^+ BLA neurons are activated by stimuli that elicit negative behaviors, while Ppp1r1b^+ BLA neurons are activated by stimuli that elicit
positive behaviors. Rspos2+ BLA neurons are crucial for negative behaviors and associations, while Ppp1r1b+ BLA neurons are crucial for positive behaviors and associations. Rspos2+ and Ppp1r1b+ neurons are antagonistic at the behavioral, neuronal population, and electrophysiological levels. They not only drive opposing behaviors, but also antagonize valence-specific behaviors, antagonize the overall activation of the opposing neurons and interact through reciprocal feedforward inhibition. Collectively, these results support a model in which mutually inhibitory Rspos2+ and Ppp1r1b+ neurons are the principle neurons that represent and elicit negative and positive behaviors, respectively.

Previous inactivation studies have implicated a greater contribution of the aBLA in contextual fear conditioning4, and the pBLA in reward conditioning73. Here, we dissociated, using specific genetic markers for cell-type specific manipulations, the aBLA and pBLA in negative and positive behaviors, respectively. Although Rspos2+ and Ppp1r1b+ neurons constitute virtually all BLA pyramidal neurons, there may be further functional, genetic, and or structural diversity within each of these two cell-types. Other genetic markers found as candidates on our screen were not further pursued in this study, but further studies could be performed to examine the role of other genetically distinct BLA neurons. However, from the examination of Rspos2+ and Ppp1r1b+ neurons in a set of behavioral assays, we found no evidence suggesting that Rspos2+ and Ppp1r1b+ neurons participate in behaviors or associations across valence.

Previous in vivo electrophysiology and stimulus-dependent studies suggested that negative and positive BLA neurons may be intermingled34,35. However, our results suggest that negative and positive neurons are spatially segregated into the aBLA and pBLA, respectively. At the transition between the aBLA and pBLA, these two types of neurons can be considered intermingled; however, examination of the entire BLA showed that Rspos2 and Ppp1r1b are
spatially segregated in the BLA and define what has previously been defined as the anterior and posterior subfields of the BLA and correspond to magnocellular and parvocellular neurons, respectively.

Previous studies have targeted BLA neurons for the study of negative and positive behaviors using projection target-based criteria. These studies suggested that nucleus accumbens (NAc) projections may be a defining feature of positive BLA neurons. However, retrograde and anterograde projection experiments showed that ~30% of BLA neurons that project to NAc, are *Rspo2* BLA neurons. Furthermore, stimulation of *Rspo2* somas or their NAc projections resulted in negative behaviors (Figure 15). These findings demonstrate that such a projection-based definition is insufficient for distinguishing negative and positive BLA neurons.

Previous observations that BLA to NAc projections mediate positive behaviors are likely due to the observation that a larger proportion of NAc-projecting BLA neurons are *Ppp1r1b* (Table 1).

It is widely thought that the amygdala fear circuit involves direct transmission of negative information from BLA principle neurons to CeL neurons and/or effector neurons in the CeM. Contrary to these previous hypotheses, our data suggest that positive, but not negative BLA neurons project to the CeM and CeL, while negative, but not positive BLA neurons, project to the CeC. Here, previous projection-based definition of BLA neurons—namely that the neurons projecting to the CeM drive negative behavior—is not supported by our findings. In regards to CeM and CeL projections, our findings are consistent with anatomical studies demonstrating that parvocellular BLA neurons (which are *Ppp1r1b*) send strong projections to the CeL and CeM and provide further support for the role of the central amygdala in appetitive behaviors. In regards to connections from negative BLA neurons to effector neurons in the CeM, our findings suggest that this may be indirect route.
through the CeC. A recent study identified a population of Calcr* neurons in the CeC/CeL, which supports similar negative behaviors as Rpso2* BLA neurons, and, thus, may be an intermediate between negative BLA neurons and the putative CeM effector neurons52.

II. Central Amygdala

Our findings suggest that the CeA has an integral role in appetitive behaviors. The PAG has been shown to be a key site for executing defensive behaviors 81,82 and several amygdala models hypothesized that the PAG-projecting CeA neurons mediate defensive behaviors 8,50,83,84. Although Cre transgenics and targeted virus injections would not necessarily give absolute selectivity when it come to the functional study of neurons, none of the CeA neurons that were identified in this study that project to the PAG (Figure 28A-D)—CeL Sst*, CeL Crt*Nts*Tac2*, CeM Nts*, CeM Sst*, and CeM Tac2* neurons—elicit or are required for defensive behaviors or respond (using the expression of Fos) to stimuli that elicit defensive behaviors (Figure 18-20). Rather, these PAG-projecting populations elicit appetitive behaviors (though not necessarily through their projections to the PAG) and were activated by stimuli that elicit or are associated with appetitive behaviors (Figure 18,19). Furthermore, although the global action of several of the genes (Crh, Nts, Pdyn, Sst, Tac2, Tac1) that were found to be expressed in these PAG-projecting CeA neurons have been traditionally thought to be involved in negative behaviors and affective states, several studies that have examined the roles of these genes in the CeA and/or PAG have shown an opposing effect of these neuropeptides on negative behaviors or a positive role in appetitive behaviors, which is consist with our findings 85-90. Collectively, the populations that were examined in this study constitute almost all neurons (>90%) in the CeL and CeM, reinforcing the idea that the CeA participates in appetitive behaviors. Moreover, these data suggest that the main route for conveying defensive information from the BLA to PAG may involve an alternative circuit that does not involve the
CeL or CeM. Nevertheless, we do not disregard the role of the CeA in defensive behavior, as CeC \textit{Prkcd}^+ neurons participate in defensive behaviors (Figure 18A,20A). In addition, neither do we preclude the role of PAG-projecting CeA neurons in defensive or conditioned defensive behaviors; nor the possibility of an unidentified neuronal population or a subpopulation of one of these populations for regulating defensive behaviors. However, in light of our findings and previous conflicting reports on the role of the CeA in defensive behavior \textsuperscript{16,47,53,65}, further studies will be required to resolve how, if at all, defensive behaviors are positively mediated by the pathway from the CeA to PAG. Nevertheless, the involvement of several distinct CeA projection neurons as positive mediators of appetitive behavior validates the integral role of the CeA in reward-related function \textsuperscript{44,59,60,80,91,92}.

Studies on \textit{Prkcd}^+ neurons in the CeA have suggested that there may be functional diversity among \textit{Prkcd}^+ CeA neurons. An early study on \textit{Prkcd}^+ neurons suggested a role in inhibition of defensive behavior \textsuperscript{51}. More recent studies have shown that CeA \textit{Prkcd}^+ neurons inhibit feeding behavior \textsuperscript{65} and that a subpopulation of \textit{Prkcd}^+ neurons, CeA \textit{Calcr}^+ neurons, elicit and are required for defensive behavior \textsuperscript{52}. Here, based on the gene expression patterning of \textit{Calcrl} and \textit{Prkcd}, \textit{Calcrl}^+ neurons define neurons in the CeC (Figure 24B) \textsuperscript{93} rather than what was previously reported as the CeL in the study that described CeA \textit{Calcrl}^+ neurons in defensive behaviors \textsuperscript{52}. \textit{Prkcd}^+ neurons reside in the CeL as well as define a subpopulation of \textit{Calcrl}^+ neurons in CeC (Figure 17A) \textsuperscript{52}. Considering this structural distinction, experiments between these two types of \textit{Prkcd}^+ CeA neurons yielded behavioral, functional, and connectivity dissociations. CeC \textit{Prkcd}^+ neurons elicit and are required for defensive behaviors and are activated by stimuli that drive defensive behaviors (Figure 18A and 19A). This is consistent with the reported role of CeA \textit{Calcrl}^+ neurons in defensive behaviors \textsuperscript{52}. In contrast, CeL \textit{Prkcd}^+ neurons do not drive defensive behaviors, but are activated by contextual fear.
extinction (Figure 18B and 19B). This is consistent with the initially hypothesized role of inhibition of defensive behaviors \(^{51}\). Based on findings from retrograde rabies experiments, CeC Prkcd\(^+\) and CeL Prkcd\(^+\) are reciprocally connected (Figure 21A). Thus, Prkcd\(^+\) neurons in the CeA represent two distinct populations that have opposing functions on defensive behavior and we speculate that CeL Prkcd\(^+\) neurons and CeC Calcrl\(^+\) neurons, rather than CeL Prkcd\(^-\) and CeL Sst\(^+\) neurons \(^{8,50,53}\), may represent the electrophysiological opposing units for fear-related responses in the CeA \(^{51,94}\).

With regard to appetitive behaviors, CeC Prkcd\(^+\) and CeL Prkcd\(^+\) neurons both directly inhibit mediators of appetitive behavior in the CeL and CeM (Figure 31A). Both Prkcd\(^+\) populations are connected in such a way that they can support an inhibitory role on appetitive behaviors, but may be functionally distinct based on differences in Fos activation profile and anatomical inputs. CeC Prkcd\(^+\) neurons are activated by threatening stimuli and aversive tastes (Figure 19A and 19D) and receive input from neurons that respond to aversive stimuli, BLA Rspo2\(^+\) neurons (Figure 31A) and calcitonin-related polypeptide, alpha (Calca) expressing neurons of the lateral parabrachial nucleus (by consideration that CeC Prkcd\(^+\) neurons are a subpopulation of CeC Calcrl\(^+\) neurons) \(^{25,52,96}\). In contrast, CeL Prkcd\(^+\) neurons are activated by states of satiety \(^{65}\) (Figure 19E) and receive input from BLA Ppp1r1b\(^+\) neurons (Figure 31A), which respond to reward-related stimuli and Calca\(^+\) neurons of the lateral parabrachial nucleus, which in addition to responding to threat, also respond to states of satiety \(^{25,96}\). Therefore, furthering previous findings and proposals on the role of CeA Prkcd\(^+\) neurons \(^{65}\), these results suggest that the role of CeC Prkcd\(^+\) (and by extension CeC Calcrl\(^+\)) neurons is to signal the inhibition of appetitive behaviors mainly in response to aversive stimuli such as threat and aversive tastes, while the role of CeL Prkcd\(^+\) neurons is to signal the inhibition of appetitive behaviors in response to positive states such as satiety.
CeL Sst* and CeL Chr+Nts+Tac2* neurons are Prkcd neurons of the CeL. CeL Prkcd neurons have been hypothesized to be positive mediators of fear-related behaviors. A previous study has demonstrated that CeL Sst* neurons elicit and are required for defensive behaviors. Contrary to these early findings, a more recent study suggested that activation of CeL Prkcd neurons promotes feeding, while another study demonstrated that activation of CeL Prkcd neurons suppresses defensive behaviors evoked by innate threatening odors. Here, we were unable to find evidence for CeL Sst* or CeL Chr+Nts+Tac2* neurons as mediators of defensive behavior (Figure 18-20). We speculate the main differences in our results from the original work on CeL Sst* neurons in defensive behaviors is likely attributed to our use of lower volume of virus, 100 nL of ~10^{12} vs 300 to 800 nL of ~10^{12} viral particles, resulting in more specific targeting of the CeL. Moreover, a following study on CeL Sst* demonstrated that posterior paraventricular thalamic neurons (pPVT) neurons mediate defensive behaviors and directly project to CeL Sst* neurons. Examination of the cell-type connection of the PVT to CeA, showed that anterior PVT (aPVT) neurons, regulators of appetitive behaviors, mainly project to CeL Sst* neuron, while pPVT neurons mainly project to CeC Prkcd* neurons (Data not shown). Although a disinhibitory pathway that supports defensive behaviors from BLA Rspo2* neurons (positive mediators of defensive behaviors) to CeL Prkcd* neurons is anatomically identifiable (BLA Rspo2* to CeC Prkcd* to CeL Prkcd* to CeL Prkcd*), a disynaptic inhibitory connection is also identifiable from BLA Rspo2* neurons, through CeC Prkcd* neurons to CeL Prkcd* neurons, which does not support defensive behaviors (Figure 31A). Moreover, CeL Prkcd* neurons receive direct innervation from BLA Ppp1r1b* neurons (Figure 31A), which suppress defensive behaviors and elicit appetitive behaviors. Thus, the anatomical and functional connectivity of Prkcd neurons in relation to
BLA neurons does not fully support a positive role in defensive behavior, rather emerging evidence supports the role in appetitive behaviors.

With regard to appetitive behaviors, CeL $Sst^+$ and CeL $Crh^+ Nts^+ Tac2^+$ neurons are capable of eliciting appetitive behaviors, are critical for drinking, and are strongly activated by water in addition to food (Figure 18C,18D,19D,20C,20D). Moreover, inhibition of CeL $Prkcd^+$ neurons, which are reciprocally connected to CeL $Prkcd$ neurons, results in the enhancement of drinking behavior (Figure 20B) and a previous study demonstrated that activation of CeL $Prkcd^+$ neurons suppresses drinking behavior $^{65}$. Together, these results indicate that CeL $Sst^+$ and CeL $Crh^+ Nts^+ Tac2^+$ neurons are positive mediators of appetitive behavior and mainly participate in drinking-related function. These results bring insight to previous studies that have implicated the CeL in drinking and alcohol-related behaviors $^{99-103}$. With regard to the function of the two types of $Prkcd$ neurons, examination of CeL $Sst^+$ and CeL $Crh^+ Nts^+ Tac2^+$ neurons revealed no functional, behavioral, or connectivity differences. CeL $Sst^+$ and CeL $Crh^+ Nts^+ Tac2^+$ are highly overlapping populations; $\sim50\%$ of CeL $Sst^+$ are CeL $Crh^+ Nts^+ Tac2^+$ and $\sim70\%$ of CeL $Crh^+ Nts^+ Tac2^+$ are CeL $Sst^+$ (Figure 17B). Therefore, it was not unexpected that a structural or functional dissociation was not found. Nevertheless, a previous study demonstrated that CeL $Crh^+$ neurons are mediators of active avoidance behaviors $^{104}$. This study did not take into consideration amygdala striatal transition zone (Astr), which is juxtaposed above the CeL and consists of $Crh^+$ neurons (Figure 24B). With that and based on preliminary evidence on the role of the lateral amygdala (LA) (data not shown) and its projections to the Astr, it is likely that Astr $Crh^+$ neurons are mediators of active avoidance.

Genetically distinct CeM populations—$Nts^+$, $Sst^+$, $Tac2^+$—were found to participate in appetitive behaviors. Though gene expression of striatal markers in the CeM suggests there may be alternative ways to divide CeM populations, these markers define 3 major well
segregated Drd1+ neurons (Figure 17C, 23D). Behaviorally, these populations elicit appetitive behaviors and collective silencing of these neurons results in reduced feeding and drinking (Figure 27). This is in contrast to CeL mediators of appetitive behavior, which appear to have a specific role in appetitive drinking behavior. Therefore, CeM neurons may have a more general role in appetitive behaviors. Alternatively, but not exclusively, the mediators of appetitive behavior in the CeL and CeM may function together to execute and regulate distinct behavioral programs for different types of appetitive behaviors and reward-related states. Although we did not assess the nuances of the different aspects of appetitive behaviors and reward-related phenotypes or evaluate more long-term effects of silencing these neurons, further studies will be required to further dissociate the role of these distinct populations in appetitive behaviors.

III. Genetically-defined amygdala circuit model

Here, a new model amygdala circuit model based on genetically distinct cell-type types has been developed. This model describes the amygdala circuits for defensive and appetitive behaviors. The findings from investigation of the BLA (Figure 16) and CeA (Figure 31) have been integrated in to a broader model (Figure 32). In addition, preliminary findings and data not described in this work have also been incorporated into the circuit model (Figure 32). Such features have not been published and will be denoted hypothetical and can be considered speculative. Nevertheless, such hypothetical features of the new circuit model will be described briefly.

Features in addition to the findings from investigation of the BLA and CeA circuit included:
1. Two genetically-distinct, spatially segregated population of lateral amygdala neurons
2. Genetically-defined neurons in amygdala extended circuits
3. Genetically-defined neurons in the periaqueductal gray

The main features in the new model:
1. LA neurons are not involved in defensive behavior, TFC, or CFC
2. LA neurons mediate active avoidance or place preference
3. LA neurons do not project to the CeA, rather they project to the NAc, BMA, and Astr
4. vIPAG neurons mainly mediate appetitive behaviors
5. If a vIPAG population mediates defensive behaviors such population is likely Penk$^+$ and inhibitory, rather than excitatory
6. NAc Drd2$^+$ and dorsal PFC neurons mediate defensive behaviors
7. BNST is genetically analogous to the CeA and mainly mediates appetitive behaviors

The prevailing amygdala circuit model has long provided a framework for investigating amygdala structure and function$^7$. Criticism about the old model and criticism undermining the premise of tone fear condition protocol has been largely ignored$^{106-111}$. However, regardless of the hypothetical components of this model (Figure 32), the findings based on the BLA and CeA (Figure 16, 31) have brought new light to amygdala in negative and positive behaviors. Namely, the role of the CeA in negative behaviors has been challenged (discussed in previous section II) and the nature of negative and positive BLA neurons have been elucidated (discussed in previous section I).

One main point in the distinct between the predominant amygdala circuit model and the cell-type specific genetic amygdala circuit model is the emphasis on the role of CeA neurons that project to the PAG. The original hypothesis and evidence suggested that since the PAG
mediates autonomic and “reflex”-related functions, projections from the CeA to PAG likely mediated defensive behaviors. A previous study demonstrated a microcircuit in the PAG that mediates defensive behaviors. This microcircuit claims that inhibitory neurons from the CeA send projections to inhibitory neurons in the ventrolateral PAG (vIPAG), in turn disinhibiting excitatory mediators of defensive behaviors. However, preliminary examination of PAG neurons by genetic classification and cFos expression demonstrated several genetically distinct populations beyond a simple inhibitory/excitatory classification (Figure 33). Moreover, cFos expression demonstrated that vIPAG Penk+ inhibitory neurons as the only species that responds to negative stimuli and that the excitatory neurons in the vIPAG and IPAG are mainly activated by positive stimuli. Also, the projections from the CeA mainly innervate the IPAG, rather than the vIPAG (Figure 28D), which is the “hotspot” for defensive behaviors. This data combined with the results from examination of the CeA (Chapter 211.) suggest at the least that the PAG likely has an important role in positive behaviors and that the CeA-to-PAG projection is not solely for defensive behaviors, but if the CeA-to-PAG pathway participates in defensive behaviors, the vPAG and vIPAG must be taken into consideration. Thus, the circuit model previously proposed is, at the least, incomplete, if not false in regards to the function of CeA-to-PAG pathway. Overall, the implementation of genetically-defined neuronal populations and the integration of positive behaviors into an amygdala circuit model challenge several structural and functional aspects of the current prevailing model and brings to light to future directions and understanding of the role of the amygdala and its circuits.

Chapter 4. CIRCUIT THEMES

I. Spatial segregation of valence-specific behaviors
One pattern or theme regarding the organization underlying neural circuits of valence-specific behaviors is the spatial segregation of negative and positive valence-related behaviors. The nucleus accumbens\textsuperscript{113}, prefrontal cortex\textsuperscript{114-116}, paraventricular thalamus\textsuperscript{97,98}, the medial amygdala\textsuperscript{117}, cortical amygdala\textsuperscript{118}, gustatory cortex\textsuperscript{119}, ventral tegmental area\textsuperscript{120} in mice, and dopaminergic neurons and mushroom bodies in \textit{Drosophila}\textsuperscript{121-123} are a few examples demonstrating the spatial segregation of negative and positive valence-related behaviors. The identification of spatially segregated representation of negative and positive valence-related behaviors in the BLA further this pattern\textsuperscript{25}. Thus, spatially segregated representation of negative and positive information may be a common motif throughout the central nervous system and across invertebrate and vertebrate species.

II. Antagonism of valence-specific neurons

Behaviors, for the most part (depending on the definition), are expressed through the motor neurons. The expression of particular behaviors or behavioral programs required the precise coordination of distinct behavior modules given that several different behavioral programs are executed through the same motor neurons. Hence, it is not unexpected that inhibition or mutual inhibition in neural circuits is a means of coordinating between distinct behaviors and behavioral programs. Nevertheless, such inhibitory circuits have only recently been demonstrated utilizing genetic techniques and prior to these works\textsuperscript{51}, it was not fully known whether negative and positive valence-related neurons interact in such a way.

Here, two distinct valence-opposing circuits can be found in the amygdala that utilizes mutual inhibitory connections. CeA \textit{Prkcd}\textsuperscript{+} and CeA \textit{Prkcd}\textsuperscript{-} neurons (inhibitory neurons) make reciprocal inhibitory connections and mediate negative and positive behaviors, respectively\textsuperscript{26,51,65}. Moreover, valence-specific neurons of the BLA (\textit{Rpso2}\textsuperscript{-} and \textit{Ppp1r1b}\textsuperscript{+} neurons) make reciprocal inhibitory connections\textsuperscript{25}. The distinction between the CeA and BLA is
that the reciprocal inhibitory valence circuit is mediated between two inhibitory neurons in the CeA and two excitatory neurons in the BLA. Nevertheless, these two types of mutual inhibitory circuits can be described as homotypic reciprocal inhibition, reciprocal inhibition between the same species (excitatory or inhibitory) neurons. Thus, homotypic reciprocal inhibition may be a common mechanism regulating valence-specific behaviors through valence-specific circuits or other types of neural circuits. Whether homotypic reciprocal connections or even heterotypic reciprocal connections are a common motif will require further dissection of valence-specific circuits and neural circuits of various kinds.

III. Genetic Circuit Motif

The complexities of biological systems develop through evolutionary mechanisms. Duplication and specialization are means in which new structures and function can be derived from preexisting structures\textsuperscript{124-126}. Evidence of duplication and specialization events is represented in homologies or motifs between structures. For instance, G-protein coupled receptors are a large and diverse class of transmembrane receptors. G-protein coupled receptors are generally distinct from one another by a specialized binding site for distinct ligands and similar to one other in 7 transmembrane structures and subclasses of intracellular regions for intracellular signaling\textsuperscript{127,128}. The motifs underlying molecular structure and function provide an effective way for understanding principles underlying proteins and biological molecules. Thus, do such motifs or organizing principles also apply at the neural circuit level?

In the brain, there are several regions that appear to have, at least, organizational motifs. For instance, the majority of the cortex of the mammalian brain is organized in 6 layers. In different sensory regions such as the auditory, visual, somatosensory, etc areas the 6-layer organization is seen\textsuperscript{129}. Although the specialized sensory regions of the cortex have distinct
inputs and projections, the fact that a 6-layer organization is utilized suggest an organizing principle for processing sensory input. Moreover, the distinct layers of the cortex can be defined by distinct genetic markers. Thus, one can say a genetic motif exists across the sensory cortices. Does the amygdala share any organizing principles with other brain regions?

Here, examination of the amygdala revealed that the BLA to CeA circuit that promotes and suppresses movement is analogous to the direct and indirect pathway of the basal ganglia that promotes and suppresses movement. CeM mediators of appetitive behavior express Drd1, Pdyn, and Tac1 (Figure 23D). Although Penk is expressed in a subset of CeM mediators of appetitive behavior, Drd2 is only minimally expressed (Figure 23D). CeM mediators of appetitive behavior directly receive monosynaptic input from excitatory $Ppp1r1b^+$ parvocellular neurons of the BLA (Figure 31A), which are also capable of promoting appetitive behaviors. Hence, the pathway for promoting appetitive behaviors from the BLA to CeA is genetically and structurally analogous to cortex and striatum of the direct pathway, which involves the direct innervation of Drd1+, Pdyn+, and Tac1+ striatonigral neurons from excitatory IT-type neurons of the cortex. The BLA to CeA pathway that supports the suppression of appetitive behavior emanates from BLA $Rsopo2^+$ neurons and also may involve a subset of BLA $Ppp1r1b^+$ neurons in consideration of the connection from BLA $Ppp1r1b^+$ to CeA Prkcd+ neurons (Figure 31A). Although BLA $Rsopo2^+$ neurons are capable of driving defensive behaviors, they are also capable of suppressing appetitive behaviors and form disynaptic inhibitory connections to mediators of appetitive behavior in the CeM and CeL (Figure 31A). The pathway from BLA $Rsopo2^+$ neurons to CeA mediators of appetitive behavior involves an intermediate step in the CeC that express Drd2 and Penk, but does not express Drd1, Pdyn, or Tac1 (Figure 23D). Hence, the pathway for suppressing appetitive behaviors, from excitatory $Rsopo2^+$ magnocellular neurons of the BLA to the CeA, is functionally and genetically analogous to the
cortex and striatum of the indirect pathway, which involves the direct innervation of *Drd2* and *Penk* striatopallidal neurons from PT-type neurons of the cortex. Interestingly, the IT-type cortical neurons (direct pathway) are smaller in soma size than PT-types cortical neurons (indirect pathway), while BLA *Ppp1r1b* parvocellular neurons are smaller in soma size as BLA *Rpso2* magnocellular neurons. This suggests that the two types of BLA neurons may also be morphologically analogous to the two types of corticostriatal neurons. Although an analogy can be described between the BLA and CeA with the cortex and striatum, the output structures and circuitry of the CeA were not fully examined in this study. Thus, future studies will be required to examine if the output of the CeA share any organizing principle with the output circuitry of the striatum of the direct and indirect pathway.

In addition to this corticostriatal circuit motif within the intra-amygdala circuitry, an organizational genetic motif was identified between the CeA and bed nucleus striata terminalis (BNST) (Figure 34). Based on the genetic classification of the CeA, 7 major populations of genetically distinct neurons were identified in 3 subdivisions (Figure 34). Expression of the same set of genetic markers that was previously characterized in the CeA was characterized in the BNST. Interestingly, at least 7 populations that were found in the CeA were also found in the BNST. The organization of neuronal populations in the anterolateral and anteromedial area of the BNST (BNSTal, BNSTam) is analogous to the CeM. The organization of neuronal populations in the oval nucleus of the BNST (BNSTov) is analogous to the CeL. Cholecystokinin (*Cck*) was uniquely express in the principle nucleus of the BNST (BNSTpr) suggesting the BNSTpr to be analogous to the CeC. This is because CeC *Prkcd* or CeC *Calcrt* neurons also express *Cck* (Data not shown). Although the cell-type connectivity of the BLA to BNST was not examined, based on this apparent genetic organizational motif, we speculate that the connection from the BLA to BNST likely resembles the connection from the BLA to CeA.
Furthermore, if this motif holds consist in the BNST, we could also predict that function of these BNST neurons are similar to the CeA and that the BLA-to-BNST connectivity is also analogous to the corticostriatal circuit. Finally, although the genetic organization of the nucleus accumbens (NAc) was not examined in details, the expression of the markers described in the CeA (Figure 17) are also found in the NAc in addition to the classic striatal markers. Thus, if this circuit motif holds further, the BLA-to-NAc connectivity could also be analogous to the BLA-to-CeA.

The dissection of the amygdala circuit provides evidence that these valence-related circuits utilize structural, genetic, and circuit motifs at several levels. At the organismal and macrostructural level, valence-related functions may be represented in a spatially segregated motif. At a mesostructural circuit level, a genetic circuit motif may be found in the organization and connectivity of neurons. At a microstructural circuit level, reciprocal inhibitory connections may occur as a motif between negative and positive valence-mediating populations. The extent in which these circuit motifs are utilized in these circuits will require further investigation. A conceptual understanding of neural circuit motifs provides a helpful framework for developing new hypothesis for new circuits and a broader understanding of the organization and function of the brain. This is because the evolutionary process of duplication and specialization appears to be a fundamental organizing principle that has given rise to the circuits of the brain.
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Figure 1. Activity-dependent transcriptional profiling of BLA neurons. a, Viral-based genetic scheme for activity-dependent transcriptional profiling. c-Fos promoter activity drives the expression of tTA, which in turn, binds TRE and drives the expression of PABP-FLAG in the absence of doxycycline (Dox). b, PABP-FLAG expression in the BLA in mice kept on a Dox diet (On Dox), taken off a Dox diet and exposed to home cage (Off Dox), Shock, Female, Seizure, (one-way ANOVA, P<0.0001, n = 6 per group). Significance for multiple comparisons, **P<0.01, ****P<0.0001, not significant (N.S.). c, PABP-FLAG expression in soma and varicosities of a BLA neuron. FLAG expression in the BLA of On Dox (d), Off Dox (e), Seizure (f), Shock (g,h), and Female (i,j) group. FLAG expression and nuclear marker, DAPI, in Shock (h), and Female (i) group. Scale bar 25μm (c), 250μm (d,e,f,g,i), 80 μm (h,j). k, RMA normalized RNA expression values from microarray from RNA purified from Shock (n = 3) and Female (n =3) groups. Red and green points represent enriched genes (>1.25 fold, ANOVA p<.05, log2 scale). l, Quantification of in situ hybridization of BLA expression of candidate genetic markers enriched in shock group (green) and female group (red) (n = 3 mice per group). Positive control genes (black). Results show mean ± s.e.m (b,l).
Figure 2. Rspo2⁺ and Ppp1r1b⁺ BLA neurons define spatially segregated populations of BLA pyramidal neurons. a, Quantification of smFISH of Rspo2 (green) and Ppp1r1b (red) expression across the AP axis (coronal distance from bregma - 0.8mm to -2.8mm) of the BLA (n = 3). b, Two sagittal views (ML distance from midline, 3.2mm, 3.4mm) of double smFISH of Rspo2 and Ppp1r1b with nuclear marker, DAPI, in the BLA. c, Coronal view of double smFISH of Rspo2 and Ppp1r1b across the AP axis of the BLA. Double smFISH of Camk2a and Rspo2 (d), Camk2a and Ppp1r1b (e), Gad1 and Rspo2 (f) Gad1 and Ppp1r1b (g), in the BLA (Larger micrograph in Fig. 10). Scale bar 500µm (b), 200µm (c), 25µm (d-g). h, Biocytin-filled magnocellular (top) and parvocellular (bottom) BLA neuron, scale bar 50µm. i, Single-cell qPCR traces of Rspo2 (green) and Ppp1r1b (red), of magnocellular (top) and parvocellular (bottom) BLA neurons. j, Electrophysiological response to current steps in a Rspo2⁺ (top) and Ppp1r1b⁺ (bottom) BLA neuron. k, Comparison of mean soma diameter, membrane resistance (Rm), and membrane capacitance (Cm) of qPCR-confirmed Rspo2⁺ (green, n = 11) and Ppp1r1b⁺ (red, n = 12) neurons. Significance for unpaired
Figure 3. Rspo2* and Ppp1r1b+ BLA neurons are activated by valence-specific stimuli. c-FOS expression across the AP axis (coronal distance from bregma -0.8mm to -2.8mm) of the BLA in response to shock (n = 8), context (n = 8), female (n = 6) (a); TMT (n = 6), BA (n = 7), peanut oil (n = 6) (b); quinine water (n = 8), no water (n = 8), water (n = 6), sucrose water (n = 8) (c). The total number of c-FOS+ cells is represented for each coronal section of a unilateral BLA (a-c), micrographs found in Extended Data Fig. 4. d. Relative c-FOS expression in the aBLA and pBLA in response to shock, context, female (one-way ANOVA, P<0.0001). e. Relative c-FOS expression in response to TMT, BA, peanut oil (one-way ANOVA, P<0.0001). f. Relative c-FOS expression in response to quinine water, no water, water, sucrose water (one-way ANOVA, P<0.0001). Significance for multiple comparisons (d-f), *P<0.05, **P<0.01, ****P<0.0001, not significant (N.S.). Double-label smFISH (n = 5 in each group) of c-Fos/Rspo2* (g,k,m) or c-Fos-/Ppp1r1b+ (h,l,n) in response to shock (S) or context (C). Double-label smFISH of c-Fos/Rspo2* (i,o,q) or c-Fos/Ppp1r1b+ (j,p,r) in response to water (W) or no water (NW). Significance for unpaired t-test (g-j). **P<0.01, not significant (N.S). Scale bar 125μm (k-r). Results show mean ± s.e.m (a-j).
Figure 4. RsplO+ and Ppplrb+ BLA neurons participate in valence-specific behaviors. a, Optogenetically targeting RsplO+ and Ppplrb+ BLA neurons. Scheme and results for RsplO-ChR2 and Ppplrb-ChR2 mice during shocks (l,m), or water consumption (n,o). b, Ppplrb-ChR2 mice (n = 8) displayed greater levels of nose pokes on Day 1 and 2 compared to EYFP controls (n = 6), no difference between RsplO-ChR2 (n = 8) and RsplO-eYFP (n = 6) mice. c, RsplO-neurons (n = 11) displayed greater preference to light stimulation compared to EYFP controls (n = 8), while Ppplrb-ChR2 (n = 7) mice displayed greater preference to light stimulation compared to EYFP controls (n = 7). Scheme and results for activating BLA neurons in RsplO-ChR2 and Ppplrb-ChR2 mice during shocks (l,m), or water consumption (n,o). m, Ppplrb-ChR2 mice (n = 8) displayed lower freezing levels compared to EYFP controls (n = 6), no difference between RsplO-ChR2 (n = 6) and RsplO-eYFP (n = 6) mice. e, Ppplrb-ChR2 mice (n = 10) displayed lower total nose pokes and cue-reward association in nose port (z-score) compared to EYFP controls (n = 11), no difference between RsplO-ChR2 (n = 9) and RsplO-eYFP (n = 8). Scheme and results for RsplO-ChR2 and Ppplrb-ChR2 mice in an optogenetic freezing test (f,g), optogenetic self-stimulation test (h,i), and optogenetic place preference test (j,k). g, RsplO-ChR2 mice (n = 7) displayed greater freezing levels on Day 1 and 2 compared to EYFP controls (n = 6), no difference between Ppplrb-ChR2 (n = 5) and Ppplrb-eYFP (n = 5) mice. i, Ppplrb-ChR2 mice (n = 6) displayed greater levels of nose pokes on Day 1 and 2 compared to EYFP controls (n = 6), no difference between RsplO-ChR2 (n = 8) and RsplO-eYFP (n = 6) mice. k, RsplO-ChR2 mice (n = 7) displayed greater preference to light stimulation compared to EYFP controls (n = 8), while Ppplrb-ChR2 (n = 7) mice displayed greater preference to light stimulation compared to EYFP controls (n = 7). Scheme and results for activating BLA neurons in RsplO-ChR2 and Ppplrb-ChR2 mice during shocks (l,m), or water consumption (n,o). e, Ppplrb-ChR2 mice (n = 8) displayed lower freezing levels compared to EYFP controls (n = 6), no difference between RsplO-ChR2 (n = 6) and RsplO-eYFP (n = 6) mice. o, RsplO-ChR2 mice (n = 6) displayed lower total nose pokes and cue-reward association compared to EYFP controls (n = 5), no difference between Ppplrb-ChR2 (n = 9) and Ppplrb-eYFP (n = 7) mice. Significance for unpaired t-test between experimental groups compared to corresponding EYFP controls, *P< 0.05, **P< 0.01, ***P<0.001, ****P<0.0001, not significant (N.S).
Figure 5. *Rspo2* and *Ppp1r1b* BLA neurons antagonize valence-specific behaviors. a, Scheme of activation of BLA neurons in *Rspo2-CHR2* and *Ppp1r1b-CHR2* mice during shocks (Day 1). b, Time course of freezing during day 1 and day 2 in *Ppp1r1b-CHR2* (*n* = 8) and *Ppp1r1b-eYFP* (*n* = 8) mice. c, On day 1 and day 2, *Ppp1r1b-CHR2* (*n* = 6) displayed lower freezing levels compared to eYFP controls (*n* = 6) and *Rspo2-eYFP* (*n* = 6) mice. d, Scheme of activation of BLA neurons in *Rspo2-CHR2* and *Ppp1r1b-CHR2* mice during reward conditioning. e, Time course of z-score of poking in *Rspo2-CHR2* (*n* = 6) and *Rspo2-eYFP* (*n* = 5) mice. f, *Rspo2-CHR2* mice (*n* = 6) displayed lower total nose pokes and cue-reward association compared to eYFP controls (*n* = 5), no difference between *Ppp1r1b-CHR2* (*n* = 9) and *Ppp1r1b-eYFP* (*n* = 7) mice. Significance for unpaired *t*-test between experimental groups compared to corresponding eYFP controls, *P* < 0.05, **P** < 0.01, ***P*** < 0.001, ****P*** < 0.0001, not significant (N.S). Results show mean ± s.e.m.  

Quantification of smFISH of c-Fos in *Ppp1r1b* and *Rspo2* neurons in *Ppp1r1b-CHR2* and *Ppp1r1b-eYFP* mice that received shock simultaneously with blue light stimulation. Quantification of smFISH of c-Fos in *Rspo2* and *Ppp1r1b* neurons in *Rspo2-CHR2* and *Rspo2-eYFP* mice that received water simultaneously with blue light stimulation. Significance for unpaired *t*-test (g,i), **P** < 0.01, ***P** < 0.001, ****P*** < 0.0001, not significant (N.S). Results show mean ± s.e.m. Scale bar, 300µm (h,i,k,l).
Figure 6. Rs1o2+ and Ppp1r1b+ BLA neurons establish reciprocal inhibitory connections. a,b, Scheme for the experimental setup for recording in magnocellular (Rspo2+) (a) and parvocellular (Ppp1r1b+) (b) neurons, while stimulating Ppp1r1b+ (Ppp1r1b-ChR2 mice) and Rs1o2+ (Rspo2-ChR2 mice) neurons, respectively. c,d, Sagittal view of biocytin-filled magnocellular BLA neurons in Ppp1r1b-ChR2 mice (c) and parvocellular BLA neurons in Rs1o2-ChR2 mice (d). Scale bar 200μm, inset: 50 μm(c,d). AsterisksA denotes the electrophysiological traces in e and f.f Inhibitory postsynaptic potentials (IPSPsP) recorded in magnocellular (e) and parvocellular (f) BLA neurons by 10Hz optogenetic stimulation of Ppp1r1b-ChR2 (e) and Rs1o2-ChR2 (f) fibers. Green,G (e) and red (f) traces represent average trace of 20 sweeps recorded during periods without spikes. Inhibitory postsynaptic currents (IPSCs) recorded in Rs1o2+ (magnocellular) (g) and Ppp1r1b+ (parvocellular) (h) BLA neurons (clamped at 0 mV) in response to optogenetic stimulation (10Hz train) of Ppp1r1b-ChR2 (g) and Rs1o2-ChR2 (h) fibers. Currents are blocked by bath application of gabazine (GBZ, 10 μM), insets: IPSCs amplitude before (GBZ-) and after GBZ (GBZ+) application (for both magnocellular (n = 6) (g) and parvocellular (n = 6) (h), Wilcoxon signed-rank test, *P < 0.05. Probability of connection, Ppp1r1b+ to Rs1o2+ connection (i) and Rs1o2+ to Ppp1r1b+ connection (j). The two groups interact predominately by mutual inhibition rather than excitation, Fisher exact test, ***P <0.001 (i,j). IPSC onset in Rs1o2+ (green) and Ppp1r1b+ (red) neurons were similar (k). IPSC amplitude was greater in Ppp1r1b+ (red) than in Rs1o2+ (green) neurons (l), unpaired two-tailed paired t-test *P<0.05. Recorded magnocellular (green) and parvocellular (red) neurons were confirmed using soma diameter and anatomical position (m); membrane resistance (Rm) and membrane capacitance (Cm) (n). Rs1o2+ and Ppp1r1b+ cells were statistically distinct in all four parameters and consistent with values characterized in Fig. 2, significance for unpaired two-tailed paired t-test *P<0.05, **P<0.01, ***P<0.0001 (n, m). Results show mean ± s.e.m (g,h,k,l).
Figure 7. *Rspo2* and *Ppp1r1b*+ BLA neurons project to distinct amygdaloid nuclei and prefrontal areas. Quantification of CTB+ neurons across the AP axis (coronal distance from bregma -0.8mm to -2.8mm) of the BLA from CTB targeted to the amygdala and extended amygdala areas (a)—CeC (c,d), CeL/CeM (e,f), NAc (g,h), or dual CTB targeted to prefrontal cortex (b)—PL and IL (i,j) (n = 3 per group). Injections site of CTB (c,e,g,i) and CTB+ BLA neurons (d,f,h,j). Co-labelling of *Rspo2* mRNA in the BLA with CTB targeted to the CeC (k) and NAc (m). Co-labelling of *Ppp1r1b* mRNA in the BLA with CTB injected into the CeL/CeM (l) and NAc (n), quantification in Table 1, micrographs in Fig.14. Rspo2-ChR2+ fibers are found in the CeC, NAc, and PL (o). Ppp1r1b-ChR2+ fibers are found in the CeL, CeM, NAc, and IL (p). Scale bar 250 μm (c-j,o,p), 25 μm(k-n). Results show mean ± s.e.m.
Figure 8. RNA analysis of activity-dependent transcriptional profiles from BLA neurons. a, Example bioanalyzer traces of RNA samples collected from footshock (green) \((n=3)\), female (red) \((n=3)\), on dox (black) group \((n=1)\). Bioanalyzer traces was used to test the quality of RNA sample for RNA microarray, the graph shows the fluorescence levels, which corresponds to RNA levels, of different RNA species of different size (nt). Bioanalyzer traces showed that footshock and female samples yielded RNA samples with RNA quality number (RQN) >6 \((n=6)\), while the on dox RNA sample RQN <4 \((n=1)\). Peaks at .02kb, 1.9kb and 4.7kb correspond to the marker, 18S rRNAs, and 28S rRNAs, respectively. b, Analysis of MAS5 normalized data of arrays from the footshock \((n=3)\) and female \((n=3)\) group.
Figure 9. In situ hybridization of candidate genetic markers of BLA neurons. Gene expression of candidate genetic markers in the BLA using in situ hybridization. a-g, Genes that were enriched in the array of the footshock group (green). h-p, Genes that were enriched in the array of the female group (red). q-t, Positive control for interneurons (yellow). u,v, Positive control for excitatory neurons (yellow). Micrographs represent FISH with the exception of Ppp1r1b (smFISH). a-v, nuclear marker, DAPI (blue). Scale bar 100µm.
Figure 10. *Rspo2* and *Ppp1r1b* BLA neurons collectively constitute all BLA pyramidal neurons. smFISH of *Rspo2/Camk2a* (a), *Rspo2/Gad1* (b), *Ppp1r1b/Camk2a* (c), *Ppp1r1b/Gad1* (d), coronal BLA, scale bar 200 µm. e, smFISH of *Rspo2+Ppp1r1b/Camk2a*, sagittal BLA, scale bar 250 µm. f, higher magnification expression of *Rspo2+Ppp1r1b/Camk2a*, scale bar 50µm.
Figure 11. Spatial distribution of *c-Fos* expression in the BLA in response to valence-specific stimuli. *c-Fos* protein was visualized using IHC by an Alexa Fluor 555 secondary antibody. For improved graphical representation, images were inverted and saturation removed. a, *c-Fos* expression across the AP-axis of the BLA in response to shock, context, female. b, *c-Fos* expression across the AP-axis of the BLA in response to olfactory stimuli. c, *c-Fos* expression across the AP-axis of the BLA in response to gustatory stimuli. Scale bar 250μm.
Figure 12. Validation of Cre-driver mouse lines for targeting Rspo2+ and Ppp1r1b+ BLA neurons. Rspo2-Cre and Cartpt-Cre mice were injected with a Cre-dependent eYFP virus into the BLA and smFISH was performed against Rspo2 and Ppp1r1b, respectively. a, Quantification of the percentage of Rspo2+ BLA neurons that express eYFP (eYFP/Rspo2) and the percentage of eYFP+ BLA neurons that express Rspo2 (Rspo2/eYFP) (n = 4). b, eYFP (green) and Rspo2 (red) expression in the BLA of virus injected Rspo2-Cre mice. c, Quantification of the percentage of Ppp1r1b+ BLA neurons that express eYFP (eYFP/Ppp1r1b) and the percentage of eYFP+ BLA neurons that express Ppp1r1b (Ppp1r1b/eYFP) (n = 4). d, eYFP (green) and Ppp1r1b (red) expression in the BLA of virus injected Cartpt-Cre mice. Though Ppp1r1b is endogenously expressed outside of the BLA, such as in the intercalated cell mass, choroid plexus, and striatum (Fig. 2c), Cre-dependent virus targeted in the Cartpt-Cre mice does not express in these off targeted cells. Scale bar 250µm.
Figure 13. Fiber placement for targeting Rspo2+ and Ppp1r1b+ BLA neurons. Example of optic fiber placement in Rspo2-Arch (a) and Ppp1r1b-Arch (b) mice. Scale bar, 500μm.
Figure 14. Retrograde tracing from putative projection targets of Rspo2+ and Ppp1r1b+ BLA neurons. smFISH of Rspo2 and Ppp1r1b in CTB injected brains. Rspo2 (a) and Ppp1r1b (b) expression in the BLA of CeC-CTB mice. Rspo2 (c) and Ppp1r1b (d) expression in the BLA of CeL/M-CTB mice. Rspo2 (e) and Ppp1r1b (f) expression in the BLA of NAc-CTB mice. Scale bar 250μm.
Figure 15. Activation of NAc fibers of Rsop2+ BLA neurons elicits negative behaviors. Optic fiber was unilaterally implanted above the NAc of Rsop2-ChR2 mice (NAc Rsop2-ChR2). NAc Rsop2-ChR2 underwent behavioral assays. a, Optogenetic freezing test (n = 9). b, Optogenetic self-stimulation test (n = 11). c, Optogenetic place preference test (n = 9). Behavioral performance was compared against Rsop2-ChR2 (Fig. 4) using an unpaired t-test. No significant difference was observed across all assays. d, Optic fiber placement in the NAc of Rsop2-ChR2 mice. Scale bar 500μm.
Figure 16. Circuit model of the BLA. a, Anatomical connections of genetically identifiable populations of amygdala neurons. Projections identified, but cell-type unknown*, hypothetical**. b, The negative circuit of the amygdala (colored). CeC and PL projections are key distinguishing features of Rspo2* BLA neurons to Ppp1r1b* BLA neurons. Rspo2* BLA neurons project the CeC, but the genetic identity of the neurons that are innervated has yet to be identified; one possibility is CeL Calcr* neurons. Nevertheless, if Rspo2* BLA neurons ultimately activate the effector neurons of freezing in the CeM, then an indirect route must be taken through the CeC and/or possibility the intercalated cell (not depicted). c, The positive circuit of the amygdala (colored). CeL, CeM, and IL projections are distinguishing features of Ppp1r1b* BLA neurons to Rspo2* BLA neurons. Ppp1r1b* BLA neurons send dense fibers to the CeL and CeM. Therefore, a population in the CeL and/or CeM may mediate appetitive behaviors; possibly, CeM Tac2 neurons and/or a subpopulation of CeL Prkcd* neurons, which are Penk.
Figure 17. Identification of genetically distinct populations in the CeA. (A-C) Quantification of overlap of Prkcd and Calcr/ in the CeC (A). Quantification of overlap of Prkcd, Sst, Crh, Tac2, and Nts in the CeL (B). Quantification of overlap of Sst, Nts, Tac2, and Crh in the CeM (C). Values represent percent labelling of overlap of genes in column amongst genes in rows.

For example, 56% of CeC Calcr/ neurons coexpress Prkcd (A). Values represent percentage of labeling from totaling all cells counted from n = 3 mice. Hierarchical clustering was performed in the CeL using the percent overlap profile of each gene (B). (D-O) Representative histology of CeA expression of Prkcd and Calcr/ (D), Prkcd and Sst (E), Prkcd and Nts (F), Prkcd and Tac2 (G), Prkcd and Crh (H), Sst and Calcr/ (I), Nts and Sst (J), Nts and Tac2 (K), Nts and Crh (L), Sst and Tac2 (M), Sst and Crh (N), Tac2 and Crh (O) in the anterior CeA (anterior-posterior AP distance from Bregma -0.8mm) and posterior CeA (AP distance from Bregma -1.6mm).

Scale bar, 250μm. (P) 7 major population of neurons that were selected for examination and color selection for subsequent data presentation, CeC Prkcd+ neurons (green), CeL Prkcd+ (light blue), CeL Sst+ (dark blue), CeL Crh+Nts+ Tac2+ (teal), CeM Sst+ (light purple), CeM Nts+ (dark purple), and CeM Tac2+ (magenta) neurons.
Figure 18. Genetically distinct CeA neurons drive appetitive and defensive behaviors (A-G) Behavioral assessment of percent freezing without (OFF) or with (ON) photostimulation (first column) and total number of nose pokes in unstimulated (OFF) or photostimulated (ON) port in self-stimulation experiments (second column) from optogenetic activation of CeC Prkcd+ (A), CeL Prkcd+ (B), CeL Sst+ (C), CeL Crh Nts Tct2+ (D), CeM Sst+ (E), CeM Nts+ (F), and CeM Tac2+ (G) neurons. Representative histology of ChR2 expression in the targeted CeA neurons (third column). Anterior-posterior distribution of ChR2 expression found in Figure S2. ChR2 expression was pseudocolored in correspondence with selected color scheme (Figure 1P). Significance for paired t-test, *P<0.05, **P<0.01, ***P<0.001 (A-G). Anterior-posterior (AP) distance from Bregma (mm), scale bar, 250µm.
Figure 19. Genetically distinct CeA neurons are activated by distinct stimuli (A–E) The percent overlap of Fos within CeC Prkcd+, CeL Prkcd+, CeL Sst+, CeL Crh+Nts*Tac2, CeM Nts*, CeM Sst*, and CeM Tac2- neurons in response to Shock (+) or No Shock (-) (A); Contextual Extinction Recall (+) or Contextual Fear Recall (-) (B); *ad libitum Food (+) or No Food (-) in food-deprived mice (C); Quinine Water (Q), No Water (-) or *ad libitum Water (+) in water-deprived mice (D); Cholecystokinin (CCK) (+) or saline (-) injection in food-deprived mice (E). CeL Crh+Nts*Tac2- neurons were measured by quantifying Fos in Tac2- neurons in the CeL. Significance for unpaired t-test (A, B, C, E) and one-way ANOVA with Bonferroni's multiple hypothesis correction comparing experimental groups with no water control (D), *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (A-G). Values are mean ± s.e.m. from 1-2 sections per mouse from up to n = 4 mice, individual values are shown by black dots, some values that are too large and beyond the limit of the y-axis are not shown.
Figure 20. Inhibition of genetically defined CeA neurons during feeding, drinking, and defensive behaviors (A-G). Behavioral assessment of percent feeding during food presentation in food-deprived mice (first column); percent drinking during water presentation in water-deprived mice (second column); percent freezing during presentation of footshocks on Day 1 (third column) and contextual recall without optogenetic inhibition on Day 2 (fourth column) from optogenetic inhibition of CeC Prkcd* (A), CeL Prkcd* (B), CeL Sst* (C), CeL Crh*Nts*Tac2* (D), CeM Sst* (E), CeM Nts* (F), and CeM Tac2* (G) neurons. Representative histology of eArch3.0 expression and optic fiber placement in the targeted CeA neurons (fifth column). eArch3.0 expression was pseudocolored in correspondence with selected color scheme (Figure 1P). Significance for unpaired t-test, *P<0.05, **P<0.01, ***P<0.001 (A-G). Anterior-posterior (AP) distance from Bregma (mm), scale bar, 250μm.
Figure 21. Monosynaptic retrograde tracing from genetically defined CeA neurons

(A) Quantification of rabies-mediated retrograde labeled $Ppp1r1b^+$ and $Ppp1r1b^-$ neurons in the BLA from genetically defined CeA neurons. Individual points represent the number of retrograde labeled neurons per section in the BLA from $n = 8$ sections per mouse from $n = 3$ mice, bars represent mean ± s.e.m. Arbitrary threshold of 2.5 neurons per section was used to construct a connectivity model (Figure S5E).

(B) Quantification of rabies-mediated retrograde labeled $Prkcd^+$ and $Prkcd^-$ neurons in the CeC from genetically defined CeA neurons. Individual points represent the number of retrograde labeled neurons per single section in the CeC from $n = 3$ sections from 3 mice, bars represent mean ± s.e.m. Arbitrary threshold of 2.5 neurons per section was used to construct a connectivity model (Figure S5E).

(C) Quantification of rabies-mediated retrograde labeled $Prkcd^+$ and $Prkcd^-$ neurons in the CeL from genetically defined CeA neurons. Individual points represent the number of retrograde labeled neurons per single section in the CeL from $n = 3$ sections from 3 mice, bars represent mean ± s.e.m. Arbitrary threshold of 5 neurons per section was used to construct a connectivity model (Figure S5E).

(D-J) Representative histology of rabies-mediated retrograde tracing from CeC $Prkcd^+$ neurons (D), CeL $Prkcd^+$ neurons (E), CeL $Sst^+$ neurons (F), CeL $Crh^+ Nts^+ Tac2^+$ neurons (G), CeM $Nts^+$ neurons (H), CeM $Sst^+$ neurons (I), CeM $Tac2^+$ neurons (J) in the CeA and BLA. Starter cells are labeled with eGFP (green) and mouse line of starter cells are noted (italicized), Rabies virus is labeled with mCherry (red). $Prkcd$ protein (purple) was labeled in the CeA. $Ppp1r1b$ protein (purple) was labeled in BLA. The anterior-posterior (AP) distance from Bregma (mm), scale bar, 250μm.
Figure 22. BLA Ppp1r1b+ and Rspo2+ neurons make monosynaptic excitatory connections to distinct CeA neurons (A-B) The proportion and number of CeC, CeL, and CeM neurons that receive excitatory (black), excitatory and inhibitory (yellow), inhibitory (red), or no response (white) from blue light stimulation of BLA Ppp1r1b-ChR2 fibers (A) or BLA Rspo2-ChR2 fibers (B). Numbers inside bars represent total number of neurons for each case. (C-D) Example voltage clamped traces of IHC or qPCR-confirmed CeC Prkcd+, CeL Prkcd+, CeL Prkcd-, CeM Nts+, CeM Sst+, and CeM Tac2- neurons in response to blue light stimulation of BLA Ppp1r1b-ChR2 fibers (C) or BLA Rspo2-ChR2 fibers (D). Bottom traces represent responses at ~-70mV and top traces represent responses at ~-50mV. Counts of genetically confirmed neurons found in Figure S6J and S6K. (E-F) Representative histology of IHC confirmation of CeL Prkcd+ neurons in BLA Ppp1r1b-ChR2 slices (E) and CeL Prkcd+ neurons in BLA Rspo2-ChR2 slices (F). The anterior-posterior (AP) distance from Bregma (mm), scale bar, 100μm.
The document contains a series of tables and diagrams related to gene expression patterns in different regions of the brain. The tables and diagrams illustrate the expression levels of various genes in different areas, such as CeC, CeL, CeM, Drd1, Drd2, Drd3, Penk, Pdyn, Prkcd, Sat, Nts, Tac1, Crl, Drd1, Drd2, Sat, Prkcd, Penk, Pdyn, Tac1, and Crh. The expressions are quantified using color codes and numerical values, indicating the intensity and distribution of gene expression across different brain regions.
Figure 23. BLA to CeA pathway for appetitive behavior is genetically analogous to corticostriatal circuits. (A-C) Expression of striatal genetic markers in the CeA. Quantification of overlap of Drd2 and Penk in the CeC (A). Quantification of overlap of Pdyn and Penk in the CeL (B). Quantification of overlap of Drd2, Penk, Drd1, Tac1, and Pdyn in the CeM (C). Values represent percent labelling of overlap of genes in column amongst genes in rows. For example, 35% of CeL Penk labeled neurons coexpress Pdyn (B). Values represent percentage of labeling from totaling all cells counted from n = 3 mice. Hierarchical clustering was performed in the CeM using the percent overlap profile of each gene (C). (D-E) Quantification of overlap of striatal markers—Drd1, Drd2, Pdyn, Penk and Tac1—amongst Prkcd+ neurons in the CeC; Prkcd+, Sst+, Nts+, Tac2+, and Crh+ neurons in the CeL; Sst+, Nts+, Tac2+, and Crh+ neurons in the CeM (D). Quantification of overlap of Prkcd, Sst, Nts, Tac2, Crh amongst Drd1+ and Penk+ neurons in the CeC; Pdyn+ and Penk+ neurons in the CeL; Drd1+, Drd2+, Pdyn+, Penk+ and Tac1+ neurons in the CeM (E). Values represent percentage of labeling from totaling all cells counted from n = 3 mice. (F-G) Overlap matrix of genes expressed in the CeL (F) and CeM (G). Values represent percentage of labeling from totaling all cells counted from n = 3 mice and include the percentage of labeling values found in Figure 1. Hierarchical clustering was performed from using the overlap profile of each gene. (H) Graphical summary of genetically distinct CeA populations and their spatial distribution within the CeA. The CeM and CeL populations, though not portrayed intermingled, are intermingled. (I) Representative histology of CeA expression of CeA genetic markers (Prkcd, Sst, Nts, Tac2, Crh) with striatal markers (Drd1, Drd2, Pdyn, Penk, Tac1). Scale bar, 50μm.
Figure 24. Expression of genetic markers in the CeA (A-B) Expression of Calcr, Htr2a, Nts, Prkcd, Sst, and Tac2 in the CeA (A). Quantification of Calcr, Htr2a, Nts, Prkcd, Sst, and Tac2 smFISH expression in the CeC, CeL, anterior CeM (aCeM) and posterior CeM (pCeM) (A). Genes with no obvious expression in a CeA subdivision was not quantified for that subdivision. Boundaries for the CeC, CeL, aCeM, and pCeM depicted in cartoon (B). Representative histology of expression of gene markers in the CeA (B). Values represent mean ± s.e.m of percentage of labeling out of total number of cells using the nuclear marker DAPI from n = 3 mice (A). Anterior-posterior (AP) distance from bregma (mm), scale bar, 250µm. Astr, amygdala striatal transition zone. (C) Proportion of CeL Gad1+ neurons that are CeL Prkcd+, CeL Sst+, and CeL Crh+Nts+ Tac2+ neurons and the proportion of CeM Gad1+ neurons that are CeM Nts+, CeM Sst+, and CeM Tac2+ neurons. Prkcd, Sst, and Tac2 label 96% of CeL Gad1+ neurons, while 100% of CeL Prkcd, Sst, and Tac2 neurons are Gad1+. Nts, Sst, and Tac2 label 94% of CeM Gad1+ neurons, while 100% of CeM Nts, Sst, and Tac2 neurons are Gad1+. Representative histology of Prkcd (red), Sst (red), Tac2 (red), and Gad1 (green) in the CeL and Nts (red), Sst (red), Tac2 (red), and Gad1 (green) in the CeM (lower panel). Values represent mean ± s.e.m from n = 3 mice (A). Scale bar, 125µm (B). (D) Htr2a colabelling with Crh, Prkcd, Sst, Nts, and Tac2 in the CeL and/or CeM. White circles circles represent Htr2a+ double labelled with corresponding genetic marker. Scale bar, 50µm.
Figure 25. Genetic targeting of distinct CeA neurons (A) Represented histology of viral targeting of distinct CeA neurons. The expression of ChR2 is shown across the anterior-posterior axis of the CeA using 100nL of a Cre-dependent ChR2 containing viral vector for targeting distinct CeA nuclei in Cre-expressing mice. Scale bar, 250μm. (B) Optogenetic assessment of freezing (first column) and self-stimulation (column 2) in Cre-+ mice of implants targeting the CeC (first row), CeL (second row), and CeM (third row). *P<0.05.
Figure 26. *Fos* expression in genetically defined CeA neurons (A) Representative histology of *Fos* expression in response to several of the stimuli from quantified data in Figure 3. Experimental groups (odd rows) and control groups (even rows). (B) Quantification of *Fos* expression in CeC *Calcrt* neurons in response to shock or no shock (B). Values are mean ± s.e.m. from 2 sections per mouse from n = 3 mice. Significance for unpaired t-test, *P* < 0.05. (C) Representative histology of *Fos* expression in CeC *Calcrt* neurons in response to Shock or No Shock (C). White circles represent *Calcrt* neurons double labelled with *Fos* (C). Scale bar, 50µm.
Figure 27. CeM Drd1+ neurons are required for feeding and drinking (A-C) Feeding (A), drinking (B), and freezing (C) behavior in response to inhibition of CeM Drd1+ neurons. Significance for paired t-test, *P<0.05, ***P<0.001. (D) Representative histology of eArch3.0 expression in the CeM of Drd1-cre mice. Scale bar, 250μm. (E-F) Raster plots of feeding (E) and drinking (F) bouts from inhibition of CeM Drd1+ neurons (Cre+ mice 9-16) or controls (Cre- mice 1-8). Time bins, 1s.
Figure 28. Anatomical model of genetically defined CeA neurons
(A) Quantification of retrogradely labelled PAG-projecting CTB+ neurons with genetic markers in the CeA. Values represent mean ± s.e.m. the percentage of CTB+ neurons that the corresponding gene labels from n = 3 mice. No CTB+ neurons were found in the CeC, thus CTB quantification was not performed for CeC Prkcd+ neurons. (B) Representative histology of PAG CTB injection site. Scale bar, 1mm. (C) Representative histology of CTB+ neurons in the CeA with Prkcd, Crh, Nts, Sst and Tac2 expression. Magnified micrographs of the CeL, white circles represent double labelled cells (fourth column). Scale bar 200 μm (column 1-3), 50 μm (column 4). (D) Anterograde fibers from genetically distinct CeA populations. Anterograde fibers in the PAG of several CeA ChR2-eYFP mice are shown. Scale bar, 250 μm. (E) Helper virus and rabies virus injected into the CeA of Cre- mice, no visible immunofluorescence of helper (eGFP) or rabies virus (mCherry) was found in n = 3 mice. (F) Targeting of rabies virus in distinct CeA cell populations. The values represent the mean ± s.e.m of percentage of accurately targeted starter cells (mCherry*eGFP+) amongst all starter cells. Cell counts are from 2 sections (anterior and posterior) per mice from n = 3 mice.
Figure 29. Intrinsic electrophysiological properties and BLA connectivity of genetically defined CeA neurons (A-B) Resting membrane resistance of neurons in the CeC, CeL, and CeM (A) and genetically confirmed neurons in the CeC, CeL, and CeM (B). Red points represent neurons recorded from Ppp1r1b-cre mice green points represent neurons recorded from Rspos2-cre mice. Resting membrane resistance of CeC neurons is significantly lower than that of CeL and CeM neurons. Significance for one-way ANOVA with Bonferroni's multiple hypothesis correction, ****P<0.0001. (C-D) Resting membrane potential of neurons in the CeC, CeL, and CeM (C) and genetically confirmed neurons in the CeC, CeL, and CeM (D). Red points represent neurons recorded from Ppp1r1b-cre mice green points represent neurons recorded from Rspos2-cre mice. Resting membrane potential of CeC neurons is significantly lower than that of CeL and CeM neurons. Significance for one-way ANOVA with Bonferroni's multiple hypothesis correction, ****P<0.0001. (E) qPCR traces for confirming genetic identity of CeA neurons. Single cell qPCR traces for Prkcd from CeC neurons. Single cell qPCR traces for Nts, Sst, and Tac2 from CeM neurons. rfu, relative fluorescence units (units are scaled down 10^6). (F-G) Latency of connection from BLA Ppp1r1b+ neurons (F) and BLA Rspos2i neurons (G) to CeC, CeL, and CeM neurons. (H-I) Latency of excitatory (E) and inhibitory (I) responses from BLA Ppp1r1b+ (H) and BLA Rspos2i (I) in the CeC, CeL, and CeM. (J-K) The type of connectivity from BLA to genetically defined populations of CeA neurons. The number and type of genetically confirmed neuron sorted by the type of response from BLA Ppp1r1b+ (J) and BLA Rspos2i (K) neurons. Each circle represents a single genetically confirmed neuron.
A

CeC

CeL

aCeM

pCeM

B

C

D

E

Drd1+/Nts+Sst+Tac2+

Nts+Sst+Tac2+/Drdl+

Colabelling (%)
Figure 30. Expression of striatal markers in the CeA (A-B) Expression of Drd1, Drd2, Pdyn, Penk, and Tac1 in the CeA. Quantification of Drd1, Drd2, Pdyn, Penk, and Tac1 smFISH expression in the CeC, CeL, anterior CeM (aCeM) and posterior CeM (pCeM) (A). Genes with no obvious expression in a CeA subdivision was not quantified for that subdivision. Boundaries for the CeC, CeL, aCeM, and pCeM depicted in cartoon (B). Representative histology of expression of gene markers in the CeA (B). Values represent mean ± s.e.m of percentage of labeling out of total number of cells using the nuclear marker DAPI from n = 3 mice (A). Anterior-posterior (AP) distance from bregma (mm), scale bar, 250μm (B). Astr, amygdala striatal transition zone. (C-D) Representative histology of double labelling of striatal markers in the CeA. Magnified micrographs of Drd1 expression with Drd2, Pdyn, Penk, and Tac1 (D). Circles represent double labelled neurons (D). Anterior-posterior (AP) distance from bregma (mm), scale bar, 250μm (C), 50μm (D). (E) Proportion of CeL Drd1+ neurons that are CeM Nts+, CeM Sst+, and CeM Tac2+ neurons. Nts, Sst, and Tac2 label 95% of CeM Drd1+ neurons, while 97% of CeM Nts, Sst, and Tac2 neurons are Drd1+. Quantification from 2 section per mouse from n = 3 mice.
Figure 31. Summary of anatomical and genetic results. (A) Structural and functional model of cell-type specific BLA to CeA connectivity derived from monosynaptic rabies tracing experiments (Figure 2 and 5). BLA Rsps2 neurons mainly innervate CeC Prkcd neurons, which in turn innervate several CeA neurons that mediate appetitive behaviors. BLA Ppp1r1b neurons innervate all CeA neurons in the model, CeA neurons that mediate appetitive behaviors as well as CeA Prkcd neurons, which in turn innervate CeA neurons that mediate appetitive behaviors. (B) Graphical summary of genetically distinct CeA populations and their spatial distribution within the CeA. CeM neurons (Figure 1, 7), though not portrayed intermingled, are intermingled. CeL neurons are overall intermingled but also have a slight spatial segregation as depicted in the cartoon.
Figure 32. Long range circuit model for valence-related behaviors. This model takes into consideration published works, anatomical data, preliminary data to derive a circuit model for valence-related behaviors. The gray lines denote hypothetical connections and gray arrows denote hypothetical function or less resolved hypothetical connection. LA, lateral amygdala; BLA, basolateral amygdala; CeC, capsular nucleus of the central amygdala; CeL, lateral nucleus of the central amygdala; CeM, medial nucleus of the central amygdala; mplTC, medial paracapsular intercalated cells; Astr, amygdala-striatal transition zone; LPBN, lateral parabrachial nucleus; IPAG, lateral periaqueductal gray; vIPAG, ventrolateral periaqueductal gray; PFC, prefrontal cortex; aPFC, anterior prefrontal cortex; pPFC, posterior prefrontal cortex; PVT, paraventricular thalamus; aPVT, anterior paraventricular thalamus; pPVT, posterior paraventricular thalamus; BNSTpr, principle nucleus of the bed nucleus of the stria terminalis; BNSTov, oval nucleus of the bed nucleus of the stria terminalis; BNSTal/am, anterolateral and anteromedial nucleus of the bed nucleus of the stria terminalis; NAc, nucleus accumbens; aNAc, anterior nucleus accumbens; pNAc, posterior nucleus accumbens.
Figure 33. Genetic diversity and c-Fos activation in the PAG (A) *In situ* hybridization of expression of *Gad1*, *Penk*, *Sst*, and *Tac1* in the PAG. (B) Quantification of cells per section that were positive for corresponding gene. Values represented by heat gradient from 0, lowest, to 36, highest, cells per 20µm section, genes were quantified in the posterior 2/3s “hotspot” of the PAG. (C) Double *in situ* hybridization of expression all combinations between *Gad1*, *Penk*, *Sst*, and *Tac1* in the PAG. (D) Quantification, percentage (%) of double labelling of genes. The percent labelling of overlap (%) is shown. For instance, 85% of *Gad1*+ neurons are co-labelled with *Sst*. (E) Example micrographs of *in situ* hybridization of double labelling between gene markers and c-Fos in response and shock. (F) Quantification of c-Fos+ cells per 20µm section, c-Fos was quantified in the posterior 2/3s “hotspot” of the PAG. (G) Quantification of double labelling with c-Fos in the iPAG (top row) or vIPAG (bottom row) in response to shock (+) or no shock (-) (column 1-4) or water (+) or no water (-) in water deprived mice (column 5-8). Values represent the percentage of co-labelled neurons as a proportion to the total number of corresponding gene expressing neurons (column 1-3,4-7). The number of *Gad* cells that were c-Fos+ per 20µm section was also quantified as a reflection of c-Fos+ excitatory neurons (column 5,8). **P < 0.01, ***P < 0.001, ****P < 0.0001. iPAG, lateral periaqueductal gray; vIPAG, ventrolateral periaqueductal gray; dIPAG, dorsolateral periaqueductal gray.
Figure 34. The genetic organization in the BNST is analogous to the CeA (A) In situ hybridization expression of Cck, Crh, Nts, Prkcd, Sst, Tac2 in the BNST. (B) Double label in situ hybridization expression of genetic markers in the BNST. (C) Anatomy of boundaries and quantification of expression of genetic markers in the BNST. Values represent the total number of cells per 20μm section as a heat map. (D) Percent overlap between genetic marker representing in the same fashion as Figure 17a and Figure 33d. Based on the overlaps between genetic markers we find genetically distinct species in the BNST similar to that of the CeA. More specifically, the BNSTpr is analogous to the CeC; BNSTov is analogous to the CeL; BNSTal and BSTam is analogous to the CeM. CeC, capsular nucleus of the central amygdala; CeL, lateral nucleus of the central amygdala; CeM, medial nucleus of the central amygdala; BNSTpr, principle nucleus of the bed nucleus of the stria terminalis; BNSTov, oval nucleus of the bed nucleus of the stria terminalis; BNSTal, anterolateral nucleus of the bed nucleus of the stria terminalis; BSTam, anteromedial nucleus of the bed nucleus of the stria terminalis.
Table 1.

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<th>Genetic and Anatomical Characterization of Rspo2* and Ppplrlb* BLA neurons</th>
<th>( Rspo2^* )</th>
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<th>( Rspo2^<em>Ppplrlb^</em> )</th>
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<td>Total Neurons (n = 3)</td>
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<td>2311</td>
<td>54</td>
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<td>Mean Proportion (%)</td>
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<td>39.1 ± 1.14</td>
<td>0.970 ± 0.191</td>
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<th>( Rspo2^* )</th>
<th>( Gad1^* )</th>
<th>( Rspo2^<em>Gad1^</em> )</th>
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<td>112</td>
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<th>( Ppplrlb^* )</th>
<th>( Gad1^* )</th>
<th>( Ppplrlb^<em>Gad1^</em> )</th>
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<td>Total Neurons (n = 1)</td>
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<td>116</td>
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<tr>
<th>((Rspo2+Ppplrlb)^<em>Camk2^</em>)</th>
<th>((Rspo2+Ppplrlb)^<em>Camk2^</em>)</th>
<th>((Rspo2+Ppplrlb)^<em>Camk2^</em>)</th>
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<td>Mean Proportion (%)</td>
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<td>Mean Proportion (%)</td>
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Morphological and Electrophysiological Characterization of Rspo2* and Ppplrlb* BLA neurons

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<th>Vm (mV)</th>
<th>Rm (MO)</th>
<th>Cm (pF)</th>
<th>Spike threshold (mV)</th>
<th>Rheobase (pA)</th>
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<td>Magnocellular (n = 37)</td>
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<td>( Rspo2^* ) (n = 10)</td>
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<td>108.9 ± 9.6</td>
<td>190.3 ± 19.1</td>
<td>-37.6 ± 0.9</td>
<td>198.7 ± 18</td>
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<td>( Ppplrlb^* ) (n = 11)</td>
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<td>-57.1 ± 1.5</td>
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<td>99.5 ± 5.9</td>
<td>-34.8 ± 1.1</td>
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<th>Vm (mV)</th>
<th>Rm (MO)</th>
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<th>Rheobase (pA)</th>
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<td>( Rspo2^* ) (n = 10) vs ( Ppplrlb^* ) (n = 11)</td>
<td>0.00002</td>
<td>0.051</td>
<td>0.0016</td>
<td>0.0009</td>
<td>0.06</td>
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<td>Magno. (n = 27) vs ( Rspo2^* ) (n = 10)</td>
<td>0.5</td>
<td>0.4</td>
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<td>0.7</td>
<td>0.9</td>
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<td>0.6</td>
<td>0.3</td>
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METHODS

Subjects. Wild-type C57BL/6J (Stock #000664), mice were obtained from Jackson Laboratory. Cartpt-Cre (Stock #036659-UCD), produced through the GENSAT project, was obtain from Mutant Mouse Resource and Research Center (MMRRC). Cartpt-Cre mice were backcrossed to C57BL/6J for 2 generations. Rspos2-cre mice was generated using a bacterial artificial chromosome (BAC) clone (RP32-39M21) with a Cre construct driven by the regulatory elements of Rspos2. Experiments were performed in mice 8-16 weeks of age. All subjects were male mice. All subjects were cared and maintained in accordance with protocols approved by the Massachusetts Institute of Technology (MIT) Committee on Animal Care (CAC) and guidelines by the National Institutes of Health (NIH).

Viruses. The mouse minimal fos promoter (-623 to +1050 from the transcriptional start site) followed by advanced tTA was cloned into an adeno-associated virus (AAV) backbone to generate the pAAV-cfos-tTA vector. cDNA clone of mouse Pabpc1 with a C-terminal Myc-DDK tag (Origene, Cat. #MR209653) was subcloned into the pAAV-TRE-ChR2-EYFP plasmid to generate the pAAV-TRE-PABP-FLAG plasmid. AAV plasmids were packaged into AAV9 vectors by the Gene Therapy Center and Vector Core at the University of Massachusetts Medical School. AAV5-Ef1a-DIO-eArch3.0-eYFP (AV5257), AAV5-Ef1a-DIO-ChR2-eYFP (AV5226B), and AAV5-Ef1a-DIO-eYFP (AV431OD) was obtained from University of North Carolina at Chapel Hill Vector Core. AAV5-Ef1a-ChR2-eYFP (CS0633-3CS) was obtained from University of Pennsylvania School of Medicine Vector Core.

Stereotactic injections. Subjects undergoing stereotactic injections were anaesthetized under isoflurane. Standard stereotactic procedures were used. Viruses were injected using a mineral
oil filled glass micropipette attached to a 1μL microsyringe. For activity-dependent transcriptional profiling, 200nL of ~2.0×10⁸ GC of AAV9-cfos-tTA and AAV9-TRE-PABP-FLAG (1:1 mixture) were bilaterally injected into the BLA (distance from bregma, AP-1.4mm, ML±3.3mm, DV-4.85mm) of doxycycline (Dox) fed mice and incubated for 7 days prior to downstream experiments. For behavioral experiments, 200μL of viral stocks of AAV5 cre-dependent viruses was injected into the BLA of Rsps2-cre (AP-1.6mm, ML±3.3mm, DV-4.85mm) and Cartpt-cre mice (AP-2.0mm, ML±3.4mm, DV-4.9mm) and incubated for 3-4 weeks prior to behavioral experiments. For retrograde tracing, Alexa Fluor 555-conjugated cholera toxin subunit B (CTB) (1μg/μL) was unilaterally injected into the CeC (50nL, AP-1.0, ML+2.9, DV-4.5), CeL/M (100 nL, AP-1.34mm, ML+2.9mm, DV-4.6), NAc (300nL, AP+1.0, ML+.75, DV-4.8) and incubated for 7 days prior to sacrifice. Alexa Fluor 555 and 647-conjugated CTB was injected into the PL (200nL, AP+1.75, ML+.3, DV-2.3) and IL (200nL, AP+1.75, ML+.3, DV-3.0) and incubated for 10 days prior to sacrifice. For brain slice electrophysiological experiments, 200nL of AAV9-Ef1a-DIO-ChR2-eYFP was injected into the BLA of 4-5 week old Rsps2-cre and Cartpt-cre mice and incubated 4 days prior to electrophysiological experiments.

**Fiber implantation.** 5.0mm Mono fiberoptic cannulas (Doric Lens) was implanted (unilaterally or bilaterally, depending on the experiment) above the BLA of Rpso2-cre and Cartpt-cre (AP-2.0mm, ML+3.3mm, DV-4.3), and above the NAc of Rsps2-cre (AP+1.3mm, ML+.75mm, DV-4.0). Once positioned above the BLA, the mono fiberoptic cannula was cemented using dental cement (Teets cold cure; A-M Systems) to the skull, which contained 2 screws that lied medially to the implant site. Once the dental cement cured, a protective cap surrounding the implant, made using a 1.5mL black Eppendorf tube, was fixed onto the implant using dental
cement. Mice spent 3-4 week post-operation for recovery. Mice were handled by investigator 2-3 days prior to behavioral experiments.

**RNA immunoprecipitation.** 12 wild-type male mice kept on Dox diets were bilaterally injected with AAV\(_9\)-c-fos-tTA and AAV\(_9\)-TRE-PABP-FLAG virus. One week post-operation, mice were taken off a Dox diet for 2 days and underwent a fear conditioning protocol (3 shocks, 0.75mA, 2s duration) or exposed to a female mouse in the home cage for 2hrs. Immediately after, mice were returned to a Dox diet. 2 days later, mice were anaesthetized with isoflurane and were sacrificed by decapitation. 2 control mice were kept on a Dox diet. Brains were dissected, flash frozen on dried ice, and stored in -80°C until RNA immunoprecipitation. RNA immunoprecipitation was performed in a similar fashion as described by the McKnight Lab (University of Washington). Brains were thawed for 30min in a -16°C cryostat; 300μm sections across the BLA were collected. Using a razor blade, the BLA's was crudely dissected 2 mice brains and were collected into a single 1.5mL microcentrifuge tubes. This yielded ~30μg of brain tissue. 1mL of homogenization buffer (HB, 1% NP-40, 100mM KCl, 50mM Tris pH 7.4, 12mM MgCl\(_2\), 200U/mL Promega RNasin, 1mM DTT, 100μg/mL cyclohexamide, 1mg/mL heparin, 1% protease inhibitors (P8340, Sigma)). Samples were transferred to a 2mL dounce homogenizer and homogenized using pestle A and, subsequently, pestle B. Homogenized samples were transferred to 1.5mL microcentrifuge tube and were centrifuged at 10,000 rcf. Supernatant was separated into a new microcentrifuge tube, 5μL of anti-FLAG (F742S, Sigma) was added and incubated for 6hrs at 4°C. 200 μL Pierce A/G Magnetic Bead were washed in HB, added to the homogenates, and incubated overnight at 4°C. Magnetic beads were separated using a magnetic tube rack and washed 3 times in a salt buffer (.3M KCl, 1% NP-40, 50mM Tris pH 7.4, 12mM MgCl\(_2\), 100μg/mL cyclohexamide, .5mM DTT). Protein-RNA
complexes were dissociated from magnetic bead by vortexing samples in lysis buffer (RLT
lysis buffer from Qiagen RNease Kits with 10μL/mL β-Mercaptoethanol). Magnetic beads were
drawn off and RNA was isolated using the Qiagen RNAeasy Micro Kit. RNA samples were
stored in -80°C until further downstream experiments.

**RNA analysis.** RNA samples were analyzed using the Affymetrix Mouse 430 2.0 chip by MIT
BioMicroCenter. CEL files from the Mouse 430 2.0 chip were normalized by RMA or MAS5
through the Affymetrix Expression Console Software. Subsequently, CHP files were analyzed
through Affymetrix Transcriptome Analysis Console 2.0—3 samples from the shocked mice
and 3 samples from the female exposed mice were grouped. The data from this analysis has
been deposited to the NCBI Gene Expression Omnibus (GEO), accession number GSE78137.

**Screening and selecting BLA gene marker candidates.** Based on the data obtained from
the array, the top gene candidates, independent of statistical significance, enriched in either
the RMA or MAS5 normalized data set were screen on Allen Mouse Brain Expression Atlas
(http://mouse.brain-map.org/). Based on expression patterns in the BLA, 16 gene candidates
that were enriched in the shock group were selected—**Acrv1c, Cdh9, Crhbp, Gabra1, Gabra2,
Gria4, Htr2c, Htr3a, Nptx2, Nrxn3, Pthlh, Pcdh18, Rspo2, Sema5a, Slc30a1, Zfpm2.** Based on
expression patterns in the BLA, 21 gene candidates that were enriched in the female group
were selected—**Adrbk1, Aig1, Esrra, Gipc1, Gpr39, Gpr137, Gpr165, Gria1, Grin1, Oprl1,
Neurl1a, Nos1, Nos1ap, Ntrk3, Ntng2, Penk, Ppp1r1b, Slc24a4, Slc30a3, Stx1a, Synpo.**

Interneuronal markers—**Calb1, Npy, Sst, Vip, Pvalb—and pyramidal cell markers—**Camk2a,
Thy1**—were selected as positive controls.
**Tissue.** For the screening of candidate gene markers, wild-type mice 12-16 week old were anaesthetized with isoflurane and were sacrificed by decapitation. Brains were quickly dissected and immediately flash frozen on aluminum foil on dried iced and stored in -80°C. A single session of sectioning consisted of 12 wild-type brains and 60 Superfrost Plus slides (25x75mm, Fisherbrand). 30min prior to sectioning, brains were equilibrated to -16°C in a cryostat. Brains were serially sectioned coronally at 20μm and thaw-mounted onto slides. Each mouse brain produced one section on each of 60 slides—sections from AP-0.8mm to AP-2.0mm were taken from each brain. Sections from each subsequent brain started in a staggered fashion (begun on the 6th, 11th, 16th, etc. slide). Therefore, each slide resulted with 12 coronal brain sections representing .1mm intervals between AP-0.8mm to AP-2.0mm. Brains were dried at room temperature for 30min prior to storage at -80°C. In order to obtain a homogenous representation of the BLA, no more than 2 sections were lost during sectioning of a single brain.

For single molecule fluorescent in situ hybridization, mouse brains were collected through the flash frozen method (as described above). Using a cryostat, an individual brain was serially sectioned and thaw-mounted onto Superfrost Plus slides. Coronally cut brain slides were serially sectioned at 20μm onto 10 slides, each slide contained 11 to 12 brain sections, spaced .2mm apart spanning AP-0.8mm to AP-2.8mm. Sagittally cut brain slides was serially sectioned at 20μm onto 8 slides, each slide contained at least 12 brain sections, spaced .16mm apart spanning ML3.8mm to ML2.8mm. Slides were dried at room temperature for 60 min prior to storage at -80°C.
For immunohistochemistry, mice were euthanized by avertin overdose, perfused with 1X phosphate-buffered saline (PBS) and 4% paraformaldehyde. Brains were dissected and stored in 4% paraformaldehyde at room temperature for 8 to 12 hrs, prior to storage in 1X PBS at 4°C. Coronally cut brains were sectioned at 50μm on a vibratome and serially collected into 4 wells. Each well contained coronal sections spaced .2mm apart.

**Fluorescent in situ hybridization**

Single-label fluorescent in situ hybridization (FISH) was performed using RNA probes generated from the pCRII-TOPO Vector (ThermoFisher). pCRII-TOPO vector, cut with EcoRI, was used as the backbone for cloning cDNA of candidate gene markers. Mouse brain cDNA was obtained via reverse transcription using the Omniscript RT kit (Qiagen) of RNA extracted from mouse brains (Qiagen RNeasy Lipid Tissue Mini Kit). PCR primers were the same as the forward and reverse primers reported on Allen Mouse Brain Expression Atlas with the addition of 5’-CAGTGTGCTGGAATT-3’ and 5’-GATATCTGCAGAATT-3’ to the 5’ end of the forward primer and reverse primer, respectively. PCR products for each candidate gene was isolated and cloned into the pCRII-TOPO backbone using TOPO cloning (Clontech In-Fusion HD) and maintained in Stellar Competent Cells (Clontech). RNA probes were generated by cutting pCRII-TOPO plasmids with HindIII and transcribing the anti-sense strand using a T7 RNA polymerase (NEB, HiScribe T7 High Yield RNA Synthesis Kit) with digoxygenin-labelled UTP (Roche). Digoxigenin-labelled anti-sense RNA probes were isolated (Qiagen, RNeasy Mini Kit) and stored in -80°C.

Tissue preparation of single label FISH was performed similar to standard mouse brain FISH protocols. On day 1, slides were fixed in 4% paraformaldehyde at 4°C, washed twice in
phosphate buffer pH 7.4, rinsed in diethylpyrocarbonate (DEPC) water and tetraethanolamine (TEA) buffer, pretreated with acetic anhydride, washed in 2x saline-sodium citrate solution (SSC), washed in increasing concentrations of ethanol (70%, 95%, 100%), delipidated with chloroform, washed with decreasing concentrations of ethanol (100%, 95%). The probes were dried for 2hrs at room temperature. RNA probes were denatured at 100°C then cooled on ice, then were applied onto slides in a solution of hybridization buffer and tRNA and coverslipped for overnight incubation at 60°C. Day 2, slides underwent post-hybridization stringency washes—2X Saline-Sodium Phosphate-EDTA buffer (SSPE), 50% formamide in 2X SPPE at 60°C, and twice in .1X SSPE at 60°C. Endogenous peroxidase activity was removed with .3% hydrogen peroxide in Tris-NaCl-Tween (TNT) buffer, followed by 3 washes in TNT buffer. Slides were blocked in TNT buffer with blocking reagent (PerkinElmer) (TNB) prior to being incubated with 1:100 peroxidase-conjugated anti-digoxygenin FAB fragment (anti-dig-POD, Roche) for 2hrs at room temperature. Anti-dig-POD was removed by a series of 3 TNT washes. Alexa 594-conjugated tyramide signal amplification (TSA) solution was applied over the slides for 30min, and then washed away with a series of 3 TNT washes. Slides were coverslipped and mounted using VectaShield mounting solution containing DAPI (Vector Laboratories). Percent labelling was calculated as the number of Gene+ cells as a percentage of the total large (<10µm) DAPI+ BLA cells, which is an indirect indicator of BLA principle cells as shown from quantification of Camk2+ (Figure 1).

**Single molecule fluorescent in situ hybridization.** Single molecule fluorescent in situ hybridization (smFISH) was performed using RNAscope Fluorescent Multiplex Kit (Advanced Cell Diagnostics). Custom C1 and C2 DNA oligo probes were designed for Rspo2, and Ppp1r1b, Camk2a, and Gad1 probes were available on the Advanced Cell Diagnostics.
Catalog. Brain sections were fixed in 4% paraformaldehyde for 15min, and then washed in 50%, 70%, 100%, 100% ethanol for 5min each. Slides were dried for 5min. Proteins were digested using protease solution (pretreatment solution 3) for 60-90s on wild-type tissues, 30s on CTB expressing tissues, and 5-10sec on EYFP expressing tissues. Immediately following, slides were washed twice in PBS. In parallel, C1 and or C2 probes were heated in a 40°C water bath for 10min. Probes were applied to the slides, coverslipped, and placed in a 40°C humidified incubator for 3hrs. Slides were rinsed twice in Rnascope wash buffer, and then underwent the colorimetric reaction steps according to standard kit protocol using AMP4A (C1-green, C2-red) or AMPB (C1-red, C2-green) depending on the color combination of choice. After the final wash buffer, slides were immediately coverslipped using ProLong Diamond Antifade mounting medium with DAPI (ThermosFisher).

Immunohistochemistry. Free floating brain sections were washed in PBST (1X PBS, 3% TritonX) 3 times for 10min, blocked for 1 hour in blocking buffer (PBST, 5% normal goat serum), incubated in primary antibody in blocking buffer overnight at 4°C. Next day, brains were washed in 3, 10min washes of PBST and incubated in secondary antibody in blocking buffer at room temperature for 2hrs. Primary antibodies used were rabbit anti-FLAG (F7425, Sigma, 1:1000), chicken anti-GFP (Invitrogen, A10262, 1:1000), rabbit anti-fos (Santa Cruz, sc-52, 1:2000). Secondary antibodies used were goat anti-chicken Alexa Fluor 488 (Invitrogen, A11039, 1:1000), goat anti-rabbit Alexa Fluor 555 (Invitrogen, A21428, 1:1000). After 3 additional 10 min PBST washes, slides were coverslipped and mounted using VectaShield mounting solution containing DAPI (Vector Laboratories).

For immunohistochemistry of electrophysiological experiments, brain slices were washed in PBST 3 times for 10min, blocked for 1 hour, and then incubated with chicken anti-GFP
(Invitrogen, A10262, 1:1000), to visualize ChR2-eYFP fibers, and CF555 Streptavidin (Biotium, 29038, 1:100), to visualize recorded neurons, overnight at 4°C. Next day, slices were washed in PBST 3 times for 10min, and then incubated in chicken anti-GFP (Invitrogen, A10262, 1:1000) for 2hrs at room temperature. After 3 additional 10 min PBST washes, slides were dried at room temperature for at least 6hrs prior to coversliping and mounting using VectaShield mounting solution containing DAPI (Vector Laboratories).

**Microscopy and histological representation.** Micrographs were obtained using a Zeiss fluorescent microscope or Zeiss AxioImager M2 confocal microscope using Zeiss ZEN (black edition) software. Main figure representations were colored green for Rspo2+ neurons and red for Ppp1r1b+ irrespective of native fluorescent labelling. CTB labeling were distinctly colored in order for visually distinguish different CTB experiments (Figure 15).

**c-Fos experiment.** Wild-type mice were handled by investigator once each day for 3 days prior to stimulus exposure. Stimulus exposure experiments occurred within 1 hour of the dark cycle. Shocked mice, were exposed to a fear conditioning chamber (Med Associates) for 500 s and received 3 footshocks (0.75mA, 2s duration), then returned to home cage where water and food were removed. Female exposed mice were transported in home cages to an experimental room and were exposed to a wild-type female mouse. Context-exposed mice were exposed to the fear conditioning chamber for 500 s then returned to the home cage water and food were removed. Odor exposed mice were transported in home cages to an experimental room; water and food were removed; and habituated for 4hrs prior to odor exposure. 1mL of TMT (10% TMT in dH₂O), 1mL of Peanut Oil, or 1mL of BA (0.25% benzaldehyde in 70% ethanol) were pipetted into the center of the home cage. Water deprived (overnight) wild-type mice were
Transported in home cages to an experimental room, food removed, and habituated for 4hrs prior to hydration. A bottle of water, quinine water (.05% quinine hydrochloride dihydrate), sucrose water (5% sucrose), or an empty water bottle without a spout, was carefully placed into the home cage. 90min after initial exposure, mice were sacrificed using avertin overdose and perfused for immunohistochemical analysis of c-FOS. For smFISH of c-fos, mice underwent the same stimulus exposure protocol, but were sacrificed using the flash freezing method (described above) 15min after end of the stimulus exposure or in the case of water exposure, 15min after satiety, which took <5min after water exposure. Background signals levels of c-FOS and c-fos/Rspo2 or Ppp1r1b were adjusted on ZEN, and were exported into image files for quantification in a blind fashion.

The relative c-FOS expression for the aBLA was calculated by \( \frac{\text{number of c-FOS}^+ \text{ cells in the aBLA}}{\text{total number of c-FOS}^+ \text{ cells in the BLA}} \), likewise the pBLA was calculated by \( \frac{\text{number of c-FOS}^+ \text{ cells in the pBLA}}{\text{total number of c-FOS}^+ \text{ cells in the BLA}} \). The aBLA and pBLA was determined using mouse brain atlas boundaries of the aBLA and pBLA. The relative c-FOS expression of the aBLA and pBLA are mutually exclusive. Thus, when statistically comparing values between different conditions, significance values for comparison of the aBLA and comparison of the pBLA were redundant. Because of this, only the statistics for the aBLA was graphically represented (Figure 3d-f). IHC C-FOS counting was performed for unilateral BLAs of 50\( \mu \)m sections. smFISH c-Fos counting was performed for unilateral BLAs of 20\( \mu \)m sections. The AP position was determined by a mouse brain atlas and was accurate within \( \pm .2 \)mm.

**Fear conditioning.** On day 1, mice were placed in to a fear conditioning chamber (Med Associates) while being bilaterally hooked up to optic fiber patchcords (Doric Lens) for 500s
and received shocks during the 198s, 278s, 358s time point. For optical inactivation experiments, simultaneously with the shocks at the 198s, 278s, 358s time points, a constant pulse of 532nm light (10-15mW) was delivered through the optical cannulas for duration of 20s; for optical activation experiments, 20Hz 473nm (10-15mW) light was used. On day 2, mice were hooked up to optic fiber patchcords and placed to the fear conditioning chamber for 180s, where no shock or laser was delivered. Freezing behavior was scored manually using JWatchers1.0 in a blind fashion.

**Reward conditioning.** Water-restricted mice were placed in an operant conditioning chamber (Island Motion) with one reward port equipped with a cue light. At the start of each trial, the onset of the cue light signals the availability of water reward contingent on a nose poke, lasting 5s. Upon a successful nose-poke any time during the 5s, a reward was immediately delivered through a water spout in the port and the cue light is turned off. A TTL triggering a laser pulse, bilaterally delivered to the implanted optic cannula, was issued at the same time as the reward delivery. For optical inactivation experiments, a constant 10 second pulse of 532nm light (10-15mW) was used; for optical activation experiments, a 10s 20Hz pulse train of 15ms pulses of 473nm light (10-15mW) was used. During the following inter-trial interval of randomly distributed between 10 and 15s, nose pokes did not elicit water rewards. Timestamps for cue light, nose pokes and reward deliveries were logged and analyzed with Matlab. Data arrays were constructed from the first rewarded trial to 150 trials thereafter. Total number of pokes was counted for the period. Percent in-port time during the cue presentation were calculated in 100ms time bins and quantified with a z-score procedure \( z = (x - \mu) / \sigma \) where \( x \) is the average percent time spent in the reward port, \( \mu \) and \( \sigma \) are the mean and standard deviation of percent time spent in the reward port during the 5s baseline period before cue onset.
Optogenetic freeze test. On day 1, mice were placed in to a fear conditioning chamber for 360s. 20Hz 473nm light (10-15mW) was unilaterally delivered through the optic cannula at the 180s time point for 180s. On day 2, mice were hooked up to fiber optic patchcords and returned to the fear conditioning chamber for 180s without light stimulation. Freezing behavior was scored manually using JWatchers1.0 in a blind fashion.

Optogenetic self-stimulation test. On day 1, food-deprived mice were placed into an operant conditioning chamber (Med Associates) equipped with a single nose port. The nose port contained a single food pellet in order to initiate the mouse into the port. 20Hz, 473nm light (10-15mW, 5s duration) was unilaterally delivered through the optic cannula contingent on a beam break in the nose port. Mice spend a total of 1 hour in the operant chamber. On day 2, mice were hooked up to fiber optic patchcords and returned to the reward conditioning chamber (with no food pellet) for 15min without light stimulation. Total number of pokes was quantified by MED-PC (Med Associates) software on day 1 and day 2.

Optogenetic place preference test. Mice were placed into the center of a rectangular box (70x25x31cm) where each end of the box contained distinct wall cues. Immediately upon entry into the box, mice received continuous 20Hz 473nm light (10-15mW) stimulation contingent on entry into a randomly pre-selected half of the box for 5min. The position of the mice was tracked using EthoVision XT video tracking software (Noldus). The difference score (s) was calculated by (duration in the stimulated side) - (duration in the non-stimulated side). All behavioral experiments were performed by a set of mice in cohorts of 4-16 mice. Animals were selected for surgery and behavior in a pseudorandom fashion in that mice were, as much
as possible, divided equally based on age and parents into experimental and control groups. For unilateral implants, mice received implants randomly and counterbalanced in the left or right hemisphere. For all behavioral experiments, two-tailed unpaired Student’s *t*-test was performed between experimental groups and control groups. Mice lacking expression or misplaced fibers were excluded from analysis. Experimenters were blind during data analysis and whenever possible during the experimentation.

**Anatomical Experiments.** CTB experiments were performed as described above (Stereotactic injections). Percent labelling of CTB in the BLA was quantified by the number of CTB+ cells as a percentage of the total large (<10µm) DAPI+ cells. For anatomical projection of BLA neurons, Rspo2-ChR2 and Ppp1r1b-ChR2 mice tissue underwent immunohistochemistry for eYFP using an anti-GFP antibody to amplify the eYFP signal.

**Optogenetic slice electrophysiology.** Male mice (mean-PND 45 days) were anesthetized by isoflurane and their brains were dissected. By using a vibratome (VT1000S, Leica) 300 µm-thick parasagittal slices containing the basolateral amygdala were prepared in oxygenated cutting solution at ~4 °C. Slices were then incubated at ~23 °C in oxygenated artificial cerebrospinal fluid (ACSF). The cutting solution contained 3 mM KCl, 0.5 mM CaCl², 10 mM MgCl², 25 mM NaHCO³, 1.2 mM NaHPO⁴, 10 mM d-glucose, 230 mM sucrose, saturated with 95% O/5% CO (pH 7.3, osmolarity 340 mOsm). The ACSF contained 124 mM NaCl, 3 mM KCl, 2 mM CaCl², 1.3 mM MgSO⁴, 25 mM NaHCO³, 1.2 mM NaHPO⁴, 10 mM d-glucose, saturated with 95% O/5% CO (pH 7.3, osmolarity 300 mOsm). Slices were transferred into a submerged experimental chamber and perfused with oxygenated 36 °C ACSF at a rate of 3 ml min⁻¹.
Whole-cell recordings in current-clamp or voltage-clamp mode were performed by using an infrared differential interference contrast microscope (BX51, Olympus) with a water immersion 40X objective (N.A. 0.8), and equipped with four automatic manipulators (Luigs & Neumann) and a CCD camera (Orca R2, Hamamatsu). Borosilicate glass pipettes were fabricated (P97, Sutter Instrument) with a resistances of 3–5 MΩ, and filled with the following intracellular solution 110 mM potassium gluconate, 10 mM KCl, 10 mM HEPES, 4 mM ATP, 0.3 mM GTP, 10 mM phosphocreatine and 0.5% biocytin (pH 7.25, osmolarity 290 mOsm). Recordings in voltage clamp were performed by using the following intracellular solution (in mM): 117 cesium methansulfonate, 20 HEPES, 0.4 EGTA, 2.8 NaCl, 5 TEA-Cl, 4 Mg-ATP, 0.3 Na-GTP, 10 QX314, 0.1 spermine and 0.5 % biocytin (pH 7.3, osmolarity 290 mOsm). Access resistance was monitored throughout the duration of the experiment and data acquisition was suspended whenever the resting membrane potential was depolarized above –50 mV or the access resistance was beyond 20 MΩ. Recordings were amplified using up to two dual channel amplifiers (Multiclamp 700B, Molecular Devices), filtered at 2 kHz, digitized (20 kHz) and acquired using custom made software running on Igor Pro (Wavemetrics). Gabazine was obtained from Tocris.

Optogenetic stimulation was achieved through a 460-nm LED light source (XLED1, Lumen Dynamics) driven by TTL input with a delay onset of 25 μs (subtracted off-line). Light power on the sample was 33 mW mm–2, and only the maximum power was employed. Slices were stimulated by single 2 ms light pulse repeated 20 times every 4 s or train of 15 light pulses at 10 Hz repeated 20 times every 6 s. In voltage-clamp cells were held at 0 mV for IPSC measurements, whereas, in current mode, EPSP and action potentials were measured at resting potentials.
Morphological and electrophysiological criteria were established by using single cell RT-PCR to identify the molecular subtype. Magnocellular cells were identified based on location in the anterior part of the basolateral amygdala and by large soma size (13 ± 0.5 μm). Parvocellular cells were identified based on location in the posterior part of the basolateral amygdala, close to the ventral edge of the ventricle, and by small soma size (10 ± 0.3 μm). Physiological criteria such as membrane resistance and capacitance were employed to validate the cellular subtype (Table 2, Figure 6m,n).

The intrinsic electrophysiological properties were measured current mode with the cell held at −70 mV. Input resistance was estimated by linear fit of the I-V relationship (injection of 10–12 current steps of 1-s duration). Action potential threshold was tested with a current ramp injection. Membrane time constant was estimated by single exponential fit of the recovery-time from a −100 pA current step injection of 1-s duration. Synaptic connections, in voltage or current mode, were determined by averaging 20 trials. EPSC amplitude was measured from the average maximum peak response by subtracting a baseline obtained 5 ms before light pulse starts. EPSC onset was measured from the beginning of the light pulse to the starting point of the response estimated through the intercept between the baseline and a parabolic fit of the rising phase of the EPSC. To compute the probability of connection (n success / n tests) we employed only slices with reliable ChR2 expression characterized at least by one responsive postsynaptic cell (principal cell or interneuron).

Statistical analysis was performed using Igor (Wavemetrics), Graphpad (Prism), or Excel (Microsoft). The distribution of the data was tested with the Kolmogorov-Smirnov test and a
two-tailed paired or unpaired *t*-test, or a Wilcoxon signed-rank or rank-sum test was employed accordingly. Fisher exact test was employed to verify the significance of the connection probability. Data are presented as mean ± s.e.m.

Recorded slices were recovered for morphological identification as the recorded cells were filled with biocytin. Recorded slices were filled with biocytin and fixed in 4% paraformaldehyde for morphological identification.

**Single-cell quantitative polymerase chain reaction.** In wild-type mouse brain slices, at the end of the patch clamp recordings, the cytoplasm of the recorded neuron was collected by applying negative pressure to the recording pipette. Once the cytoplasmic contents were suctioned, the glass pipette was quickly transferred to 0.2mL PCR tube fill with 10μL RNase free water, 2μL oligo(dT), 1μL dNTP, 1μL RNaseOUT provided by the SuperScript III CellsDirect cDNA Synthesis Kit (ThermoFisher). Samples were placed on a 70°C heat block for 5min, and then chilled on ice. For first strand synthesis, 8μL of RT mix was added to the sample (6μL 5x RT Buffer, 1μL .1M DTT, 1μL Superscript III RT) and incubated on a 50°C heat block for 50min. After the first strand synthesis, reverse transcriptase was inactivated by 5min incubation on an 85°C heat block. Samples were stored in -20°C until quantitative polymerase chain reaction (qPCR).

qPCR was performed using the Taqman Gene Expression Assays (Applied Biosystems). The genetic identity of BLA neuron using qPCR was determined by the ratio between *RpsO2* and *Ppplrb* expression. qPCR reaction consisted of 25μL 2X TaqMan Gene Expression Master Mix, 2.5μL of the 20X TaqMan Gene Expression Assay of *Rspo2*(Mm00555790_m1) or
PPp1r1b (Mm00454892_m1), 7μL of cDNA template, 17.5μL of RNase free water. qPCR reaction was performed in an Applied Biosystems 7500 Real-Time PCR System using the Fluorescein (FAM) channel with the standard qPCR reaction protocol for 60-80 cycles. The majority of cells did not result in amplification of either Rspo2 or Ppplrlb. Therefore, Rspo2⁺ and Ppplrlb⁺ neurons were identified based on the criterion of any positive amplification. Rspo2⁺ or Ppplrlb⁺ amplification appeared at threshold cycles (Cₜ) <50 cycle for most cells (Figure 2i).

**Statistical Analysis.** Statistical analysis and statistical graphics was generated using GraphPad Prism 6.0. Sample sizes and statistical tests were determined based on previous studies examining similar behaviors and histology analyses. Variance was not significantly different between groups that were compared and met the assumptions of the statistical tests with the exception from groups where the effects of experimental manipulations were dramatic, such as in the case of Rspo2-ChR2 vs. Rspo2-EYFP in the optogenetic freeze test, or Ppplrlb-ChR2 vs. Ppplrlb-EYFP in the optogenetic self-stimulation test. All data are represented as mean ± s.e.m.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

6 to 12 week old male mice were used for all experiments, except 5 to 6 week old male mice were used for slice patch clamp experiments. All Cre transgenic mice were bred using a heterozygous male with females of C57BL/6 background. *Crh-Cre, Drd1a-Cre, Rspo2-Cre, Sst-Cre*, and *Tac2-Cre* mice had a C57BL/6 background. *Cartpt-Cre, Nts-Cre*, and *Prkcd-Cre* mice were crossed to a C57BL/6 background for at least 2 generations from their original backgrounds from JAX laboratories or GENSAT. Cre-expressing mice were genetic knock-in
mice or has been previously been validated for genetic specificity \cite{25,51}. BLA $Rspo2^+$ neurons were targeted using $Rspo2$-Cre mice. BLA $Ppp1r1b^+$ neurons were targeted using $Cartpt$-Cre mice \cite{25}. CeC $Prkcd^+$ and CeL $Prkcd^+$ neurons were targeted using $Prkcd$-Cre mice. CeL $Sst^+$ and CeM $Sst^+$ neurons were targeted using $Sst$-Cre mice. CeL $Crh^+ Nts^+ Tac2^+$ neurons were targeted using $Crh$-Cre mice. CeM $Nts^+$ neurons were targeted using $Nts$-cre mice. CeM $Tac2^+$ neurons were targeted using $Tac2$-Cre mice. CeM $Drd1^+$ neurons were targeted using $Drd1a$-Cre mice. Mice undergoing all behavioral tests (optogenetic experiments, $Fos$ experiments) were single housed for 1 week prior to experiments and kept on a 12hr light, 12hr dark light cycle. Control mice for behavioral experiments underwent identical procedures as experimental mice, but were Cre$^-$ mice of the same sex from the same litters. Mice that underwent slice patch clamp experiments or rabies tracing experiments were grouped housed. All mice were maintained and cared in accordance with protocols approved by the Massachusetts Institute of Technology (MIT) Committee on Animal Care (CAC) and guidelines by the National Institutes of Health (NIH).

**Single Molecule Fluorescent In Situ Hybridization**

For examination of gene expression and $Fos$ experiments, tissue samples underwent single molecule fluorescent in situ hybridization (smFISH). Isoflurane anesthetized mice were decapitated, brain harvested and flash frozen on aluminum foil on dried ice. Brains were stored at -80°C. Prior to sectioning, brains were equilibrated to -16°C in a cryostat for 30min. Brains were cryostat sectioned coronally at 20μm and thaw-mounted onto Superfrost Plus slides (25x75mm, Fisherbrand). Sections from a single brain would be serially thaw-mounted onto 10 slides through the CeA, anterior-posterior distance from Bregma (-0.6mm to -1.8mm). Slides were air dried for 60 to 90mins prior to storage at -80°C. smFISH for all genes examined—
Calcr (ACDBio Cat#452281), Crh (ACDBio Cat#316091), Drd1 (ACDBio Cat#406491), Drd2 (ACDBio Cat#406501), Fos (ACDBio Cat#421981), Gad1 (ACDBio Cat#400951), Htr2a (ACDBio Cat#401291), Nts (ACDBio Cat#420441), Pdyn (ACDBio Cat#318771), Penk (ACDBio Cat#318761), Prkcd (ACDBio Cat#441791), Sst (ACDBio Cat#404631), Tac1 (ACDBio Cat#410351), Tac2 (ACDBio Cat#446391)—was performed using RNAscope Fluorescent Multiplex Kit (Advanced Cell Diagnostics) as previously described. Slides were counterstained for the nuclear marker DAPI using ProLong Diamond Antifade mounting medium with DAPI (ThermosFisher).

Immunohistochemistry

For visualizing Prkcd, Ppp1r1b, Rabies helper virus expression (eGFP), ChR2-eYFP and ArchT-eYFP expression, tissue samples underwent immunohistochemistry (IHC). Mice were euthanized by averdin overdose and underwent a standard perfusion protocol using 1X phosphate-buffered saline (PBS), followed by 4% paraformaldehyde. Mouse brains removed and postfixed overnight at room temperature. Brains were vibratome sectioned at 50µm and collected through the CeA, anterior-posterior distance from Bregma (-0.6mm to -1.8mm). IHC was performed as previously described with primary antibodies anti-rabbit Prkcd (1:1000, Abcam Cat#ab18212), anti-rabbit Ppp1r1b (1:1000, Abcam Cat#ab40801), chicken anti-Gfp (Invitrogen Cat#A10262) and secondary antibodies Alexa Fluor 647-conjugated Goat anti-Rabbit (1:1000, Invitrogen Cat#A21244), Alexa Fluor 488-conjugated Goat anti-chicken (1:1000, Invitrogen Cat#A11039). In the slice patch clamp experiments, brain slices were fixed for 4 hours at 4°C prior to undergoing the IHC protocol. In the slice patch clamp experiments, brain slices were fixed for 4 hours at 4°C prior to undergoing the IHC protocol. Biocytin was labelled using CF555 Streptavidin (1:100, Biotium Cat#29038) during the secondary antibody incubation step.
Cell Counting

Images of smFISH and retrograde rabies tracing experiments were taken using a standard fluorescent microscope (Zeiss) under a 10X objective. Colors represented in micrographs are false colors and do not necessarily reflect native colors. Images were exported and counted manually using cell counting software (ImageJ). Percent labelling found in Figure 17 and 23 were quantified by counting 1 to 3 sections equal number per mouse, from \( n = 3 \) mice and sum totaling all single and double labelled neurons to yield a raw percentage value. The bounds of what considered the CeC, CeL, and CeM were used as shown in cartoons in Figure 24 and 30. Although CeC in the posterior portion of the CeA (anterior-posterior Bregma - 1.6mm) is depicted in Figures and defined by atlas boundaries lying laterally to the CeL, the posterior CeC was not quantified in any quantification of CeC neurons as the boundary between the CeC and CeL in the posterior CeA is ambiguous.

Stereotaxic Surgeries

Mice underwent standard stereotactic procedures under isoflurane anesthesia. Vectors were injected using a mineral oil filled glass micropipette attached to a 1µL microsyringe. For optogenetic experiments of the CeA, 100nL of AAV\(_9\)-Ef1a-DIO-ChR2 or AAV\(_9\)-Ef1a-DIO-eArch3.0 was bilaterally injected into the CeC (distance from Bregma, AP-0.8mm, ML±2.9mm, DV-5.0mm), CeL (AP-1.4mm, ML±2.9mm, DV-4.7mm), or CeM (AP-0.8mm, ML±2.8mm, DV-5.0mm) of the different CeA Cre mice. Both ChR2 and Arch viruses were diluted in 1X phosphate buffer (PBS, pH 7.2), one part stock virus, 3 parts PBS to give a final concentration of ~1.0x10^{12} GC. Subsequent to injections, 5.0mm Mono fiberoptic cannulas (Doric Lens) were implanted above the site of injection of the CeC (DV-4.4mm), CeL (DV-4.2mm), or CeM (DV-4.4mm). Once positioned above the CeA, the mono fiberoptic cannula was cemented using
dental cement (Teets cold cure; A-M Systems) to the skull, which contained 2 screws that were posterior and medial to the injection site. Once the dental cement cured, a protective cap surrounding the implant, made using a 1.5mL black Eppendorf tube, was fixed onto the implant using dental cement. Post-operation, mice received an injection of slow release buprenorphine (1mg/kg). Mice spent 1 week for recovery and then were handled by investigator 2-3 days prior to behavioral experiments. For slice patch clamp experiments, 200nL of AAV9-Ef1α-DIO-ChR2 was into the BLA of Rspo2-Cre (AP-1.3mm, ML±3.3mm, DV-4.85mm) and Cartpt-Cre mice (AP-4.8mm, ML±0.5mm, DV-3.0mm) and incubated for 1 week prior to sacrifice for the slice patch clamp experiments. For CTB retrograde tracing, Alexa Fluor 647-conjugated cholera toxin subunit B (1μg/μL, Thermofischer Cat#C34778) was unilaterally injected into the PAG (300nL, AP-1.0mm, ML+2.9mm, DV-4.5mm). 1 week later, mice were sacrificed and brains underwent smFISH.

Optogenetic Activation Experiments

ChR2 virus-injected mice underwent an optogenetic freezing test followed by an optogenetic self-stimulation test. These optogenetic activation experiments were performed on cohorts of 6 to 12 mice and took place during first half of the dark cycle. The optogenetic freezing test involved exposing mice to conditioning chamber (Med Associates) for 6mins. During the 0-3min period, mice did not receive any optogenetic stimulation. During the 3-6min period, mice received continuous optogenetic stimulation, 10-15mW 20Hz 473nm light stimulation. 1 day later, mice were food deprived for 24hrs prior to the start of the optogenetic self-stimulation test. The optogenetic self-stimulation test took place in an operant conditioning chamber (Med Associates) equipped with a two nose ports. Prior to the trial, one of the two nose ports was randomly assigned to deliver optogenetic stimulation, 5s duration 10-15mW 20Hz 473nm light
stimulation, upon nose poke (ON port), while the other one did not deliver optogenetic stimulation (OFF). Each of the nose points contained a single food pellet to initiate the mouse into the port. Mice were then placed into the operant chamber for 60min. For the optogenetic freezing test, freezing was scored manually and blind to the condition of the mouse using behavioral scoring software (Solomon Coder). For the optogenetic self-stimulation test, total numbers of pokes on the OFF and ON port were automatically counted and obtained through MED-PC (Med Associates) software. Mice where non-specific ChR2 or improper optic fiber placement occurred were removed behavioral analysis.

**Fos Activation Experiments**

For all Fos staining, 6hrs prior to sacrifice, food and water were removed from the home cages of C57BL/6 wild-type mice in order to reduce any unintended activation in the Fos experiments. Mice were exposed to a fear conditioning chamber (Med Associates) for 500s in which 3 footshocks (.75mA) were delivered at the 198s, 278s, 358s time points, while control mice underwent the same procedure, but did not receive any footshocks. Mice were returned to their home cages and 30mins later sacrificed. Mice undergoing fear extinction underwent the same 3 shock fear conditioning protocol, then 24hrs later, were exposed to the fear conditioning chamber without any footshocks 3 times, for 15 minutes. Mice spent 1hr in between these 3 15min extinction sessions. 24hrs later, mice were exposed to the fear conditioning chamber for 5 minutes, returned to their home cages, and sacrificed 30mins later. Control mice underwent similar procedures, but did not undergo the 3 15min extinction sessions. Hence, control mice can be considered mice undergoing contextual fear recall, rather than contextual fear extinction recall. 24hr food-deprived mice were transported into an experimental room on a cart and then exposed to *ad libitum* food, in their home cages. After
30min from the end of the first feeding bout, mice were sacrificed. Control mice underwent identical procedures but did not receive food. This involved carting the mice to the experimental room and opening the lid of their home cages. 24hr water-deprived mice that were exposed to water in an analogous way food was presented to food-deprived mice. Quinine water exposed mice were given quinine water (.01% quinine, Sigma) rather than water. Control mice underwent identical procedures but did not receive water. Mice were sacrificed 30min after the exposure to the stimulus. 24hr food-deprived mice were given CCK injections (5µg/kg of CCK dissolved in .9% sodium chloride saline solution, Tocris), while control mice received saline injections. Mice were sacrificed 30min after the injections. Fos expression in CeC Prkcd+ and CeL Prkcd+ neurons was examined using Prkcd (ACDBio, Cat#441791). Fos expression in CeL Sst+ and CeM Sst+ neurons was examined using Sst (ACDBio, Cat#404631). Fos expression in CeL Crh+Nts+Tac2+ and CeM Tac2+ neurons was examined using Tac2 (ACDBio, Cat#446391). Fos expression in CeM Nts+ neurons was examined using Nts (ACDBio, Cat#420441).

Optogenetic Inhibition Experiments

Arch virus-injected mice underwent a feeding test, followed by a drinking test, then followed by a contextual fear conditioning test. For the feeding test, mice were food-deprived for 24hrs prior to the start of the experiment, which took place during the first half of the dark cycle. For the feeding test, mice were exposed to ad libitum food in their home cages while receiving constant 10-15mW 532nm light inhibition for 20mins. After the feeding test, mice returned to their normal diet until the start of the light cycle (approximately 12hrs later) in which mice were water-deprived. Approximately 18hrs later, in the second half of the dark cycle, mice underwent the drinking test. In the drinking test, mice were exposed to ad libitum water for
5mins in a chamber identical to their home cages but without bedding. During the entirety of the 5min drinking test, mice received constant 10-15mW 532nm light inhibition. After the test, mice were returned to their normal diet. The next day, mice underwent contextual fear conditioning. On day 1 of contextual fear conditioning, mice were placed into a fear conditioning chamber (Med Associates) for 500s and received footshocks (.75mA) during the 198s, 278s, 358s time points. Simultaneously with the onset of the footshock, a constant pulse of 532nm light (10-15mW) was delivered through the optical cannulas for duration of 30s. On day 2, mice were returned to the fear conditioning chamber for 180s, where no shock or laser was delivered. Feeding, drinking, and freezing were scored manually and blind to the condition of the mouse using behavioral scoring software (Solomon Coder). Mice where non-specific Arch or improper optic fiber placement occurred were removed behavioral analysis.

Retrograde Rabies Tracing

For monosynaptic retrograde rabies experiments, 100nL of rabies helper virus, AAV1-synP-FLEX-sTpEpB, was injected into the CeC, CeL, or CeM or CeA of Cre-expressing mice. 3 weeks later, 100nL EnvA G-protein deleted rabies virus, SADΔG-mCherry, was injected into the same location. 1 week later, mice were sacrificed and brains underwent IHC using antibodies against Prkcd (Abcam, Cat#ab182126) or Ppp1r1b (Abcam, Cat#ab40801) and visualized using Alexa Fluor 647-conjugated secondary antibody (Invitrogen, Cat#A21244).

Optogenetic Slice Electrophysiology

Mice were anesthetized by isoflurane and their brains dissected. Using a vibratome (VT1000S, Leica), we prepared 300-µm-thick coronal slices containing the basolateral and central amygdala in oxygenated cutting solution at ~4 °C. Slices were then incubated at ~23 °C in
oxygenated artificial cerebrospinal fluid (ACSF) for 45 mins to an hour. The cutting solution contained 3 mM KCl, 0.5 mM CaCl2, 10 mM MgCl2, 25 mM NaHCO3, 1.2 mM NaHPO4, 10 mM D-glucose, 230 mM sucrose, saturated with 95% O/5% CO (pH 7.3, osmolarity 340 mOsm). The ACSF contained 124 mM NaCl, 3 mM KCl, 2 mM CaCl2, 1.3 mM MgSO4, 25 mM NaHCO3, 1.2 mM NaHPO4, 10 mM D-glucose, saturated with 95% O/5% CO (pH 7.3, osmolarity 300 mOsm). Slices were transferred to a submerged experimental chamber and perfused with oxygenated 36 °C ACSF at a rate of 3 ml min⁻¹.

Whole-cell recordings in current-clamp mode were performed using a patch clamp setup with an infrared differential interference contrast microscope (BX51, Olympus) with a water immersion 40× objective (N.A. 0.8), four automatic micro-manipulators (Luigs & Neumann) and a CCD camera (Orca R2, Hamamatsu). Borosilicate glass pipettes were fabricated (P97, Sutter Instrument) with a resistances of 8–10 MΩ and filled with the following intracellular solution: 110 mM potassium gluconate, 10 mM KCl, 10 mM HEPES, 4 mM ATP, 0.3 mM GTP, 10 mM phosphocreatine and 0.5% biocytin (pH 7.25, osmolarity 290 mOsm). Biocytin was excluded from the intracellular solution if the cytosol of the patched neuron was aspirated for PCR. Access resistance was monitored throughout the duration of the experiment and data acquisition was suspended whenever the resting membrane potential was depolarized above –50 mV or the access resistance was beyond 20 MΩ. Recordings were amplified using up to two dual-channel amplifiers (Multiclamp 700B, Molecular Devices), filtered at 2 kHz, digitized (20 kHz) and acquired using custom software running on Igor Pro (Wavemetrics). Software and code are available upon request. Gabazine was obtained from Tocris.
Optogenetic stimulation was achieved through a 460-nm LED light source (XLED1, Lumen Dynamics) driven by TTL input with a delay onset of 25 µs (subtracted offline). Light power on the sample was 33 mW mm⁻², and only the maximum power was employed. Slices were stimulated by a train of 15 light pulses at 10 Hz repeated 20 times every 6 s. EPSPs, IPSPs and action potentials were measured at resting membrane potential of the patched cell.

The intrinsic electrophysiological properties were measured current mode with the cell held at −70 mV. Input resistance was estimated by linear fit of the I-V relationship (injection of 10–12 current steps of 1-s duration). Action potential threshold was tested with a current ramp injection. Synaptic connections were verified by taking an average of 10 to 20 individual trials. EPSP amplitude was measured from the average maximum peak response by subtracting a baseline obtained 5 ms before light pulse starts. EPSP onset was measured from the beginning of the light pulse to the starting point of the response estimated through the intercept between the baseline and a parabolic fit of the rising phase of the EPSC. To compute the probability of connection (n successes/n tests) we used only slices with reliable ChR2 expression characterized at least by one responsive postsynaptic cell (principal cell or interneuron).

CeL neurons were filled with biocytin, slices were recovered and fixed in 4% paraformaldehyde for verification of genetic identity using IHC against Prkcd. Analysis for CeL Prkcd⁻ neurons in addition to CeL Prkcd⁺ neurons was taken into consideration.

CeC and CeM neurons were harvested for subsequent qPCR analysis. In order to harvest the RNA of recorded neurons, a negative pressure of 250 mbar was applied for 5 mins followed by a stronger negative pressure of 500 mbar also for 5 mins. Successful suction of the cytosolic contents was visually confirmed by observing the patched cell shrink on the microscope.
screen. The negative pressure was maintained until the glass pipette was quickly withdrawn. It was then carefully lowered into a 0.2ml PCR tube until its tip was felt against the bottom of the tube. Finally, gentle positive pressure was applied to expel the contents on the pipette into a qPCR buffer solution (see below).

**Single Cell qPCR Genetic Confirmation**

Cytosolic harvests was quickly transferred to .2mL PCR tube fill with 10µL RNase free water, 2µL oligo(dT), 1µL dNTP, 1µL RNaseOUT provided by the SuperScript III CellsDirect cDNA Synthesis Kit (ThermoFisher). Samples were placed on a 70°C heat block for 5min, and then chilled on ice. For first strand synthesis, 8µL of RT mix was added to the sample (6µL 5x RT Buffer, 1µL .1M DTT, 1µL Superscript III RT) and incubated on a 50°C heat block for 50min. Next, the reverse transcriptase was inactivated by 10min incubation on an 85°C heat block. Samples were stored in -20°C until quantitative polymerase chain reaction (qPCR).

qPCR was performed using the Taqman Gene Expression Assays (Thermofisher) for Prkcd in CeC neurons or for Nts, Sst, and Tac2 in CeM neurons. Each qPCR reaction consisted of 25µL 2X TaqMan Gene Expression Master Mix, 7µL of cDNA template, 17.5µL of RNase free water, and 2.5µL of the 20X TaqMan Gene Expression Assay of Prkcd (Cat#Mm00440891_m1), Nts (Cat#Mm00481140_m1), Sst (Cat#Mm00436671_m1), or Tac2 (Cat#Mm01160362_m1). qPCR reaction was performed in an Applied Biosystems 7500 Real-Time PCR System using the Fluorescein (FAM) channel with the standard qPCR reaction protocol for 60 cycles. Any positive amplification signal (ΔRn) within the 60 reaction cycles was considered to be positive confirmation for such gene. In contrast to IHC confirmation of CeL neurons, negative results from CeC and CeM qPCR confirmation was not considered because of the high levels of false negatives in qPCR amplification.
QUANTIFICATION AND STATISTICAL ANALYSIS

Data are represented as mean ± s.e.m. All histograms display individual points, which represent the values and total number of individual samples. What the individual samples represent is indicated within the figure legends for all experiments. Student's *t*-test, paired or unpaired wherever appropriate, was performed on all comparisons with exceptions being the one-way ANOVA with Bonferroni's correction between the comparison between water and quinine with no water groups and one-way ANOVA with Bonferroni's correction for multiple comparison. 95% confidence interval was used to determine significance. Significance was displayed as *P*< 0.05, **P*< 0.01, ***P*< 0.001, ****P*> 0.0001, not significant values were not denoted. Statistical tests were performed using GraphPad Prism 6.0. Hierarchical clustering was performed by generating a matrix containing the percent overlap profile of each gene in a given CeA subdivision, then calculating the distance using the pairwise distance function (*pdist*), dendrograms were made using the linkage function (*linkage*) on Matlab8.3. The diagonals on the percent labelling matrices corresponded to identical gene pairs. Therefore, for the cluster, the diagonals of the matrices were denoted as 100%.