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Encoding of environmental cues in central amygdala neurons during foraging

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1   **Encoding of environmental cues in central amygdala neurons during foraging**

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Abstract

In order to successfully forage in an environment filled with rewards and threats, animals need to rely on familiar structures of their environment that signal food availability. The central amygdala (CeA) is known to mediate a panoply of consummatory and defensive behaviors, yet how specific activity patterns within CeA subpopulations guide optimal choices is not completely understood. In a paradigm of appetitive conditioning in which mice freely forage for food across a continuum of cues, we found that two major subpopulations of CeA neurons, Somatostatin-positive (CeA^{Sst}) and protein kinase Cδ-positive (CeA^{PKCδ}) neurons can assign motivational properties to environmental cues. While the proportion of food responsive cells was higher within CeA^{Sst} than CeA^{PKCδ} neurons, only the activities of CeA^{PKCδ}, but not CeA^{Sst}, neurons were required for learning of contextual food cues. Our findings point to a model in which CeA^{PKCδ} neurons may incorporate stimulus salience together with sensory features of the environment to encode memory of the goal location.

Significance Statement

The CeA has a very important role in the formation of memories that associate sensory information with aversive or rewarding representation. Here, we used a conditioned place preference paradigm, where freely moving mice learn to associate external cues with food availability, to investigate the roles of CeA neuron subpopulations. We found that CeA^{Sst} and CeA^{PKCδ} neurons encoded environmental cues during foraging but only the activities of CeA^{PKCδ} neurons were required for learning of contextual food cues.

Introduction

43 An organism's survival depends heavily on its ability to evaluate whether familiar cues in the environment

44 predict a threat or an opportunity. The CeA is important for this competence and contains numerous

45 genetically distinct subpopulations of neurons that can select the appropriate behavioral outputs in the

46 face of positively or negatively valenced stimuli (Ehrlich et al., 2009; Pare and Duvarci, 2012; Herry and

47 Johansen, 2014; Janak and Tye, 2015; Fadok et al., 2018). While earlier models interpreted the literature

48 in a binary mode, where CeA neurons belong either to the ''aversive'' or ''rewarding'' network, new

49 hypotheses depict the CeA as an essential site for associative learning (Wilensky et al., 2006; Ciocchi et

50 al., 2010) also contributing to the computation of prediction error signals (Ozawa et al., 2017; Yu et al.,

51 2017; Kargl et al., 2020). To understand how the surrounding environment modulates plasticity in CeA

52 circuits to drive behavioral selection, we probed the function and activity patterns of $CeA^{PKC\delta}$ and CeA^{Sst}

53 neurons during a foraging task. PKCδ- and Sst-expressing cells represent two non-overlapping populations

54 of CeA neurons (Kim et al., 2017), encompassing the majority of all cells in this region (Kim et al., 2017),

55 and are known to mediate antagonizing behaviors when studied in parallel (Kim et al., 2017; Wilson et al.,

56 2019; Kargl et al., 2020). The CeA has been previously shown to acquire responses to signals of food

57 delivery, but the contribution of these two CeA subpopulations has remained unclear (Gallagher et al.,

58 1990; Han et al., 1997, 1999; McDannald et al., 2004; Corbit and Balleine, 2005; Lee et al., 2005). In

59 particular, how these appetitive memories are represented in a setting where the animal freely navigates

60 and is forced to engage goal-oriented actions remains unknown. We hypothesized that PKCδ+ cells that

61 integrate convergent inputs from cortical, thalamic, hippocampal, and brainstem structures (Douglass et

62 al., 2017), properties that are reminiscent of the Hebbian model of learning-induced synaptic plasticity,

63 may be involved in associative learning tasks that impose valence on different tastes or link taste valence

64 to other sensory stimuli.

65   Indeed, we found that CeA$^{PKC\delta}$ neurons, previously related to aversive behavior (Haubensak et al., 2010;

66   Cai et al., 2014; Cui et al., 2017; Kim et al., 2017; Yu et al., 2017; Wilson et al., 2019), were required for

67   approach behavior in a context that signals food availability, whereas CeA^{Sst} neurons were not. We

68   performed Ca^{2+} imaging to evaluate neuronal activity changes when the mice freely decided to switch

69   between environments. We observed that after learning, a subset of these two populations had

70   developed specific activities to the positive context. Our findings suggest a model in which CeA^{Sst} neuron

71   activity encodes information directly related to food consumption, whereas CeA$^{PKC\delta}$ cells endow sensory

72   features of the environment with representation of the food reward salience to ultimately drive correct

73   behavioral choices.

74   **Materials and Methods**

75   **Animals**

76   Prkcd-Cre (Tg(Prkcd-glc-1/CFP,-Cre)EH124Gsat) BAC mice were imported from the Mutant Mouse

77   Regional Resource Center. Sst-Cre (Ssttm2.1(cre)Zjh) transgenic mice were acquired from the Jackson

78   Laboratory (https://www.jax.org/). Rosa26R Cre dependent reporter mice that express LacZ (B6.129S4-

79   Gt(ROSA)26Sortm1Sor/J) have been described previously (Soriano, 1999). Mice were backcrossed onto a

80   C57BL/6NRj background (Janvier Labs - http://www.janvier-labs.com). 3-6 month old male Prkcd-cre;LacZ

81   or Sst-Cre;LacZ mice were used for the place preference assay combined with optogenetic manipulation

82   of CeA$^{PKC\delta}$ and CeA^{Sst} cells, as well as for the open field task. 8 to 18 month old male and female Prkcd-

83   cre;LacZ or Sst-Cre;LacZ mice were used for the place preference assay combined with Ca^{2+} imaging of

84   CeA$^{PKC\delta}$ and CeA^{Sst} cells. The older age of mice used for the Ca2+ imaging experiments was primarily due

85   to the experimental design. In some cases, a waiting period of several months was necessary for the

86   inflammation to decrease and to clearly see fluorescent cells through the lens. Mice were kept on a 12-h

4

87   light/dark cycle. All behavior experiments were conducted during the light phase of the cycle and under

88   dimmed light in the behavioral boxes.

89   **Viral Constructs**

90   The following AAV viruses were purchased from the University of North Carolina Vector Core

91   (https://www.med.unc.edu/genetherapy/vectorcore: AAV5-ef1a-DIO-eNpHR3.0-mCherry, AAV5-ef1a-

92   DIO-mCherry. The AAV5-Syn.Flex.GCaMP6s virus was obtained from Addgene

93   (http://www.addgene.org/).

94   **Stereotaxic surgeries**

95   Viral injections in the CeA

96   Mice were anaesthetized using isoflurane (Cp-Pharma) (induction, 3%; maintenance, 1.5%) in oxygen-

97   enriched air and head-fixed on a stereotaxic frame (Model 1900 – Kopf Instruments). Body temperature

98   was maintained at 37^0C using a heating pad. Carprofen (Rimadyl – Zoetis) (5 mg/kg body weight), and an

99   analgesic, were given via subcutaneous injection. Mice were bilaterally injected with 0.3 µl of virus in the

100   CeA by using the following coordinates calculated with respect to the bregma: −1.22 mm anteroposterior,

101   ± 2.9 mm lateral, −4.7 to -4.8 mm ventral. Viral particles were delivered using glass pipettes (708707 -

102   Blaubrand intraMark) connected to a Picospritzer III (Parker Hannifin Corporation) and controlled by a

103   Master-8 pulse stimulator (A.M.P.I) at a flow rate of ~50 nL/min. After delivery of the virus, the pipette

104   remained in the brain for 5 min to prevent spread of the virus. Virus was allowed to be expressed for a

105   minimum duration of 4.5 weeks and a maximum of 10 weeks before behavioral experiments.

106   Optic fibers implants

107   Mice used in optogenetic experiments were, in the same surgery, bilaterally implanted with optic fibers

108   (200-µm core, 0.22 NA, 1.25-mm ferrule - Thorlabs) above the CeA (−4.35 mm ventral from bregma). The

5

109   skull was first protected with a thin layer of histo glue (Histoacryl, Braun), the fibers were then fixed to

110   the skull using UV light-curable glue (Loctite AA3491 - Henkel), and the exposed skull was covered with

111   dental acrylic (Paladur - Heraeus).

112   GRIN lens implantation and baseplate fixation.

113   For gradient index (GRIN) lenses implantation, 3 weeks after viral injection, mice expressing GCaMP6s in

114   PKCδ+ or SST+ cells were anesthetized using the same procedure as described above. A small craniotomy

115   was made above the CeA using the same coordinates as for the injection of the viral preparation. Debris

116   were removed from the hole and a customized blunted 23G needle (0.7mm in diameter) was slowly

117   lowered down into the brain at a speed of 150 μm/min to a depth of -4.6 mm from bregma. After

118   retraction of the needle, a GRIN lens (ProView Lens; diameter, 0.5 mm; length, ~8.4 mm, Inscopix)

119   mounted on a GRIN lens-holder was slowly (150 μm/min) implanted above the CeA. The skull was first

120   protected with a thin layer of histo glue (Histoacryl, Braun), the lens was then fixed to the skull using UV

121   light-curable glue (Loctite AA3491 - Henkel), and the exposed skull was covered with dental acrylic

122   (Paladur - Heraeus). The exposed top of the lens was protected by a covering of a silicone adhesive (Kwik-

123   cast - World Precision Instruments). 4 to 8 weeks after GRIN lens implantation, mice were anesthetized

124   and placed in the stereotaxic setup. A baseplate (BPL-2; Inscopix) attached to the miniature microscope

125   was positioned above the GRIN lens. Concentration of the anesthetic gas was lowered and the focal plane

126   was adjusted until neuronal structures and GCaMP6s dynamics were clearly observed. Mice were then

127   fully anaesthetized again and the baseplate was fixed using C&B Metabond (Parkell). A baseplate cap

128   (BCP-2, Inscopix) was left in place until imaging experiments.

129 **Behavioral assays**

130 Conditioned place preference with food reward

131 The conditioned place preference behavior was motivated by previous work (Stern et al., 2018). It was

132 conducted in a custom-built arena made of two-chambers: a rectangular shaped chamber (45*15 cm) and

133 a triangular shaped chamber (45*30 cm) separated by a corridor (Fig. 1A). Chambers additionally differed

134 based on the texture of the floor and pattern on the walls. The area size of the two chambers were

135 identical. On the first day of the behavioral experiment, mice were allowed to explore the arena for 20

136 min. Preferences for the two chambers were measured and the least preferred one was chosen as the

137 food-paired chamber (context +) for the rest of the paradigm. From then on, and until the end of the

138 experiment, mice were singly housed and food restricted. They were weighed daily and supplied with

139 necessary food to maintain at least 85% of their initial body weight. Conditioning was then conducted

140 over four consecutive days. For this, mice were first sequestered in the neutral context for 15 min and

141 then manually transferred to the positive context for 15 min in which they had access to a food pellet of

142 approximately 1 g. The remaining food was weighed at the end of each conditioning session to determine

143 food consumption. On the 5th and final day of the experiment, mice were allowed to freely navigate

144 between the two chambers in the absence of food. The preference for the positive context was measured

145 for a period of 10 min as a readout for contextual appetitive conditioning. For Ca^{2+} imaging experiments,

146 mice were subjected to the same behavioral task and at the end of the 10 min recall session, we added a

147 food pellet to the positive context and recorded the activity of CeA cells for another 10 min.

148 We had previously performed pilot experiments with WT animals in which 3 groups of 5 mice were

149 conditioned either for 2, 4, or 5 days and the preference of each group for the positive chamber was

150 tested 24hr after the last conditioning day. We found that preference for the appetitive chamber

151 increased from 53.6% +/-1.3 to 71.9% +/-7.6 when we increased the conditioning from 2 to 4 days but did

152   not increase further when performing the experiment for 5 conditioning days (PI = 61.7% +/- 5) (data not

153   shown). We therefore chose to perform 4 days of conditioning because a preference of 70% for the food-

154   paired chamber would give us a perfect range to identify both increases and decreases in our optogenetic

155   experiment. In our imaging experiment, we also needed animals that would move a lot between the two

156   chambers and spend a sufficient amount of time in both contexts so that we could see both an increase

157   in activity in the positive chamber and a decrease in activity in the neutral one.

158   For the optogenetic experiments, PKCδ-eNpHR3.0, PKCδ-mCherry, Sst-eNpHR3.0 and Sst-mCherry mice

159   bilaterally received constant 561-nm intracranial light (15 mv) either during the whole 30 min of the

160   conditioning (both in the positive and neutral context) and during all 4 conditioning sessions, or during

161   the whole recall session only. For this, mice were tethered to optic-fiber patch cables (Doric Lenses or

162   Thorlabs) connected to a 561 nm CNI laser (Cobolt) via a rotary joint (Doric Lenses) and mating sleeve

163   (Thorlabs). During habituation and recall, mice were free of the optic-fiber patch cables.

164   Animals that did not explore both chambers at least once during recall were excluded. This includes 1

165   PKCδ-eNpHR3.0, 1 PKCδ-mCherry and 1 Sst-eNpHR3.0 animals used for the experiment in Fig. 1G-O and 1

166   PKCδ-eNpHR3.0, 1 Sst-mCherry and 2 Sst-eNpHR3.0 animals used for the experiment in Fig. 3.

167   Open-field

168   PKCδ-eNpHR3.0, PKCδ-mCherry, Sst-eNpHR3.0 and Sst-mCherry mice were allowed to explore a custom-

169   built Plexiglas arena (40 cm × 40 cm × 25 cm) for 10 min. During the experiment, mice bilaterally received

170   constant 561-nm intracranial light through optic-fiber patch cords (Doric Lenses or Thorlabs) connected

171   to a 561 nm CNI laser (Cobolt) via a rotary joint (Doric Lenses) and mating sleeve (Thorlabs).

8

172 Real-time place preference (RTPP)

173 RTPP assays were carried out over two consecutive days. On the first day, PKCδ-eNpHR3.0 and PKCδ-

174 mCherry mice were allowed to explore a custom-built arena made of two chambers (each: 25*25 cm) for

175 20 min. Mice received constant 561-nm intracranial light in one chamber, the photoinhibition-paired

176 chamber. The laser was triggered on the basis of the location of the animal by using Ethovision XT 14

177 software (Noldus). On the next day, the experiment was repeated under the same conditions as on day

178 one, except that the photoinhibition-paired chamber was switched.

179 Conditioned place preference without food reward

180 Conditioned place preference experiments without food reward were carried out in a similar manner as

181 conditioned place preference experiments with food reward except that the inhibition of CeA^{PKCδ} neurons

182 was not paired with delivery of a food reward. Briefly, the experiment was conducted in a custom-built

183 square arena (26.5 x 26.5 cm), divided in the middle by a wall with an opening to form two equally sized

184 triangular compartments which the mice could freely navigate between. Chambers differed based on

185 the texture of the floor. On the first day of the behavioral experiment, PKCδ-eNpHR3.0 and PKCδ-mCherry

186 mice were allowed to freely explore the arena for 20 min. Preferences for the two chambers were

187 measured and the least preferred one was chosen as the photoinhibition-paired chamber for the rest of

188 the paradigm. Conditioning was then conducted over four consecutive days in which mice were

189 sequestered for 15 min in each chamber but received constant 561-nm intracranial light only in the

190 photoinhibition-paired chamber in the absence of food. On the 5th and final day of the experiment, mice

191 were able to freely navigate between the two compartments and the preference for the photoinhibition-

192 paired chamber was measured over a 20 min period.

193 **In vivo Ca^{2+} imaging of freely moving mice**

194 All imaging experiments were conducted on freely behaving mice PKCδ-GCaMP6s and Sst-GCaMP6s mice.

195 GCaMP6s fluorescence signals were acquired using a miniature integrated fluorescence microscope

196 system (nVoke - Inscopix). Before each imaging session, the miniscope was secured in the baseplate

197 holder. Mice were habituated to the miniscope attachment procedure 3 days before behavioral

198 experiments. The analog gain and LED output power were adjusted such that the best dynamic

199 fluorescence signals were at the focal plane. Settings were kept constant within subjects and across

200 imaging sessions. Imaging acquisition and behavior were synchronized using the data acquisition box of

201 the nVoke Imaging System (Inscopix), triggered by the Ethovision XT 14 software (Noldus) through a TTL

202 box (Noldus) connected to the USB-IO box from the Ethovision system (Noldus). Compressed images were

203 obtained at 1200 pixels by 800 pixels and 10 frames per second using the Inscopix acquisition software

204 (Inscopix). We recorded the activities of CeA neurons during habituation, on day 1 and day 3 of

205 conditioning, and during recall. To minimize photo-bleaching, we limited the duration of acquisition to 3

206 times 2 min during habituation, 1 times 2 min during conditioning in the neutral context, and 3 times 2

207 min during conditioning in the positive context. Each recording bout was evenly spaced in time. On recall

208 day, Ca^{2+} transients were recorded for the full 20 min of the behavioral experiment.

209 **Histology**

210 Animals that underwent behavioral experiments combined with optogenetic manipulations of CeA cells

211 were anesthetized with ketamine/xylazine (Medistar and Serumwerk) (100 mg/kg and 16 mg/kg,

212 respectively) and transcardially perfused with phosphate-buffered saline (PBS), followed by 4%

213 paraformaldehyde (PFA) (1004005, Merck) (w/v) in PBS. Extracted brains were postfixed at 4 °C in 4% PFA

214 (w/v) in PBS for 12 h, embedded in 4% agarose (#01280, Biomol) (w/v) in PBS and sliced using a Vibratome

215 (VT1000S - Leica) into 50 or 100-μm free-floating coronal sections.

216   Mice that underwent calcium imaging experiments were anesthetized with ketamine/xylazine (Medistar

217   and Serumwerk) (100 mg/kg and 16 mg/kg, respectively) and decapitated. The head together with GRIN

218   lens implant and baseplate, were fixed at 4 °C in 4% PFA (w/v) in PBS for a minimum of 4 days before

219   dissection of the brain. Extracted brains were sliced using a Vibratome (VT1000S - Leica) into 100-μm free-

220   floating coronal sections.

221   **Microscopy**

222   Epifluorescence images were obtained with an upright epifluorescence microscope (Zeiss) with 5×/0.15

223   or 10×/0.3 objectives (Zeiss). Images were minimally processed with ImageJ software (NIH) to adjust for

224   brightness and contrast for optimal representation of the data. A median filter was used to decrease noise.

225   **Quantification and statistical analyses**

226   Extraction of behavioral data for the conditioned place preference assay with food reward

227   In the conditioned place preference assay, the animal location was recorded at 15Hz using a webcam

228   (Logitech) suspended above the arena and velocity as well as the preference index and number of entries

229   to the positive context and the food zone were automatically analyzed by Ethovision XT 14 software

230   (Noldus).

231   For analysis of the calcium recordings, we could not directly score feeding bouts, since we only used a

232   camera that was suspended above the arena. Therefore, we manually scored the amount of time when

233   the animals were on top of the food and chose a criteria of 2.5s as a minimum time to define a feeding

234   bout.

235 Extraction of behavioral data for the open-field task

236 Animal location was recorded at 15Hz using a webcam (Logitech) suspended above the arena and the

237 number of entries to the center of the arena (20 cm × 20 cm square), cumulative duration in center, as

238 well as velocity were assessed with Ethovision XT 14 software (Noldus).

239 Extraction of behavioral data for the RTPP

240 Animal location was recorded at 15Hz using a webcam (Logitech) suspended above the arena and the

241 location of the animal as well as the velocity were assessed using Ethovision XT 14 (Noldus). Cumulative

242 duration in the photoinhibition-paired chamber was averaged across two sessions.

243 Extraction of behavioral data for the conditioned place preference assay without food reward

244 Animal location was recorded at 15Hz using a webcam (Logitech) suspended above the arena and the

245 preference index for the photoinhibition-paired chamber as well as the velocity were assessed using

246 Ethovision XT 14 (Noldus).

247 Extraction of ΔF/F and temporal registration with behavioral data

248 For imaging data processing and analysis, all videos recorded from one imaging session were combined

249 into a single image stack using the Inscopix data processing software (version 1.3.0 – Inscopix) and saved

250 as a tiff. Tiff files were then processed using the miniscope 1-photon imaging signal extraction pipeline

251 (MIN1PIPE) (Lu et al., 2018), which returns fully processed ROI components with spatial footprints and

252 temporal calcium traces as outputs. Briefly, the data go through different steps of neural enhancing,

253 hierarchical movement correction, and neural signal extraction that combine a first seeds-cleansing step

254 followed by a simplified spatiotemporal CNMF. Behavioral data were finally temporally aligned to the

255 calcium traces using linear interpolation and unix time stamps as references for both datasets.

12

256   Longitudinal registration of ROIs

257   ROIs from several recording sessions were longitudinally registered using CellReg Matlab GUI (Sheintuch

258   et al., 2017) (https://github.com/zivlab/CellReg). In brief, the roifn output variable from the Min1pipe that

259   contains the processed vectorized ROI footprints for each session was transformed in Matlab using:

260   roi_use = permute(reshape(roifn, pixh, pixw, n), [3, 1, 2]), where n is the number of ROIs.

261   Transformed ROIs footprints were then registered using CellReg and the following parameters: alignment

262   type: translations and rotations (max rotation in degrees: 30). A maximal distance of 12 microns was used

263   to compute the probabilistic model. The initial and final cell registrations were performed using spatial

264   correlation models. The resulting cell_to_index_map file was used to identify identical ROIs between

265   different days, calculate the total number of recorded cells per animal, and the number of overlapping

266   neurons during the whole recording session.

267   Regressors and correlation analyses

268   Regressors were built as previously described (Miri et al., 2011). For this, the behavior of each mouse in

269   the positive context, food zone or 'on top of the food' (square-wave data sets) was convolved with a kernel

270   with an exponential decay based on the measured half-decay time for GCaMP6s (~0.150 s) (Chen et al.,

271   2013). The resulting predicted calcium traces were then used to compute Pearson's correlation

272   coefficients with the corresponding calcium traces. To classify neurons as 'neurons encoding appetitive

273   context', 'food responsive cells', or 'unresponsive in positive context', we examined which coefficients

274   rise above or below chance by correlating our fluorescent traces to 1000 random regressors that were

275   constructed after randomly shuffling the real behavioral dataset by bouts of 20s. We required a threshold

276   of 1.96 deviation from the standard error of the random coefficients mean (corresponding to the 95%

277   confidence interval) to assign a cell to a particular functional group.

278   Classification of neurons preferentially active in the positive context

279   To quantify which neurons were preferentially active in the positive context compared to the neutral one

280   during conditioning, ΔF/F transients were z-scored at each time point using the following formula: (F(t) –

281   Fm)/SD, where F(t) is the ΔF/F value at a time t, Fm, and SD are the mean and standard deviation of the

282   baseline calculated from time point when the animals were in the neutral context. An average of the single

283   z-scored time points was then calculated for when the animal was located in the positive context. Neurons

284   were considered to be preferentially active in the positive context when the averaged z-scored value

285   exceeded the 1.96 threshold (corresponding to the 95% confidence interval).

286   Decoding of positive and neutral context locations

287   To decode the location of the mice in the positive or neutral context during recall, we used a logistic

288   regression classifier. For decoder training and testing, we used neuronal Ca^{2+} signals expressed as ΔF/F.

289   For each animal, classifiers were trained on 70% of the data during recall and tested on the remaining

290   30%. We computed the prediction score as the average of correct predictions over a 10-fold cross-

291   validation procedure. Correct predictions were defined as the ratio of TP / (TP + FP +FN) where TP is the

292   number of true positives, FP the number of false positives and FN the number of false negatives.

293   To evaluate the statistical significance of decoding performance, we trained logistic regression decoders

294   on temporally shuffled behavioral data. For this, behavioral data were split into 7 sec bouts and randomly

295   shuffled. This was repeated 5 times. The shuffled prediction score was defined as the average of correct

296   predictions of these 5 repetitions.

297   Alignment of calcium responses to positive and neutral context entries

298   ΔF/F transients were z-scored with the baseline calculated from time points when the animals were in the

299   neutral context for alignment to positive context entries, or with the baseline calculated from time points

300   when the animals were in the positive context (for alignment to neutral context entries) (see formula

301   above in: Classification of neurons preferentially active in the positive context). We omitted short bouts

302   whose duration was below 2.5 sec to exclude epochs when the animals were only shortly going in and out

303   of the corridor space without fully entering the context. Z-scored calcium responses of single neurons to

304   context entry were then averaged in a time window from 3 s before transition to 4.5 s after. Cells were

305   finally sorted in a descending order based on their activity response upon entry in the + context.

306   Heatmaps of the spatial Ca2+ activity

307   To plot a heatmap of the average spatial activity of one selected cell we used the raw ΔF/F data. The total

308   activity in a specific x-y location was normalized to the total time the animal spent in that location. x-y

309   data were discretized in 50 x 50 pixels.

310   Statistical analysis

311   No statistical methods were used to pre-determine sample sizes. The numbers of samples in each group

312   were based on those used in previously published studies. Behavioral experiments were conducted by an

313   investigator with knowledge of the animal genotype and treatment. For behavioral and *in vivo* imaging

314   experiments, behavioral-tracking software and custom-written Python scripts were used to obtain and

315   analyze the data in an automated and unbiased manner. Statistical analyses were performed with Prism

316   5 (GraphPad) and all statistics are indicated in the figure legends. t-tests were used for individual

317   comparisons of normally distributed data. When normality was not assumed, Mann-Whitney U test and

318   Wilcoxon signed-rank test (for paired observations) were performed for individual comparisons. A one-

319   way repeated measures ANOVA or Friedman's test (as a non-parametric equivalent) was used for within-

320   subject comparisons followed by Bonferroni post hoc analysis or Dunn's multiple comparison test. After

321   the conclusion of experiments, virus-expression, optic-fiber and GRIN-lens placement were verified. Mice

15

322   with null virus expression as well as mice in which both optical fibers were wrongly located or at least

323   more than 500 µm above the CeA were excluded from analysis.

324   **Data and code availability**

325   The datasets supporting the current study have not been deposited in a public repository but are available

326   from the corresponding author on request. This study used custom-built Python 3.0 programmed scripts

327   that are also available from the corresponding author upon request.

328   **Results**

329   **Inhibition of CeA$^{PKC\delta}$ neurons impairs contextual appetitive conditioning**

330   To assess the contributions of CeA subpopulations to the acquisition of contextual appetitive memories,

331   we trained mice in conditioned place preference experiments to associate one chamber (positive context)

332   of a two-chamber arena with a food reward (Fig. 1A). Following conditioning, we measured the amount

333   of time the mice spent in the positive context in the absence of food as a readout for contextual appetitive

334   learning. Typically, WT mice learned the task well, spending on average 71.9 +/- 7.2 % of their time in the

335   positive context (Fig. 1B).

336   We focused on CeA$^{PKC\delta}$ and CeA^{Sst} neurons, because they represent two non-overlapping populations of

337   central amygdala cells, representing the majority of all neurons in this region (Kim et al., 2017), and, when

338   studied in parallel, mediate antagonizing behaviors (Kim et al., 2017; Wilson et al., 2019; Kargl et al., 2020).

339   We optogenetically inhibited either CeA$^{PKC\delta}$ or CeA^{Sst} neurons during all conditioning sessions (in both the

340   positive and the neutral contexts) by transducing the CeAs of *Prkcd-Cre* or *Sst-Cre* animals with Cre-

341   dependent halorhodopsin-expressing virus (eNpHR3.0-mCherry) and placing optic fibers bilaterally above

342   the CeA (Fig. 1C-F). Control mice expressing mCherry behaved similarly to WT mice, although their

343   preference index (PI) after learning was slightly lower (61.4 +/- 2.9 % for PKCδ mCherry, and 69 +/- 5.1 %

344 for Sst mCherry mice) (Fig. 1G-I). Silencing of CeA^{Sst} neurons did not alter behavior (Fig.1G-H). In contrast,

345 CeA$^{PKC\delta}$ neuron-inhibited mice showed poor learning performance, lacking a preference for the positive

346 context during recall (Fig. 1G,I). In addition, CeA$^{PKC\delta}$, but not CeA^{Sst}, neuron-inhibited animals spent

347 significantly less time in the food zone of the positive context (Fig. 1J-L), even though they visited it with

348 the same frequency as control mice (Fig. 1M).

349 Several other parameters that may have influenced the outcome of this learning paradigm were found to

350 be similar between the experimental groups, including velocity during recall (Fig. 1N), the amount of food

351 eaten during training (Fig. 1O), and the degree of anxiety as measured by duration and entries to the

352 center, as well as velocity, in an open field task (Fig. 1P-T).

353 We further controlled whether inhibition of CeA$^{PKC\delta}$ neuron activity could lead to an assignment of

354 negative or positive valence to both contexts in the absence of food, which could have interfered with the

355 contextual appetitive conditioning. In a real time place preference assay (RTPP), we found that CeA$^{PKC\delta}$

356 neuron-inhibited mice exhibited neither a preference nor an aversion for the photoinhibition-paired

357 chamber (Fig. 2A-E). We then performed similar conditioned place preference experiments as described

358 in Fig. 1A but in the absence of a food reward while photoinhibiting CeA$^{PKC\delta}$ cells in one chamber over 4

359 conditioning days. When we tested the preference of the animals for the photoinhibition-paired chamber,

360 we found that inhibition of CeA$^{PKC\delta}$ neurons alone without food, did not produce a conditioned place

361 preference, nor an aversion for the photoinhibition-paired chamber (Fig. 2A-B and F-I) indicating that

362 inhibition of these cell's activity alone did not participate in assigning a rewarding or an aversive signal.

363 We next examined the roles of these two populations during memory retrieval or expression. We used

364 the same tools as before (Fig. 1C) except this time, we selectively inhibited CeA$^{PKC\delta}$ or CeA^{Sst} cells only

365 during the recall phase. Similar to inhibition of CeA neurons during the conditioning phase, we found that

366 inactivation of CeA$^{PKC\delta}$, but not CeA^{Sst}, cells significantly decreased the preference of the animals for the

17

367   positive context compared to control mice (Fig. 3A-F), although the time spent in the food zone (Fig. 3G-

368   I), as well as the frequency of visits (Fig. 3J) remained unchanged. Control parameters such as the velocity

369   during recall (Fig. 3K), and amount of food eaten during training (Fig. 3L) were similar between groups.

370   Overall, these results suggest that CeA^PKCδ neurons may play a role in forming and retrieving associations

371   between contextual cues and food availability.

372   ***In vivo* recordings of CeA^PKCδ and CeA^Sst neuron activity during appetitive conditioning**

373   To understand how CeA^PKCδ and CeA^Sst neuron activity differentially contributes to this behavior, we

374   expressed the GCaMP6s indicator in the CeA of PKCδ+ and Sst+ cells and recorded the dynamics of

375   fluorescent signals using a miniscope during all phases of the task (Fig. 4A-C). Mice were subjected to the

376   same behavioral task and at the end of the recall, a food pellet was added to the positive context to make

377   sure that we could functionally tag 'food responsive' neurons.

378   We recorded the activities of 24–61 cells per mouse, (202 cells total) in 5 Prkcd-cre mice, and 14–60 cells

379   per mouse, (149 cells total) in 5 Sst-cre animals (Fig. 4D). The numbers of active cells during training and

380   recall were higher as compared to habituation day in both groups. Longer imaging time during training

381   and especially recall may account for this difference. Alternatively, investigation and consumption of food

382   may have recruited new sets of neurons (Fig. 4C, D). Using a ROIs alignment method (Sheintuch et al.,

383   2017), we registered cell identities across imaging sessions. We found approximately 10 % of cells in

384   common for all four recording days (Fig. 4E). When excluding the habituation day, the number of common

385   cells increased to about 17% in both Cre lines (Fig. 4E), suggesting that neurons recorded during

386   conditioning and recall may be functionally more similar than the ones initially active during habituation.

387   Of 10 recorded mice, 3 per genotype showed a PI≥ 50% during recall which is lower than observed before

388   (Fig. 4F). It is possible that the weight of the miniscope rendered movements more laborious and

389   decreased performance. Nonetheless, 8 animals out of 10 increased their preference for the positive

390 context after conditioning (Fig. 4F), spending on average 2.1 times longer in the food zone and visiting it

391 1.7 times more often compared to the other 3 corners of the arena (Fig. 4G-H). All together, these are

392 good indications that those 8 animals learned the task.

393 **Central amygdala encoding of Pavlovian appetitive learning**

394 We tested the Hebbian model of appetitive conditioning (D. O. Hebb, 1949; Brown et al., 1990; Sejnowski,

395 1999) by investigating whether the positive context which represents multisensory information, could

396 serve as a general predictor of food availability after learning. Or, in other words, could CeA neurons that

397 fire during food intake acquire a specific response to the positive context after learning?

398 Taking into account only the data of the 8 mice that learned the task (Fig. 4F-H), we convolved the positive

399 context signal with the kinetics of the calcium indicator to create a regressor and correlated the activity

400 of each CeA cell with the corresponding regressor across time (Miri et al., 2011). An example trace of a

401 positive context regressor is shown at the bottom of Fig. 5K. We found that during habituation, the

402 activities of all neurons showed little correlation to the positive context. Instead, during recall, the

403 activities of both populations shifted toward positive correlations (Fig. 5A-B), suggesting that the

404 representations of the positive chamber by CeA$^{PKC\delta}$ and CeA^{Sst} neurons were transformed after learning.

405 For each mouse, we randomly selected 70% of the data during recall to train a logistic regression classifier

406 and asked whether, in the remaining dataset, the location of the mice in the positive context, could be

407 accurately predicted based on CeA$^{PKC\delta}$ and CeA^{Sst} population activity. In all mice tested, we could reliably

408 decode the position of the animals in the positive chamber (70.7 ±4.7% for CeA$^{PKC\delta}$ and 70 ±4.1% for CeA^{Sst}

409 recorded ensembles), while performances significantly dropped when the decoder was trained on

410 temporally shuffled behavioral data (Fig. 5C), indicating that correct predictions were well above chance.

411 We also found a decreased performance of 10% and 15%, respectively, when the classifier was tested on

412 CeA$^{PKC\delta}$ and CeA^{Sst} population activity that excluded the 4 most important features (defined here as the

19

413 neurons showing the highest correlations to the positive context) (Fig. 5C). This suggests that at least a

414 fraction of both populations encoded essential information about the location of the animal in the positive

415 context. When we aligned the neuronal Ca^{2+} responses of each cell to the onset of the transitions from

416 the neutral to the positive context and averaged the data, we found that a subset of both $CeA^{PKC\delta}$ and

417 CeA^{Sst} neurons was activated upon transition to the positive context (Fig. 5D-E). During habituation, we

418 could not identify a group of neurons whose activity reliably increased upon entry in the positive chamber,

419 meaning that encoding of the appetitive context by $CeA^{PKC\delta}$ and CeA^{Sst} neurons arose after conditioning

420 (Fig. 5D-E). These cells exhibited an area-biased activity that was specifically high when the mouse was in

421 the appetitive context and preferentially within the food corner (Fig. 5F-G). Cells that displayed a

422 significant correlation toward the positive context regressor accounted for about 14.5% of $CeA^{PKC\delta}$ and

423 8.7% of CeA^{Sst} recorded cells during recall (Fig. 5H-I). Neurons encoding appetitive context were found in

424 4/4 Prkcd-cre analyzed mice and 3/4 analyzed Sst-Cre mice (Fig. 5-1A). Analysis of the ΔF/F traces revealed

425 different patterns of activity within the positive context. Some neurons fired both upon entry to the

426 positive chamber and investigation of the food zone (Fig. 5J,L, cells 4 and 6, Fig. 5K,M, cell 15). Some cells

427 were active preferentially within the food zone (Fig. 5K,M, cell 11). Other non-memory neurons showed

428 no preference for the positive context nor the food zone (Fig. 5J-M, cells 22 and 23). At this point, no

429 differences were observed between neurons encoding appetitive context within the PKCδ and Sst

430 populations. Again, using a regressor-based approach, we found that the activities of 60% of $CeA^{PKC\delta}$ and

431 all CeA^{Sst} neurons encoding appetitive context were significantly upregulated during food consumption

432 on the same recall day (Fig. 5N-O and Fig. 5-1A), demonstrating that a subset of these cells can be tagged

433 as food responsive.

434 We also looked at neurons that would be particularly unresponsive in the positive context (i.e significant

435 negative correlation to the positive context regressor), and identified a total of 9 PKCδ+ and 7 Sst+

436 neurons. The activity of these neurons was however very weak, as only 6 of them showed a significantly

20

437   elevated activity in the neutral context (Fig. 5J-M, cells 17 and 21 and Fig. 5-1B). None of these 6 identified

438   neurons nor any other recorded cells showed an increased activity upon transition to the neutral context

439   (Fig. 5P-Q). Additionally, we trained the logistic regression classifier on the presence of the mice in the

440   neutral context while omitting the neurons encoding appetitive context for each animal (Fig. 5H-I). By

441   doing so, we found that performance of the decoder was low (41.2% +/-14.3% for Prkcd-cre, and 48.4%

442   +/-19.5% for Sst-cre recorded mice) (Fig. 5R). This is a good indication that there may not be any other

443   neurons (besides the neurons encoding appetitive context), whose activity can be used to predict the

444   location of the animal in the neutral context.

445   We found no correlation between the number of neurons encoding appetitive context and the learning

446   index (data not shown), but this could be an issue inherent to the behavioral task: while the 'poor learners'

447   may associate only the precise location of the food zone with a rewarding outcome, the 'good learners'

448   may associate the whole positive context or even the whole arena with food delivery. In the latter case,

449   neurons encoding appetitive context may already fire when the animal recognizes the whole arena but

450   we do not have the means to visualize them.

451   This hypothesis is supported by the fact that neurons encoding appetitive context, whose activity best

452   represented the food zone, were found in mice that showed lower learning indices, demonstrated as a

453   negative correlation between the average correlation of neurons encoding appetitive context to the food

454   zone regressor and the PI of the mice (Fig. 5S). Additionally, when we compared the prediction scores of

455   our logistic regression decoder with the PI of the mice, we found that decoding accuracy of the classifier

456   decreased with learning performances (Fig. 5T). In other words, the classifier did not manage to find a

457   good representation of the positive context in the neural activity of the 'best learners'. It is therefore

458   possible that mice that learned best generalized the representation of the positive context to the whole

459   arena.

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460   Overall, these results suggest that contextual information associated with the positive context became

461   predictive of food delivery after learning and support a model in which groups of CeA$^{PKC\delta}$ and CeA^{Sst}

462   neurons encode contextual food cues in line with a Hebbian plasticity mechanism.

463   **Differences in calcium activity patterns between CeA$^{PKC\delta}$ and CeA^{SST} neurons**

464   Next, we took a step back and examined the activity profiles of all recorded neurons during conditioning,

465   taking into consideration all 5 Prkcd-cre and 5 Sst-cre recorded animals (Fig. 4F). On day 1 and day 3 of

466   training, we found that a large fraction of both CeA$^{PKC\delta}$ and CeA^{Sst} neurons strongly increased their activity,

467   specifically when the mice were sequestered in the positive context when the food reward was present

468   (Fig. 6A-B and 6C-D, cells A to F). These cells accounted for 30.9 ±10.3% of PKCδ and 31.1 ± 7.3% of Sst

469   recorded ensembles on day 1, and their proportion increased to 41.7 ± 6.7% and 58.3 ±7.8%, respectively,

470   on day 3 of conditioning (Fig. 6E).

471   For each cell, we calculated Pearson's correlations for their corresponding feeding regressor and

472   accordingly classified neurons as activated by food consumption. Interestingly, we observed that CeA$^{PKC\delta}$

473   cells did not always fire systematically during each feeding bout (Fig. 6C). Conversely, CeA^{Sst} neurons

474   increased their activities at the onset of a feeding bout, and more reliably for the next following ones (Fig.

475   6D). Supporting these observations, on day 1 and on day 3 of training, CeA^{Sst} neurons showed stronger

476   positive correlation to the feeding regressor (Fig. 6F-G) compared to CeA$^{PKC\delta}$ cells, and the proportion of

477   food responsive cells was higher among the CeA^{Sst} population (Fig. 6H). There was no difference in the

478   fraction of PKCδ+ and Sst+ cells active in the positive context during training (Fig. 6E), suggesting that the

479   activity of CeA$^{PKC\delta}$ cells may not directly relate to food consumption but rather to the salience of the food

480   reward. This phenotype was not because Pkcd-Cre animals ate less than Sst-Cre, as there was no positive

481   correlation between the amount of time spent eating and the proportion of food responsive cells on day

482   3 of training (Fig. 6I-J).

22

483　We then tracked neurons over time to see whether they would maintain their food responsiveness over

484　time and indeed, we found that the majority of cells that showed significant correlation to the feeding

485　regressor on one conditioning day conserved their functional tag on the subsequent day (Fig. 6C-D, cells

486　B, D, E and F, and Fig. 6K). Additionally, when tracking PKCδ+ and Sst+ cells that were classified as food

487　responsive on recall, we found that 83% of PKCδ+ and Sst+ were also found food responsive on day 3, and

488　22% of PKCδ+ and 50% of Sst+ on day 1 (Fig. 6L-N). In comparison, the majority of non-food responsive

489　neurons on recall day were classified non-responsive during the whole task (Fig. 6L-N). Most food

490　responsive neurons were also significantly active in the positive context compared to the neutral one

491　during all training sessions and were absent during habituation (Fig. 6C, cells B and C, Fig. 6D, cells D to F

492　and Fig. 6M-O), together revealing their functional selectivity.

493　When tracing back the origin of the PKCδ and Sst neurons encoding appetitive context (Fig. 5-1A) we

494　identified two different scenarios:

495　1) When detected, cells were tagged as food responsive during each step of the whole paradigm and they

496　were absent during habituation. This scenario includes 60% of the $CeA^{PKCδ}$ and all CeA^{Sst} neurons encoding

497　appetitive context.

498　2) Cells were either not food responsive or lost their 'food responsive' tag after conditioning. This includes

499　40% of the PKCδ neurons encoding appetitive context (found in 2 mice).

500　These last findings suggest that during conditioning, CeA^{Sst} cells whose activities show high positive

501　correlation with food intake behavior may be important to link environmental information with the

502　physical properties of the food. In contrast, CeA ^{PKCδ} neurons that are robustly activated upon food pellet

503　delivery in the arena, but not reliably upon food consumption, might rather form associations between

504　environmental information and the salience of the food reward.

23

Discussion

Here, we used a conditioned place preference assay allowing us to identify neuronal correlates of appetitive memories as the animal freely moves toward the food. This is noticeably different from classical studies of associative learning that looks at neuronal activity shortly following exposure to a conditioned tone applied by the experimenter (Pare and Quirk, 2017).

We found that two genetically distinct populations of CeA neurons (PKCδ+ and Sst+ neurons), developed specific activity patterns for the chamber associated with the reward. Interestingly, only the activities of CeA^{PKCδ}, but not CeA^{Sst}, cells were required for the formation and early retrieval of a preference for the appetitive context, suggesting that these neurons encode a combination of sensory, reward and contextual information to support learning that a spatial location predicts food availability.

CeA^{PKCδ} neurons were previously shown to inhibit appetitive behaviors in response to threats or aversive tastes, or in response to satiety (Cai et al., 2014; Kim et al., 2017). Moreover, CeA^{PKCδ} neurons could be described as 'broadly aversive', since these cells also promote pain-related responses, and are linked to the expression of aversive memories (Haubensak et al., 2010; Cui et al., 2017; Wilson et al., 2019). Our findings, instead, suggest that CeA^{PKCδ} cells may have functions that are more general and relate to a variety of stimuli. This is supported by findings from anesthesia neurons which show an 80% overlap with CeA^{PKCδ} neurons and attenuate (rather than promote) pain-related behavior (Hua et al., 2020). CeA^{Sst} neurons may also show multidimensional response properties, since these neurons relate to appetitive stimuli (Kim et al., 2017; Wilson et al., 2019), but also contribute to the generation of defensive behaviors (Li et al., 2013; Penzo et al., 2015; Yu et al., 2016). Although, these findings underscore the notion that molecularly distinct populations represent functionally specialized units, it remains possible that further subdivision of CeA populations using two or three genetic markers will reveal the presence of more specialized cells.

528   How could CeA[PKCδ] neurons mediate learning of contextual food cues?

529   Inhibition of CeA[PKCδ] neurons during conditioning, did not affect the number of times an animal visited the

530   precise location of the food zone, but rather the time spent within this area. This was consistent with our

531   calcium imaging data showing that CeA neurons encoding appetitive context showed a significant increase

532   in activity when the animal was either specifically in the food zone or in the positive context. We interpret

533   these results such that the activity of CeA neurons may be relevant to encode a representation of the

534   goal, i.e. reward associated with a particular location, rather than a representation of compass cues that

535   would help the animal in its search for the food. In the animals that learned best, this memory may initially

536   be linked to the location of the food zone and was then generalized to the positive context and ultimately

537   to the whole arena. Inhibition of CeA[PKCδ] neurons during recall also impaired the preference of the animals

538   for the positive context, although the effect was weaker and the time spent within the food zone was

539   unaffected. This suggests that CeA[PKCδ] cells may be involved in the early retrieval of appetitive memories

540   but these memories may be gradually transferred to different brain regions over time (Do Monte et al.,

541   2016).

542   Although contextual memory traces were observed in subsets of both CeA[PKCδ] and CeA[Sst] neurons, our

543   data uncovered differences between their activity patterns. We found for instance, that the proportion

544   of neurons active during food intake was significantly higher among the CeA[Sst] compared to the CeA[PKCδ]

545   population. We hypothesize that CeA[Sst] neurons encoding appetitive context may be essential to link a

546   context or a sensory stimulus with the physical properties of food (e.g. taste, texture, etc), but preventing

547   this association from forming does not prevent the animals from developing a preference for the food-

548   paired chamber. Conversely, CeA[PKCδ] neurons encoding appetitive context may mediate the association

549   of contextual stimuli with the general affective properties of food, a function that has been previously

550   suggested for the CeA (Balleine and Killcross, 2006). Inhibiting the formation of these memories would

551   then impair an animal's ability to develop a preference for the location of the food reward. It is however

552 important to mention here that our conclusions were drawn based on loss of function experiments of the

553 entire population but with relevant neuronal activity patterns seen in only a subset of cells. Therefore it

554 is possible that specific inhibition of memory neurons as opposed to whole PKCδ+ or Sst+ populations

555 could lead to a different phenotype. Our data are nonetheless consistent with recent evidence that

556 initially, before learning, CeA[PKCδ] neurons are tuned to stimulus salience but after conditioning, they

557 encode valence discrimination to select the appropriate defensive or appetitive responses (Kargl et al.,

558 2020). Furthermore, CeA[PKCδ] neurons seem to be part of a bottom-up network that is necessary to relay

559 reinforcement signals to higher order areas such as the insula cortex (Kargl et al., 2020) and the lateral

560 amygdala (Yu et al., 2017), which ultimately feed back to the CeA to drive associative learning.

561 In conclusion, our work reports specific activity patterns of CeA neurons that resemble contextual memory

562 traces to appetitive stimuli. We propose that salience coding in PKCδ+ cells is integrated with sensory

563 representations of the environment through Pavlovian learning mechanisms to drive correct behavioral

564 choices during foraging.

565 **Author Contributions**

566 M.P and F.F designed experiments. M.P, F.F and LG performed experiments. M.P analyzed experiments.

567 R.K supervised experiments. M.P and R.K wrote the manuscript. Funding Acquisition, R.K.

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656 **Figures and Legends**

657 **Figure 1.** Inhibition of CeA[PKCδ] neurons during conditioning impairs contextual appetitive conditioning

658 **A,** Place preference behavioral paradigm. **B**, Preference index (PI) for the positive contextf+ context before

659 and after conditioning for WT animals (n = 5 mice). **C**, Optogenetic inhibition of CeA neurons. The CeA of

660 Prkcd-Cre or Sst-Cre mice was transduced with a Cre-dependent AAV expressing eNpHR3.0-mCherry or

661 mCherry alone. CeA neurons were photoinhibited *in vivo* with constant yellow light. **D-E**, Representative

662 epifluorescent images of Prkcd-Cre (D) and Sst-Cre mice (E) injected in the CeL with an AAV-DIO-

663 eNPHR3.0-mCherry (D_1 and E_1) or AAV-DIO-mCherry (D_2 and E_2) and showing the location of the optic fiber

664 tract (in yellow). Scale bars: 100 μm. **F** Approximate optic fiber locations for each animal and expression

665 spread. Locations are shown on schematic coronal section planes with distances (anterior-posterior axis)

666 from bregma. **G,** PI for the food-paired chamber (+ context) during recall after inhibition of CeA neurons

667 during conditioning (Mann-Whitney U test, for PKCδ group comparison: U = 1, P = 0.0021 and for Sst group

668 comparison: U = 29, P = 0.7927). **H-I**, PI for the food-paired chamber before and after conditioning for

669 CeA[Sst] (H) and CeA[PKCδ] neuron-inhibited animals (I), and respective control mice. **J-K**, Representative heat

670 maps of the behavior of individual PKCδ-eNpHR3.0 and PKCδ-mCherry mice during recall. Green

671 represents the minimum and purple the maximum per-pixel frequency. **L**, Number of visits to the food

672 zone during recall (two-tailed unpaired t-test, for PKCδ group comparison: t(13) = 0.5618, P = 0.5838 and

673 for Sst group comparison: t(14) = 1.772, P = 0.0982). **M**, PI for the food zone during recall (Mann-Whitney

674 U test, for PKCδ group comparison: U = 10.5, P = 0.0489 and for Sst group comparison: U = 14, P = 0.0650).

675 **N**, Velocity during recall (two-tailed unpaired t-test, for PKCδ group comparison: t(13) = 0.6142, P = 0.5497

676 and for Sst group comparison: t(14) = 0.7520, P = 0.4645). **O**, Cumulative amount of food eaten during the

677 four training days (two-tailed unpaired t-test, for PKCδ group comparison: t(13) = 0.3398, P = 0.7394 and

678 for Sst group comparison: t(14) = 1.141, P = 0.2730). **P-Q**, Representative traces (in red) of the behavior

679 of PKCδ-eNpHR3.0 (P_1), PKCδ-mCherry (P_2), Sst-eNpHR3.0 (Q_1) and Sst-mCherry mice (Q_2) during the

31

680 openfield task. The yellow square represents the center. **R**, Cumulative duration in center during the

681 openfield task (Mann-Whitney U test, for PKCδ group comparison: U = 33, P = 0.8096 and for Sst group

682 comparison: U = 25.5, P = 0.8168). **S**, Number of visits to the center during the openfield task (two-tailed

683 unpaired t-test, for PKCδ group comparison: t(15) = 1.033, P = 0.3181 and for Sst group comparison: t(13)

684 = 0.3481, P = 0.7334). **T**, Velocity during the openfield task (two-tailed unpaired t-test, for PKCδ group

685 comparison: t(15) = 0.6166, P = 0.5467 and for Sst group comparison: t(13) = 1.348, P = 0.2006). (For

686 panels G-O, n = 8 PKCδ-eNpHR3.0 and 7 PKCδ-mCherry mice and n = 8 Sst-eNpHR3.0 and n = 8 Sst-mCherry

687 mice and for panels P-T, n = 9 PKCδ-eNpHR3.0 and 8 PKCδ mCherry mice and n = 7 Sst-eNpHR3.0 and n =

688 8 Sst-mCherry mice). Bar graphs show mean +/- s.e.m, each dot is the quantification of a single animal;

689 ns, not significant, *P < 0.05, **P < 0. 01. CeC/L: central capsular and central lateral amygdala; BLa:

690 basolateral amygdala; CeM: central medial amygdala.

691

692 **Figure 2.** Inhibition of CeA$^{PKC\delta}$ neurons does not result in assigning a rewarding or an aversive signal.

693 **A**, Representative epifluorescent images of Prkcd-Cre mice injected in the CeL with an AAV-DIO-

694 eNPHR3.0-mCherry (A$_1$) or AAV-DIO-mCherry (A$_2$) and showing the location of the optic fiber tract (in

695 yellow). Scale bars: 100 μm. **B**, Approximate optic fiber locations for each animal and expression spread.

696 Locations are shown on schematic coronal section planes with distances (anterior-posterior axis) from

697 bregma. **C**, Representative traces of the behavior of PKCδ-eNpHR3.0 (C$_1$) and PKCδ-mCherry (C$_2$) during

698 the RTPP task. **D**, Cumulative duration in the photoinhibition-paired side during the RTPP assay (Mann-

699 Whitney U test, U = 6.0, P = 0.2222). **E**, Velocity during RTPP (two-tailed unpaired t-test: t(9) = 0.1112, P =

700 0.9139). **F**, Representative traces of the behavior of PKCδ-eNpHR3.0 (F$_1$) and PKCδ-mCherry (F$_2$) during

701 the CPP without food task. **G**, PI for the photoinhibition-paired chamber during recall after inhibition of

702 CeA neurons during conditioning and in the absence of food (Mann-Whitney U test: U = 11, P = 0.5368).

703 **H**, PI for the photoinhibition-paired chamber before and after conditioning for CeA$^{PKC\delta}$ neuron-inhibited

704 animals and respective control mice. (n = 5 PKCδ-eNpHR3.0 and 6 PKCδ-mCherry). **E**, Velocity during CPP

705 without food (two-tailed unpaired t-test: t(9) = 1.350, P = 0.2099). Bar graphs show mean +/- s.e.m and

706 each dot is the quantification of a single animal; ns, not significant. CeC/L: central capsular and central

707 lateral amygdala; BLa: basolateral amygdala; CeM: central medial amygdala.

33

708 **Figure 3.** Inhibition of CeA[PKCδ] neurons during recall impairs contextual appetitive conditioning

709 **A-B**, Representative epifluorescent images of Prkcd-Cre (A) and Sst-Cre mice (B) injected in the CeL with

710 an AAV-DIO-eNPHR3.0-mCherry (A_1 and B_1) or AAV-DIO-mCherry (A_2 and B_2) and showing the location of

711 the optic fiber tract (in yellow). Scale bars: 100 μm. **C** Approximate optic fiber locations for each animal

712 and expression spread. Locations are shown on schematic coronal section planes with distances (anterior-

713 posterior axis) from bregma. **D**, PI for the food-paired chamber (+ context) during recall while inhibiting

714 CeA neurons during recall only (Mann-Whitney U test, for PKCδ group comparison: U = 19.5, P = 0.0250

715 and for Sst group comparison: U = 49, P = 0.7491). **E-F**, PI for the + context before and after conditioning

716 for CeA[Sst] (E), CeA[PKCδ] neuron-inhibited animals (F), and respective control mice. **G-H**, Representative heat

717 maps of the behavior of individual PKCδ-eNpHR3.0 and PKCδ-mCherry mice during recall. Green

718 represents the minimum and purple the maximum per-pixel frequency. **I**, PI for the food zone during recall

719 (Mann-Whitney U test, for PKCδ group comparison: U = 40.0, P = 0.4941 and for Sst group comparison: U

720 = 27, P = 0.0597). **J**, Number of visits to the food zone during recall (two-tailed unpaired t-test, for PKCδ

721 group comparison: t(18) = 0.2447, P = 0.8094 and for Sst group comparison: t(20) = 0.3566, P = 0.7251).

722 **K**, Velocity during recall (two-tailed unpaired t-test, for PKCδ group comparison: t(18) = 0.8922, P = 0.3840

723 and for Sst group comparison: t(19) = 1.707, P = 0.1041). **L**, Cumulative amount of food eaten during the

724 four training days (two-tailed unpaired t-test, for PKCδ group comparison: t(18) = 0.7204, P = 0.4806 and

725 for Sst group comparison: t(14) = 0.7520, P = 0.0686). (n = 11 PKCδ-eNpHR3.0 and 9 PKCδ-mCherry mice

726 and n = 12 Sst-eNpHR3.0 and n = 9 Sst-mCherry mice). Bar graphs show mean +/- s.e.m, each dot is the

727 quantification of a single animal; ns, not significant, *P < 0.05. CeC/L: central capsular and central lateral

728 amygdala; BLa: basolateral amygdala; CeM: central medial amygdala.

729

730 **Figure 4.** *In vivo* calcium imaging of CeA$^{PKC\delta}$ and CeA^{SST} neurons

731 **A**, Calcium imaging of CeA$^{PKC\delta}$ and CeA^{Sst} neurons via miniscope in freely moving mice. **B**, Representative

732 epifluorescent images of Prkcd-Cre (B$_1$) and Sst-Cre mice (B$_2$) injected in the CeL with an AAV-DIO-

733 GCamp6s-eYFP and showing the location of the GRIN lens tract (in yellow). Scale bars: 100 μm. **C**,

734 Maximum-projection images of PKCδ+ GCaMP6s-expressing neurons from a representative mouse

735 recorded during habituation (C$_1$), first (C$_2$) and third (C$_3$) days of training, as well as recall (C$_4$).

736 Corresponding region-of-interests (ROIs) are depicted below each panel. ROIs identified over consecutive

737 sessions are shown in identical color. ROIs detected only in one session are shown in white. Blue

738 arrowheads indicate neurons that appeared for the first time on day 1 of conditioning. Yellow arrowheads

739 indicate neurons that are common between at least 2 out of 3 days of conditioning and recall and are not

740 visible on habituation. **D**, Numbers of detected ROIs during all four sessions (One-way repeated measures

741 ANOVA, for PKCδ group comparisons: time point, $F(3,4) = 2.16$, $P = 0.1458$ and for Sst group comparisons:

742 time point, $F(3,4) = 3.98$, $P = 0.0351$). **E**, Percentage of ROIs per Prkcd-Cre and Sst-Cre mouse that

743 overlapped in all four recording sessions or from day 1 of conditioning to recall (Wilcoxon signed-rank

744 test, for PKCδ comparison: $P=0.125$, and for Sst group comparison: $P = 0.25$). **F**, PI for the + context before

745 and after conditioning for both PKCδ- and Sst-GCaMP6s recorded mice. Data points shown as crossed

746 circles represent mice that did not show an increase in learning after conditioning. **G**, Cumulative duration

747 in food zone compared to the averaged cumulative duration in the other three corners for the 4 PKCδ-

748 and 4 Sst-GCaMP6s mice that are considered to have learned the task (see Fig. 4F). Similar symbols

749 represent the same mice. **H**, Frequency of visits to the food zone compared to the averaged frequency of

750 visits in the other three corners for the 4 PKCδ- and 4 Sst-GCaMP6s recorded mice that are considered to

751 have learned the task (see Fig. 4F). Similar symbols represent the same mice. (n = 5 PKCδ-GCaMP6s and n

752 = 5 Sst-GCaMP6s recorded animals). Bar graphs show mean +/- s.e.m, each dot is the quantification of a

753 single animal. ns, not significant.

754 **Figure 5.** Central amygdala encoding of Pavlovian appetitive learning

755 *A*-*B*, Frequency distribution of Pearson's correlation to the positive context regressor in PKCδ (A) and SST

756 (B) calcium recorded neurons during habituation and recall. *C*, Prediction scores of the locations of Prkcd-

757 Cre (purple) and Sst-Cre (yellow) calcium recorded animals in the positive context during recall, after

758 temporally shuffling of behavioral data (shuffled) and after excluding 4 neurons per animal that exhibited

759 the highest correlation value to the positive context regressor (recall -) (Friedman test, for PKCδ group

760 comparisons: F=9.9, P=0.0062 with Dunn's Multiple Comparison Test and for Sst group comparisons: F =

761 9.3, P = 0.0115 with Dunn's Multiple Comparison Test, *P < 0.05). *D*-*E*, Heatmaps of average z-scored

762 calcium responses of PKCδ (D) and SST (E) recorded neurons following entry to the positive context (at 0

763 sec) during recall (D_1 and E_1) and during habituation (D_2 and E_2). Cells were sorted in descending order

764 based on their activity response upon entry in the + context (entries to the + context varied from 6 to 14

765 times depending on the mice) (n = 75 PKCδ+ and 50 Sst+ neurons). *F*-*G*, Heatmaps of the ΔF/F signal across

766 the whole arena for one representative PKCδ (F) and Sst (G) neuron encoding appetitive context. Green

767 represents the minimum and purple the maximum per-pixel frequency (see also Fig. 5-1A). *H*-*I*, Fraction

768 of CeA neurons encoding appetitive context in Prkcd-cre (H) and Sst-cre (I) recorded animals during recall.

769 *J*-*K*, Representative Ca^{2+} traces (from 7 mice) of PKCδ (J) and Sst (K) recorded neurons during recall. The

770 trace in beige at the bottom of K represents the convolved GCaMP6s regressor for the positive context of

771 cell 21 (see also Fig. 5-1A). Values indicate the Pearson's coefficients of each cell to its corresponding

772 positive context regressor. Values in green indicate a significant negative correlation. Each ΔF/F trace was

773 normalized to its maximum value. *L*-*M*, Average traces of the corresponding PKCδ (L) and Sst (M) neurons

774 shown on the left following entry to the positive context (at 0 sec). Cells 4, 6, 15 and 11 are 4

775 representative neurons encoding the appetitive context after learning. Cells 22 and 23 are non-memory

776 cells, cells 17 and 21 are neurons whose activity is negatively correlated to the positive context. Shaded

777 areas represent s.e.m. *N*-*O*, Ca2+ signals of the corresponding PKCδ (N) and Sst (O) neurons shown in

778   figure J-K upon introduction of a food pellet in the food zone (dotted line) at the end of the place

779   preference assay. Values indicate the Pearson's coefficients of each cell to its corresponding feeding bouts

780   regressor. Values in red indicate a significant correlation. Each ΔF/F trace was normalized to its maximum

781   value. **P-Q**, Heatmap of averaged z-scored calcium responses of PKCδ (P) and SST (Q) recorded neurons

782   following entry to the neutral context (- context at 0 sec). ΔF/F transients were z-scored with the baseline

783   calculated from time points when the animals were in the positive context. Cells were sorted in

784   descending order based on their activity response upon entry in the - context (n = 75 PKCδ+ and 50 Sst+

785   neurons). **R**, Prediction scores of the locations of Prkcd-Cre (purple) and Sst-Cre (yellow) calcium recorded

786   animals in the neutral context during recall. **S**, Preference index (PI) in the positive context on recall day

787   as a function of the averaged value of all Pearson's correlation coefficients to the food zone regressor for

788   all identified neurons encoding appetitive context in a given mouse. Each dot represents a single Prkcd-

789   Cre (purple) or Sst-Cre (yellow) animal. Values shown are R^2. **T**, Prediction score of the logistic regression

790   classifier as a function of the PI of the animal on recall day. Each dot represents a single Prkcd-Cre (purple)

791   or Sst-Cre (yellow) animal. Values shown are R^2. (n = 4 PKCδ- and 4 Sst-GCaMP6s recorded animals). Bar

792   graphs show mean +/- s.e.m and each dot represents a single animal. ns, not significant, *P < 0.05.

793

37

794 **Figure 6**. Differences in calcium activity patterns between CeA $^{PKC\delta}$ and CeA^{SST} neurons

795 **A**-**B**, Maximum-projection representative images of PKCδ+ (A) and Sst+ (B) GCaMP6s-expressing neurons

796 during day 3 of conditioning in the neutral (A_1 and B_1) and in the positive context (A_2 and B_2). **C-D**,

797 Representative Ca^{2+} traces (from 6 mice) of PKCδ (C) and Sst (D) recorded neurons during habituation, day

798 1, day 3 of conditioning, and recall with food. Colored boxes indicate the location of the mouse in the

799 corridor (grey) or on top of the food (green). The values indicate the Pearson's coefficients of each cell to

800 its corresponding feeding bouts regressor. Values in red indicate a significant correlation. Each ΔF/F trace

801 was normalized to its maximum value. **E**, Proportion of neurons per mouse that are significantly active in

802 + context compared to - context on days 1 and 3 of conditioning. (Mann-Whitney U test between PKCδ

803 and Sst on day1, U = 11, P = 0.8413 and on day3, U = 6, P = 0.2045). **F-G**, Frequency distribution of Pearson's

804 correlations to the feeding bouts regressor in all PKCδ and SST recorded neurons during day 1 (F) and day

805 3 (G) of conditioning. **H**, Proportion of neurons per mouse that are food responsive on day 1, day 3 of

806 conditioning, and recall. (Mann-Whitney U test between PKCδ and Sst on day1, U = 10, P = 0.6723, on

807 day3, U = 2, P = 0.0362 and on recall, U = 4, P = 0.0952). **I**, Time spent eating during day 3 for PKCδ- and

808 Sst-GCaMP6s recorded mice (two-tailed unpaired t-test t(8) = 2.242, P = 0.552). **J**, Proportion of food

809 responsive PKCδ+ and SST+ cells as a function of the time spent eating on day 3 of conditioning. **K**,

810 Heatmaps of Pearson's correlations to the feeding bouts regressor for PKCδ (K_1) and SST (K_2) neurons that

811 were classified as food responsive on at least one of the recorded days. White represents neurons that

812 were not detected during a recording session. Blue represents no significant correlations, so non-food

813 responsive cells. Strong correlation values are represented in dark red. Arrows indicate neurons that were

814 classified as food responsive on one day and lost their functional tag on the following one. (n = 40 PKCδ+

815 and 39 SST+ neurons). **L**, Fraction of food responsive (solid lines) and non-food responsive cells (dotted

816 lines) detected during recall that are tagged as food responsive on day 3 and 1 of conditioning and present

817 during habituation. **M-N**, Food responsive PKCδ+ (purple lines, M) and Sst+ cells (yellow lines, N) as well

818   as non-food responsive cells (gray lines) detected during recall and traced back to day 3 and day 1 of

819   conditioning and habituation. Each line represents a cell and its correlation value to the feeding regressor

820   on recall, day 3 and day 1 of conditioning, as well as whether it was present or not during habituation.

821   Values above 0.25 represent a significant positive correlation to the feeding regressor (n = 92 PKCδ+ and

822   59 SST+ neurons). *O*, Proportion of food responsive PKCδ+ and SST+ neurons that were active during

823   habituation and that were significantly active in the - context or the + context during day 1 and day 3 of

824   conditioning (n = 40 PKCδ+ and 39 SST+ neurons). (n = 5 PKCδ- and 5 Sst-GCaMP6s recorded animals). Bar

825   graphs show mean +/- s.e.m, each dot represents a single animal. ns, not significant,*P < 0.05.

826

827 **Extended data and legends**

828 **Figure 5-1.**

829 **A**, Ca^{2+} traces of all PKCδ and Sst identified neurons encoding appetitive context during each step of the

830 conditioning paradigm (A$_1$). Values during recall indicate the Pearson's coefficients of each cell to its

831 corresponding positive context regressor. Values during day 1, day 3, and recall with food indicate the

832 Pearson's coefficients of each cell to its corresponding feeding bouts regressor. Values in red indicate a

833 significant correlation. Each ΔF/F trace was normalized to its maximum value. **A$_2$,** Heatmaps of the ΔF/F

834 signal across the whole arena. Green represents the minimum and purple the maximum per-pixel

835 frequency. **B**, Ca^{2+} traces of the 6 identified PKCδ and Sst cells that show both a significant negative

836 correlation to the positive context regressor and a significant positive correlation to the neutral context

837 regressor (B$_1$). Values during recall indicate the Pearson's coefficients of each cell to its corresponding

838 positive context regressor. Values in green indicate a significant negative correlation. Values during day

839 1, day 3, and recall with food indicate the Pearson's coefficients of each cell to its corresponding feeding

840 bouts regressor. Values in red indicate a significant positive correlation. Each ΔF/F trace was normalized

841 to its maximum value. **B$_2$,** Heatmaps of the ΔF/F signal across the whole arena. Green represents the

842 minimum and purple the maximum per-pixel frequency.

Figure 1

Figure 2

A PKCδ NpHR PKCδ mCherry

A₁ BLa CeC/L CeM
A₂ BLa CeC/L CeM

● PKCδ eNpH3R3.0 fibers
○ PKCδ mCherry fibers
▮ Spread of eNpHR3.0 and mCherry virus in Prkcd-Cre

B Bregma -1.22 mm Bregma -1.34 mm
BLa CeC/L CeM BLa CeC/L CeM

Bregma -1.52 mm Bregma -1.7 mm
BLa CeC/L CeM BLa CeC/L

Inhibition of CeA^{PKCδ} cells during RTPP

C C₁ PKCδ NpHR C₂ PKCδ mCherry
Laser 561nm Laser 561nm

D Cumulative duration in inhibition side (%) ns
PKCδ NpH3.0 PKCδ mCherry

E Velocity during RTPP (cm/s) ns
PKCδ NpH3.0 PKCδ mCherry

Inhibition of CeA^{PKCδ} cells during CPP without food

F PKCδ NpHR
F₁ Laser 561nm
PKCδ mCherry
F₂ Laser 561nm

G PI for the photoinhibition-paired chamber (%) ns
PKCδ NpH3.0 PKCδ mCherry

H PI for the photoinhibition-paired chamber (%)
Before After Before After
●—PKCδ NpHR ●—PKCδ mCherry

I Velocity during test (cm/s) ns
PKCδ NpH3.0 PKCδ mCherry

Figure 3

A PKCδ NpHR   PKCδ mcherry

A₁ CeC/L BLa CeM   A₂ CeC/L BLa CeM

B Sst NpHR   Sst mcherry

B₁ CeC/L BLa CeM   B₂ CeC/L BLa CeM

C

Bregma -1.22 mm   Bregma -1.34 mm   Bregma -1.52 mm   Bregma -1.7 mm

BLa CeC/L CeM

● PKCδ NpH3R fibers   ○ PKCδ mCherry fibers   ● Sst NpH3R fibers   ○ Sst mCherry

Spread of NpHR and mCherry virus in Prkcd-Cre   Spread of NpHR and mCherry virus in Sst-cre

Inhibition of CeA^{PKCδ} or CeA^{Sst} cells during recall

D PI for the food-paired chamber (%)

E PI for the food-paired chamber (%) — Before / After

F PI for the food-paired chamber (%) — Before / After

G PKCδ NpHR — Food zone

H PKCδ mCherry — Food zone

● Sst NpHR   ● Sst mCherry   ● PKCδ NpHR   ● PKCδ mCherry

I PI for the food zone (%)

J Frequency for the food zone

K Velocity during recall (cm/s)

L Amount of food eaten during training (g)

Figure 4

Figure 5

A Correlation to + context

Distribution of cells (%)

Pearson's coefficient

B Correlation to + context

Distribution of cells (%)

Pearson's coefficient

C Location in + context

Prediction score (%)

ns   ns
*   *

Recall Shuffled Recall -   Recall Shuffled Recall -

D **D₁** Recall
Entry to + context
Cell 4
Cell 6
Cell 22
PKCδ
-3   0s   4.5

D₂
Habituation

E **E₁** Recall
Entry to + context
Cell 11
Cell 15
Cell 23
Sst cells
-3   0s   4.5

E₂
Habituation

z-score
3
1.8
0

— PKCδ recall   --- PKCδ hab   — Sst recall   --- Sst hab

F
Food zone

G
Food zone

H
14.5±5.8%
PKCδ memory cells

I
8.7±5.6%
Sst memory cells

J Recall
PKCδ cell 4   0.33
PKCδ cell 6   0.30
DF/F
PKCδ cell 22   -0.21
PKCδ cell 17   -0.24

K
Sst cell 15   0.49
Sst cell 11   0.24
DF/F
Sst cell 23   -0.17
Sst cell 21   -0.3

L Entry to + context
z-score
6
0
12
0
3
0
3
0
-2.5 0s 4.5

M
z-score
6
0
70
0
3
0
3
0
-2.5 0s 4.5

N Recall with food
PKCδ cell 4   0.42
PKCδ cell 6   -0.52
DF/F
PKCδ cell 22   -0.29
PKCδ cell 17   0.85

O
Sst cell 15   0.23
Sst cell 11   0.79
DF/F
Sst cell 23   -0.27
Sst cell 21   0.15

■ Corridor   ■ Context +   ■ Food zone   — + Context regressor   ⁞ Adding food   ■ Feeding bouts   1 min

P Entry to - context
Cell 16
Cell 17
PKCd cells
-3   0s   4.5

Q Entry to - context
Cell 18
Cell 20
Cell 21
Cell 19
Sst cells
-3   0s   4.5

z-score
3
1.8
0

R Location in neutral context
Prediction score (%)
100
0
Recall   Recall

S
PI for the + context (%)
70
30
0.25
0.1   0.2   0.3   0.4   0.5
Pearson's coefficients to food zone regressor

T
PI for the + context (%)
80
20
0.58*
50 60 70 80 90
Prediction score (%)

Figure 6