



OPEN Morphological and electrophysiological diversity and connectivity of principal neurons in the lateral and basal nuclei of the mouse amygdala

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The basolateral amygdala is a cortical structure involved in various cognitive functions. Despite numerous studies on amygdala operation, the properties of its excitatory principal neurons (PNs) are not fully characterized. Here, we combined neuroanatomical, electrophysiological, and tracing methods to analyze the single-cell features and morphological characteristics of PNs in the lateral (LA) and basal amygdala (BA). Using a mouse reporter line, we found that cholecystokinin (CCK) promoter-driven DsRed expression defines two distinct PN populations. PNs in the DsRed-positive area of the LA had small somata and short dendrites, consistent with their electrophysiological profiles. PNs in the DsRed-negative area of the LA and all PNs in the BA showed similarly ramified dendrites and single-cell features. Importantly, PN dendritic arbors were confined to DsRed-defined subnuclei, which correlated with distinct extra-amygdalar inputs, indicating input specificity. Axonal projections also corresponded with soma location; for example, BA PNs projecting to the medial prefrontal cortex - but not to the lateral nucleus of the central amygdala (CEAl) - were found in the DsRed-positive subnucleus, whereas those projecting to the CEAl were located in the DsRed-negative subnucleus. Our findings reveal that the basolateral amygdala contains distinct subnuclei with specific input–output patterns, enabling flexible information processing necessary for the diverse amygdala functions.

Abbreviations

AAA	Anterior amygdalar area
Alp	Agranular insular area, posterior part
AP	Anteroposterior
BAa	Basal amygdalar nucleus, anterior part
BAP	Basal amygdalar nucleus, posterior part
BLAv	Basolateral amygdalar nucleus, ventral part
BMAa	Basomedial amygdalar nucleus, anterior part
BMAp	Basomedial amygdalar nucleus, posterior part
Br.	Bregma
CEA	Central amygdalar nucleus
CEAl	Central amygdalar nucleus, lateral part
CEAm+c	Central amygdalar nucleus, medial and capsular part
CLA	Clastrum
COA	Cortical amygdalar area
CP	Caudoputamen
cpd	Cerebral peduncle

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CTB	Cholera toxin B subunit
ctx	Cortex
DV	Dorsoventral
ec	External capsule
ENTl	Entorhinal area, lateral part
ENTmv	Entorhinal area, medial part, ventral zone
EP nucl.	Endopiriform nucleus
EPd	Endopiriform nucleus, dorsal part
EPv	Endopiriform nucleus, ventral part
fi	Fimbria
FS	Fundus of striatum
GP	Globus pallidus
IA	Intercalated amygdalar nucleus
int	Internal capsule.
LA	Lateral amygdalar nucleus
LAA	Lateral amygdalar nucleus, anterior part
LAp	Lateral amygdalar nucleus, posterior part
MEA	Medial amygdalar nucleus
ML	Mediolateral
opt	Optic tract
PA	Posterior amygdalar nucleus
PAA	Piriform-amygdalar area
PFC	Prefrontal cortex
PIR	Piriform cortex
PL	Prelimbic cortex
RT	Reticular nucleus of the thalamus
SI	Substantia innominata
st	Stria terminalis
SUBv	Subiculum, ventral part
TR	Postpiriform transition area
vHipp	Ventral hippocampus
vHipp/SUB	Ventral hippocampus/subiculum
VL	Lateral ventricle
ZI	Zona incerta

The amygdala is a complex structure in the brain comprised of multiple interconnected nuclei playing roles in a broad spectrum of normal behavioral functions and pathological conditions^{1,2}. The basolateral amygdala complex (BLA) is a well-defined region composed of the lateral (LA), basal (BA) and basomedial (BMA) nuclei^{3,4}. The principal neurons (PNs) in the BLA are glutamatergic projecting cells^{4–7} accounting for ~80% of all neurons in the mouse LA and BA⁸ and sending axonal projections toward cortical and subcortical structures^{4,7,9–11}. Although developmental studies indicate that PNs in the BLA originate from the pallium^{12,13}, similarly to cortical areas¹², they are not organized into layers^{4,6}, posing a challenge in exploring their network organization principles.

BLA nuclei are heterogeneous in their morphological and electrophysiological characteristics or functionality^{3,4,14}. Early neuroanatomical studies divided the BA into anterior and posterior parts^{15,16}, which were supported by transcriptomic investigations¹⁷. The most recent mapping of the brain-wide connectivity of the BA has further delineated the anterior BA into three domains: medial, lateral and caudal parts¹¹. These and additional studies have highlighted that PNs with different projection target areas spatially segregate within the BA^{11,18–20}. For instance, neurons targeting the prelimbic cortex (PL) involved in controlling fear states^{2,21,22} and anxiety^{23–25} are more abundant in the anteromedial BA^{19,20,26}, while the lateral nucleus of the central amygdala (CEAl) projecting neurons conveying information of conditioned fear^{27–29} are mainly located in the posterolateral BA^{19,29}. Interestingly, the mouse LA has not been divided further in these recent studies¹¹, although previous studies in rats and monkeys proposed the presence of distinct subnuclei in the LA, too^{3,4,10,14,16}.

Cholecystokinin (CCK) is a neuropeptide that affects the local function of the BLA through altering fear- and anxiety-related behavior^{30–32}. In BAC-CCK-DsRed transgenic mice, DsRed fluorescent protein is expressed under the CCK promoter, resulting in fluorescent labeling of a subset of CCK containing neurons³³. Both PNs and a group of GABAergic basket cells express CCK and CB1 cannabinoid receptors in the BLA^{34,35}, with the former group characterized by weak and the latter by strong DsRed expression³⁶. The CCK promoter-driven expression of DsRed signals in PNs shows heterogeneity along the anteroposterior axis both within LA and BA, suggesting the presence of distinct structural domains in these BLA nuclei.

In this study we demonstrate that DsRed expression-defined areas within the LA and BA are structural units that may correspond to functional subnuclei. In addition, we observed that PNs in the DsRed + LA are smaller and have shorter dendritic arbors compared to those PNs located in the posterior LA and BA. Finally, our results revealed that PNs project to known target areas within the amygdala and the surrounding areas, but we also observed axons of BA PNs in the LA that have not been revealed yet.

Results

CCK promoter-driven expression of DsRed defines LA and BA subnuclei

To study the distribution of CCK-expressing PNs in the BLA, BAC-CCK-DsRed transgenic mice were used^{33,36}. In low magnification images of coronal and horizontal amygdala sections obtained from these transgenic mice, distinct subunits can be separated both in the LA and BA based on the DsRed content of the neurons: one region shows strong DsRed labeling (DsRed+ area), while the other part contains only sparsely labeled DsRed-expressing neurons (DsRed- area) (Fig. 1A, B). The DsRed+ subnucleus is confined to the anterior and lateral segments of the LA (LAa) and in the anterior and medial segments of the BA (BAa). Conversely, the posterior parts (LAp and BAp) are DsRed-. Thus, DsRed expression in this transgenic mouse line clearly divides both the LA and BA into two subnuclei.

The dendritic trees of PNs are restricted to the subnucleus where their cell bodies are located

To determine whether the dendritic tree location of PNs would match to the subnuclei defined by DsRed expression, we examined the location and extension of dendritic arborizations of PNs in coronal and horizontal slice preparations (Fig. 2A). PNs in different subnuclei were filled with biocytin followed by visualization of their biocytin content. After immunolabeling, the dendritic trees of individual PNs were manually reconstructed based on high-resolution confocal images and the proportion of dendritic arbors located within and outside of the DsRed+ regions was determined. The borders between the LA and BA were determined based on the immunostaining against vesicular acetylcholine transporter (VACHT)^{8,37} (see Supplementary Fig. S1 online). Our results show that the dendritic trees of the reconstructed PNs were restricted to the subnucleus of soma location (71–99% of the dendritic length was present in the given subnucleus on average). Accordingly, in the DsRed+ LAa and BAa subnuclei, dendritic trees were confined to the areas defined by DsRed expression, while in the DsRed- LAp and BAp, they avoided the DsRed+ regions (Fig. 2B–F). These results imply that DsRed expression can define structural units within the LA and BA, where the dendrites of PNs are limited to the given subnucleus.

DsRed expression-defined subnuclei can be considered structural units

If the subregions defined by DsRed expression form structural subunits, then it is likely that these distinct subnuclei receive different sets of afferents that specify the inputs received by PNs with subregion-confined dendritic trees. To test this hypothesis, we examined whether the termination pattern of afferents reaching the amygdala follows the borders of DsRed expression-defined subnuclei. The medial prefrontal cortical (mPFC), the insular cortical (IC), and the midline thalamic (MT) afferents were examined since these are among the primary sources of excitatory inputs to the LA and BA (Fig. 3)(Supplementary Figs S2–5 online). AAV9-Efla-DIO-eYFP was injected into the mPFC or IC of Vglut1-Cre::BAC-CCK-DsRed mice expressing Cre recombinase under the promoter of vesicular glutamate transporter type 1 (Vglut1) and DsRed fluorescent protein under the CCK promoter ($n = 2–2$) (Supplementary Fig. S2 online). This approach allowed us to trace the cortical glutamatergic inputs reaching the amygdala and compare their pattern to DsRed expression. In rostral and caudal segments of the BA, both mPFC and IC afferents targeted the BAa and the BAp but in a complementary manner. In the medial segments, the BAa was exclusively innervated by the mPFC afferents, whereas the LAp and BAp subnuclei were innervated by the IC axons (Fig. 3A–B) (Supplementary Figs S3–4 online). Since there is no Vglut1 expression in the thalamus³⁸, MT afferents were labeled using AAV5-CaMKII-eYFP ($n = 2$) (Supplementary Fig. S2 online). The axons of these thalamic inputs primarily targeted the BA, regardless of the subunits determined by DsRed expression (Fig. 3C) (Supplementary Fig. S5 online) in line with previous observations^{39–41}. These results demonstrate that DsRed expression in PNs defines structural units in the LA and BA as both the dendritic trees and cortical inputs match the subnuclei delineated by this fluorescent signal expression in the PNs.

Principal neurons of the LAa are smaller than those in the lap

Next, we asked whether the PNs in distinct subnuclei in the LA and BA have different morphological and electrophysiological properties. To this end, we compared the features of in vitro filled PNs (Fig. 4). LAa PNs were found to be smaller, having on average shorter dendritic length and less ramified dendrites than in other PNs examined in the DsRed- part of the LA, in the LAp (Fig. 4B, D). Accordingly, significant differences were observed in the length of 4th and 5th order dendrites of PNs (Fig. 4C). Furthermore, Sholl analysis revealed that at a distance of 50–100 μm from the soma, the dendritic length of LAa PNs was shorter compared to PNs in other subnuclei (Fig. 4E). As these PN labeling took place in slice preparations using whole-cell recordings allowing us to study single-cell features, we could determine the active and passive membrane properties of PNs located in distinct subnuclei (Table 1). Positive and negative step currents were injected into the PN somata and the voltage responses were recorded and subsequently analyzed (Fig. 5A). In accord with the morphological data, LAa PNs had the lowest capacitance in comparison to other PNs (Fig. 5B). The input resistance of LAa PNs was found to be larger only in contrast to BAp PNs (Fig. 5C). In contrast, the membrane time constant, which determines the integration properties of neurons⁴², was slower for LAp PNs in comparison to LAa and BAp PNs (Fig. 5D). Interestingly, the amplitude of the sag, which corresponds to the expression of h current^{43,44}, was not different in PNs studied (Fig. 5E), showing that postsynaptic potentials may be controlled similarly along the dendrites by this active conductance⁴⁵. Lastly, we compared the spiking features and found that LAa PNs have the highest spike threshold compared to BA PNs (Fig. 5F) and the steepest input-output function (Fig. 5G). Thus, LAa PNs are overall the smallest PNs in the LA and BA and the smallest excitatory inputs can activate them. Dendritic morphology and single-cell features of BAa and BAp PNs did not differ.

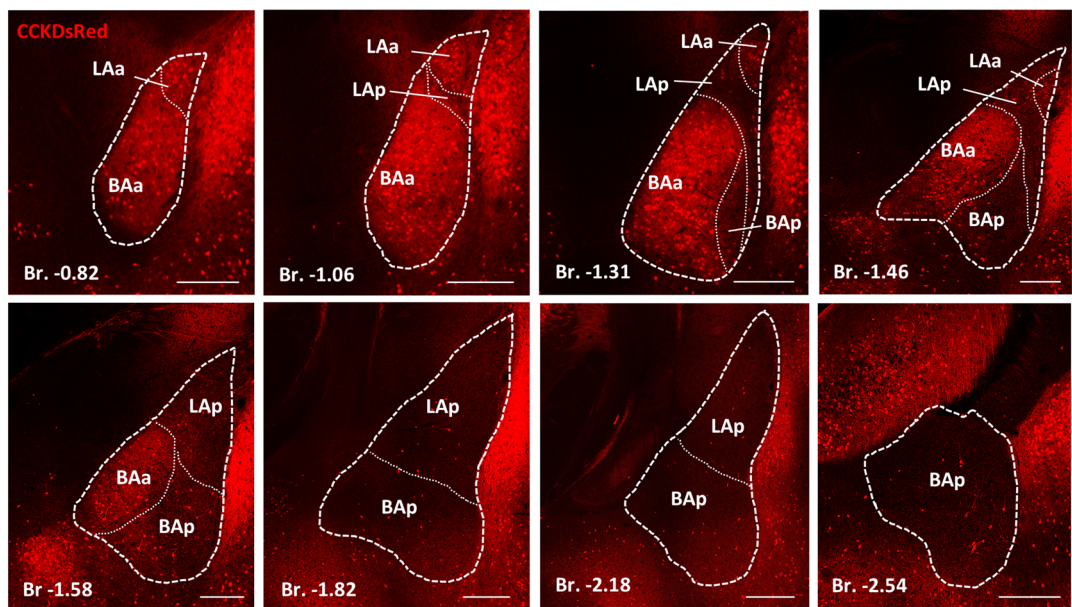
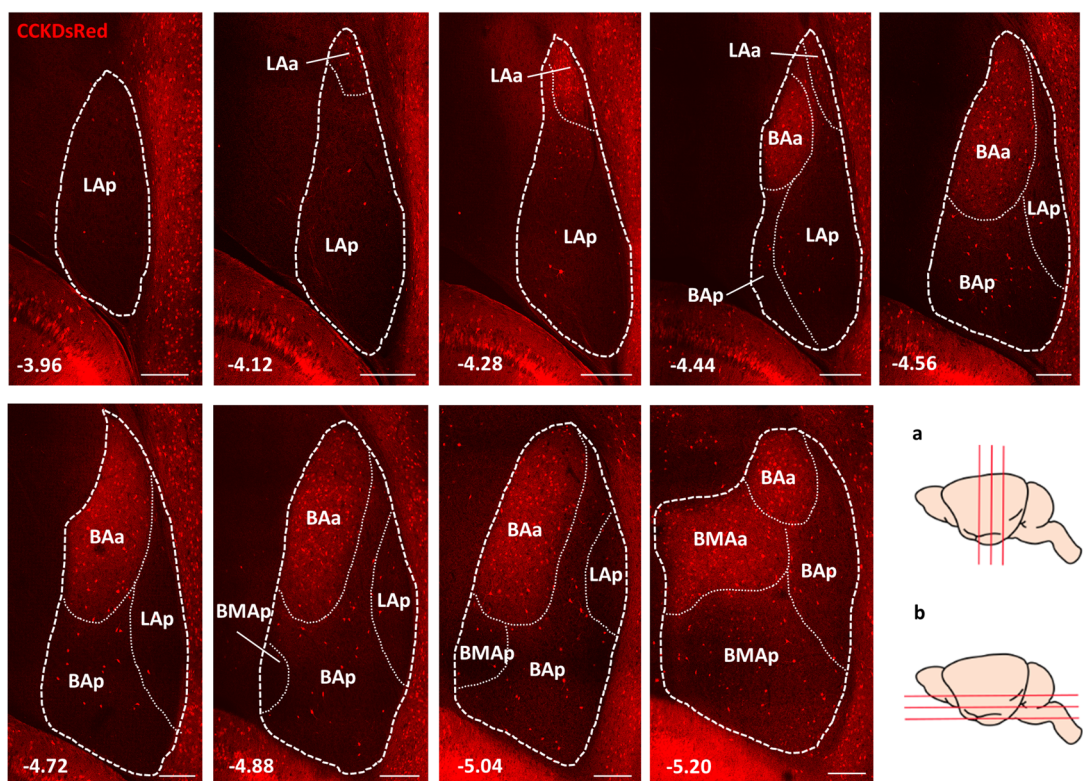
a Coronal amygdala slices in BAC-CCK-DsRed mice**b Horizontal amygdala slices in BAC-CCK-DsRed mice**

Fig. 1. Cholecystokinin (CCK) promoter-driven expression of DsRed defines LA and BA subnuclei. (**a,b**). Confocal images of coronal (**a**) and horizontal (**b**) sections of the amygdala from BAC-CCK-DsRed mice indicate the distribution of endogenously expressing DsRed fluorescent protein under the control of the CCK promoter. The density of DsRed-expressing neurons clearly divides the LA and BA into a DsRed + anterior (LAa and BAa) and a DsRed - posterior (LAp and BAp) part. White dashed and dotted lines represent the borders of the BLA and their subnuclei, respectively. Scale bar: 250 μ m. Abbreviations: LAa: lateral amygdalar nucleus, anterior part; LAp: lateral amygdalar nucleus, posterior part; BAa: basal amygdalar nucleus, anterior part; BAp: basal amygdalar nucleus, posterior part; BMAa: basomedial amygdalar nucleus, anterior part; BMAp: basomedial amygdalar nucleus, posterior part; Br.: Bregma. In coronal slices numbers refer to the distance from Bregma in mm; in horizontal slices the interaural distance is indicated.

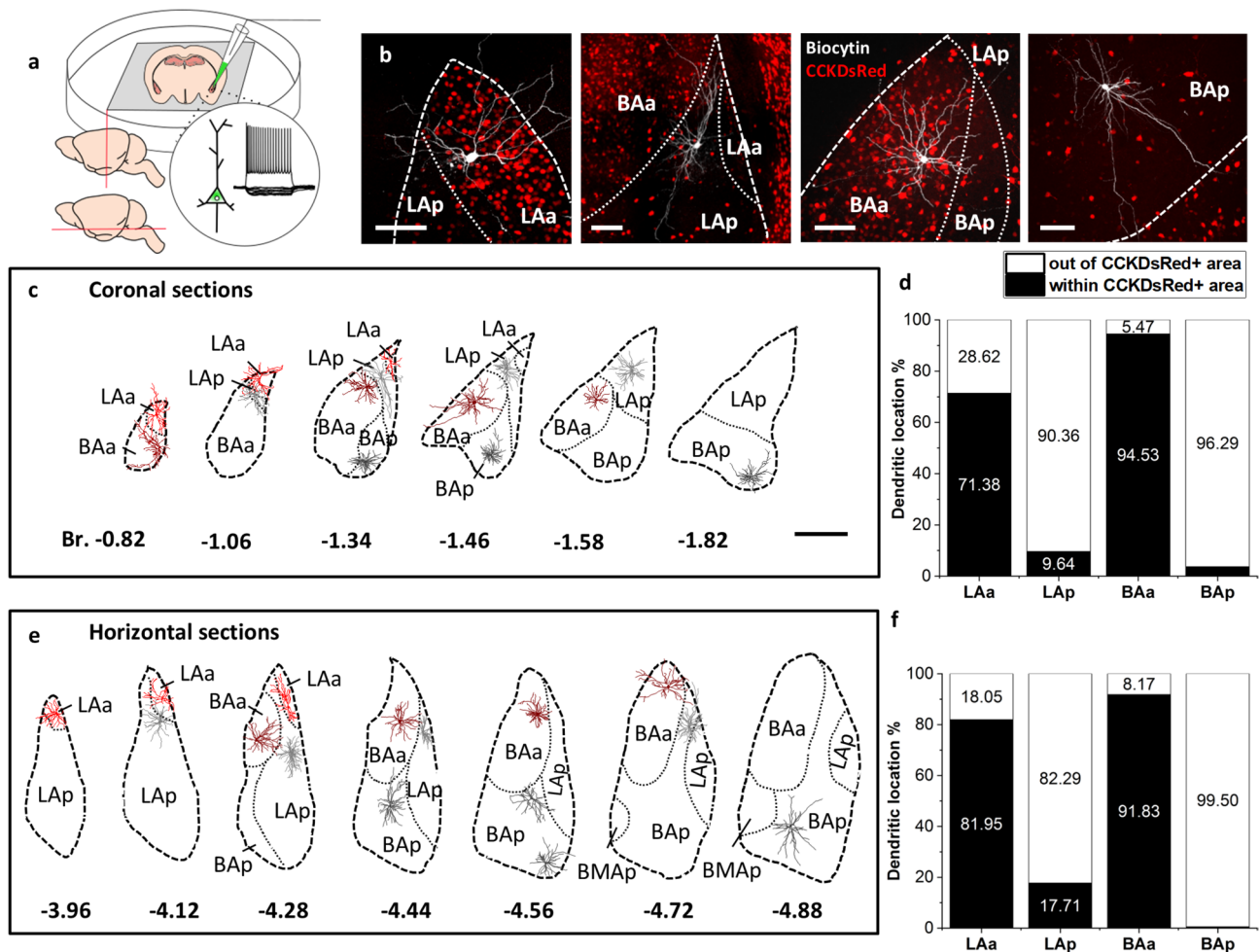


Fig. 2. The dendritic trees of principal neurons show spatial restriction to the subnucleus where the soma is located. **(a)** Experimental setup. In vitro whole-cell recording and labeling of PNs in coronal and horizontal slice preparations, focusing on distinct subnuclei of LA and BA. **(b)** Confocal images of biocytin filled example neurons showing restricted dendritic trees to the LA and BA subnuclei. Images of example PNs taken from coronal (LAp PN and BAp PN) and horizontal (LAa PN and BAa PN) slices. Scale bar: 100 μ m. **(c)** Reconstructed dendritic trees of 14 representative PNs from LA and BA subnuclei obtained from 6 different coronal sections along the anteroposterior axis of the amygdala. Scale bar: 500 μ m. **(d)** Bar graph showing the proportion of dendritic trees located within and outside of DsRed + areas in coronal slices (LAa: $n=9$ (red); LAp: $n=8$ (light gray); BAa: $n=9$ (dark red) and BAP: $n=9$ (dark gray); 35 PNs from 17 mice). Dendritic trees of PNs with somata located in the LAa and BAa are restricted to DsRed + areas. Conversely, dendritic trees of PNs with somata located in the LAp or BAP are restricted to DsRed - areas. **(e)** Reconstructed dendritic trees of 15 representative PNs from the LA and BA subnuclei obtained from 7 different horizontal sections along the dorsoventral axis of the amygdala. **(f)** Bar graph showing the proportion of dendritic trees located within and outside of the DsRed + areas in horizontal slices (LAa: $n=8$ (red); LA: $n=13$ (light gray); BAa: $n=9$ (dark red) and BAP: $n=23$ (dark gray); 53 PNs from 23 mice). Dendritic trees of PNs with somata located in the LAa and BAa are restricted to DsRed + areas. Conversely, dendritic trees of PNs with somata located in the LAp or BAP are restricted to DsRed - areas. Scale bar: 500 μ m.

The distribution of somata of PNs projecting to different target areas corresponds to the DsRed expression-defined structural subunits

Several studies have highlighted that BLA PNs with different projection targets are spatially segregated^{11,18–20}. Therefore, we examined how the distribution of these neuronal populations relates to the subnuclei defined by DsRed expression. We addressed this question by retrograde labeling of those neurons that project to the distinct target areas of the BLA. Cholera toxin B subunit (CTB), a retrograde tracer or retrograde adeno-associated virus (AAVrg) was injected into the PL, CEAl and vHipp/SUB, respectively (see Supplementary Fig. S6 online), to reveal the PN populations with different projection targets in the LA and BA. To achieve more complete labeling, tracers were administered at two anteroposterior coordinates (except for the CEAl due to its small size). Our data demonstrate that PL-projecting PNs were located almost exclusively in the BAa (Fig. 6A), while the somata of CEAl-projecting neurons were restricted to the BAP in addition to the LA (Fig. 6B). PNs projecting to the vHipp/

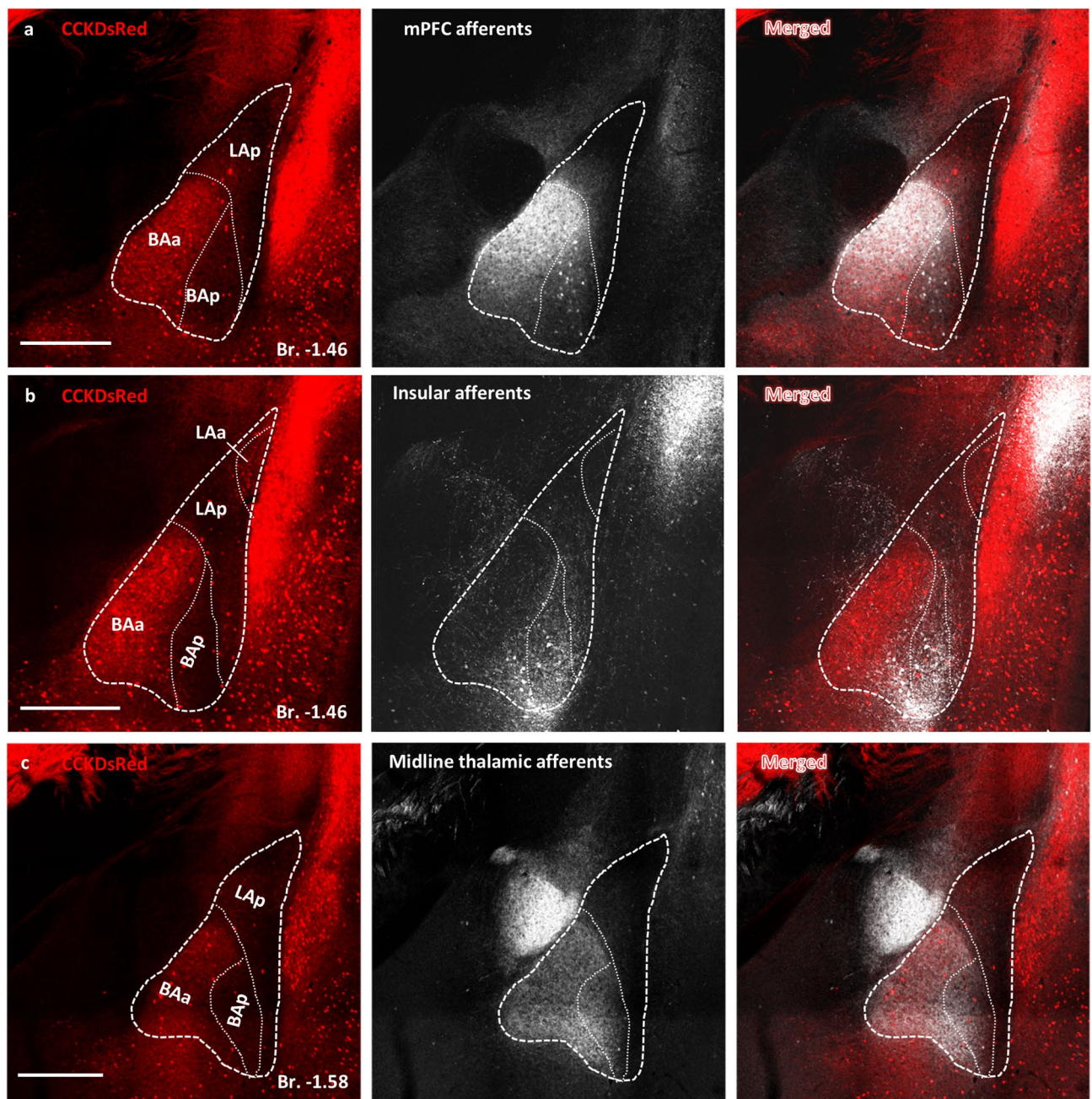


Fig. 3. CCK-defined BA subnuclei receive distinct cortical inputs. **(a)** Medial prefrontal cortical (PFC), **(b)** insular cortical (IC), and **(c)** midline thalamic (MT) afferents innervating the amygdala in BAC-CCK-DsRed mice. PFC and IC target the BAa and BAp in a complementary manner, while axons of the MT primarily target both parts of the BA, regardless of the subunits determined by CCK expression. Scale bar: 500 μ m. Numbers refer to the distance from Bregma (Br.) in mm. Note that AAV9-Ef1a-DIO-eYFP used in the experiments shown in **(a)** and **(b)** strongly labels projecting axons but also results in visualizing some somata in a retrograde manner.

SUB did not show spatial segregation within the BA (Fig. 6C). Similarly, spatial segregation within the LA was not observed in case of either projections (Fig. 6A-C). In summary, our findings imply that DsRed expression differentiates regions containing PL-projecting PNs and CEAL-projecting PNs within the BA. Conversely, BA PNs targeting the vHipp/SUB exist in both subnuclei. Moreover, there is an absence of spatial segregation in the LA for PNs projecting to either the CEAL or vHipp/SUB.

Overall, our results suggest that the DsRed expression segregates the areas where PL-projecting PNs and CEAL-projecting PNs are found in the BA, whereas BA PNs that project to the vHipp/SUB are present in both subnuclei. In addition, there is no spatial segregation of PNs in the LA that project to the CEAL or vHipp/SUB.

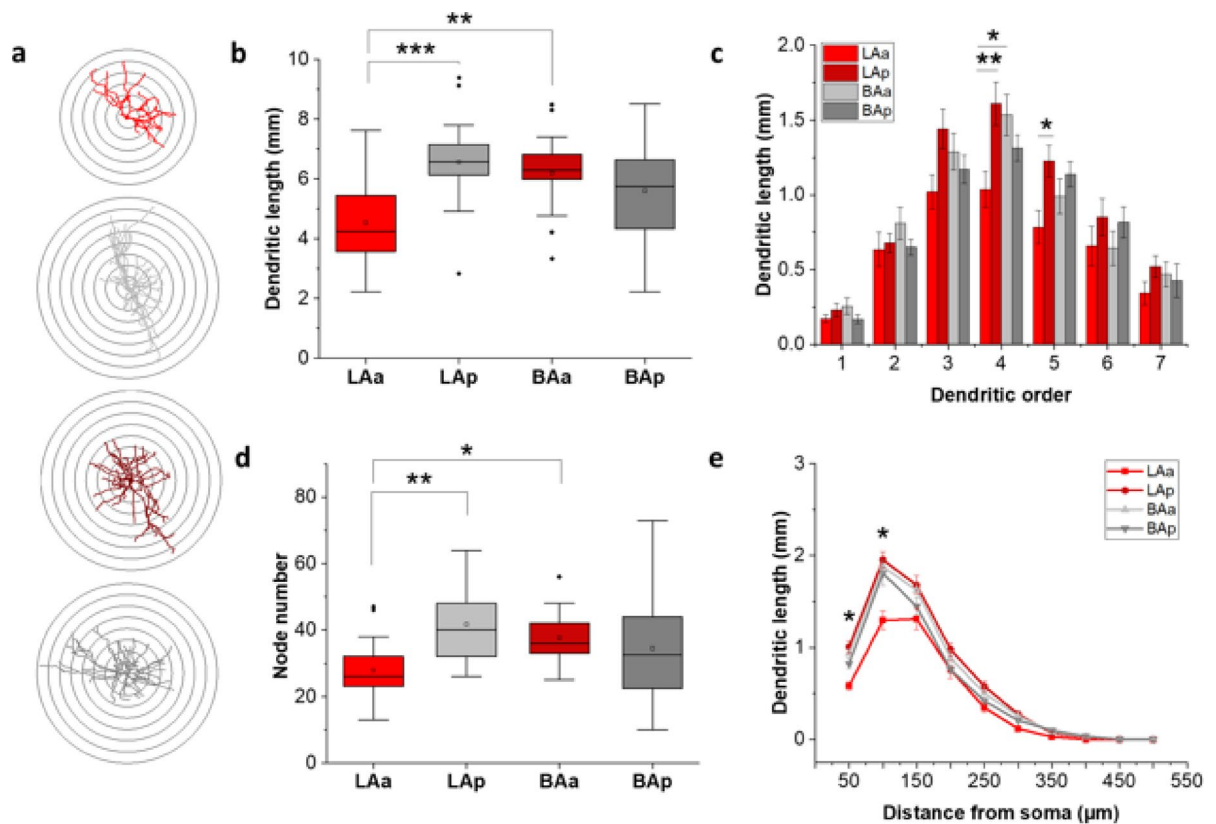


Fig. 4. Dendritic tree features of principal neurons in the subnuclei of LA and BA. **(a)** Reconstructions of dendritic trees and Sholl-analysis of representative principal neurons (PNs) in each LA and BA subnucleus. The difference between the radii of concentric circles is 50 μm . **(b)** Comparison of total dendritic length from LA and BA subnuclei. **(c)** Comparison of the dendritic length of LAa, LAp, BAa and BAp PNs as a function of dendritic order. Dunn's test showed significant differences for 4th (LAa–LAp: $p = 0.015$; LAp–BAa: $p = 0.042$) and 5th (LAa–LAp: $p = 0.021$) order dendrites. **(d)** Comparison of the number of branching nodes of dendritic trees of different PN groups. **(e)** Sholl-analysis of reconstructed dendritic trees showing the dendritic length as a function of the distance from the soma. Dunn's test revealed significant differences at 0–50 μm (LAa–LAp: $p < 0.001$; LAa–BAa: $p < 0.001$; LAa–BAp: $p = 0.021$) and at 50–100 μm distance from the soma: LAa–LAp: $p < 0.001$; LAa–BAa: $p = 0.007$; LAa–BAp: $p = 0.016$). **(a–e)** Data obtained in coronal and horizontal slices (LAa: $n = 17$; LAp: $n = 21$; BAa: $n = 18$; BAp: $n = 32$, 80 PNs from 38 mice). Statistical comparisons were performed with Kruskal–Wallis ANOVA followed by *post hoc* Dunn's test. For detailed morphological parameters see (Table 2).

Reconstructions of in vivo filled PNs reveal unique examples of connections between neighboring brain areas

Tracing studies are well-suited for revealing the long-range projections of PNs, but this method is only partially suited to uncover their axonal arborizations locally within the given brain region. Further, labeling of neural processes in brain slices also provides limited information on the within-region axonal extent. Understanding the local connectivity is crucial to comprehending the mechanisms of intra-amygdalar information processing. Consequently, we conducted in vivo juxtacellular recordings in the LA and BA in anesthetized mice (Fig. 7A), utilizing a single-cell labeling technique that allows, in addition to record spiking, visualizing the dendritic arbors and axonal projections of individual neurons^{46,47}. From 100 recorded neurons ($n = 55$ mice), 41 neurons could be reliably localized within the LA or BA. These neurons were categorized into different groups based on the success of recordings followed by Neurobiotin labeling (Supplementary Fig. S7 online) (Table 3).

First, we compared the spiking properties of PNs (Table 4). Statistical comparisons of different firing parameters detected in LAp, BAa and BAp PNs showed no significant differences (ANOVA, $p > 0.1$ for all parameters). Of labeled PNs, 21 were chosen for reconstruction that showed no or minimal weakening in staining intensity even at the distal parts of their arborizations. We then examined the morphological properties of the dendritic trees of these in vivo filled PNs (Fig. 7). By comparing the total dendritic length, structure, and the number of branching points, we observed that PNs in the LAp ($n = 4$), BAa ($n = 3$) and BAp ($n = 12$) have similar dendritic arborizations (Fig. 7B–E). In addition, we successfully labeled 2 PNs in the LAa that had shorter dendritic length and correspondingly fewer branch points (Fig. 7B–E). Overall, these data obtained in vivo are in good agreement with those obtained in slice preparations, indicating that LAa PNs are smaller with less elaborated dendritic trees in comparison to PNs in the LAp, BAa and BAp, which have similar dendritic morphology.

	LAa (n = 10–12)	LAp (n = 15–19)	BAa (n = 12–16)	BAP (n = 17–20)	Kruskal- Wallis ANOVA	LAa- LAp Dunn's test	LAa- BAa Dunn's test	LAa- BAP Dunn's test	LAp- BAa Dunn's test	LAp- BAP Dunn's test	BAa- BAP Dunn's test
AP threshold (mV)	-31.10 (-35.10, -26.40)	-34.30 (-36.40, -30.40)	-37.00 (-39.12, -32.62)	-36.40 (-37.10, -34.50)	0.003 **	0.842	0.021 *	0.005 **	0.551	0.193	1
Spike rate (Hz)	38.80 (33.45, 53.12)	31.30 (23.80, 36.30)	30.65 (25.05, 36.30)	25.65 (22.50, 28.80)	<0.001 ***	0.061	0.150	<0.001 ***	1	0.623	0.404
Accommodation ratio	3.91 (2.81, 5.25)	4.96 (3.84, 5.65)	6.49 (4.40, 7.55)	5.21 (3.32, 6.83)	0.056						
Full width at half maximum (ms)	0.65 (0.60, 0.90)	0.60 (0.60, 0.80)	0.70 (0.60, 0.87)	0.90 (0.80, 0.90)	0.007 **	1	1	0.178	1	0.005 **	0.329
AHP amplitude (mV)	0.70 (0.60, 10.80)	5.50 (0.90, 11.20)	7.45 (3.67, 21.35)	11.40 (8.67, 51.85)	0.003 **	1	0.709	0.004 **	1	0.027 *	0.384
AHP 25% decay (ms)	31.50 (2.40, 80.80)	49.05 (7.57, 84.97)	3.40 (1.70, 56.95)	57.60 (40.20, 83.6)	0.063						
AHP 50% decay (ms)	43.50 (4.60, 124.30)	68.00 (14.55, 106.62)	38.05 (3.77, 112.72)	73.10 (58.40, 131.30)	0.198						
AHP 75% decay (ms)	45.15 (26.37, 106.85)	75.40 (31.00, 117.60)	34.85 (16.57, 119.42)	83.90 (62.90, 151.00)	0.109						
Sag amplitude	0.13 (0.07, 0.20)	0.11 (3.21E-02, 0.16)	0.15 (0.08, 0.20)	0.14 (7.23E-02, 0.18)	0.442						
Input resistance (MΩ)	173.00 (120.25, 197.15)	118.50 (73.59, 144.20)	107.75 (68.79, 159.40)	76.15 (57.02, 159.40)	<0.001 ***	0.181	0.108	<0.001 ***	1	0.156	0.417
Membrane capacitance (pF)	130.80 (105.22, 205.13)	267.20 (206.10, 409.20)	278.90 (233.72, 398.60)	300.00 (220.70, 375.48)	<0.001 ***	0.003 **	<0.001 ***	0.001 ***	1	1	1
Membrane time constant (ms)	22.63 (21.34, 25.88)	32.08 (25.96, 42.51)	32.03 (22.58, 45.01)	24.02 (20.06, 29.03)	0.005 **	0.021 *	0.113	1	1	0.045 *	0.259

Table 1. Electrophysiological properties of in vitro recorded principal neurons in different LA and BA subnuclei. Data presented as the median with the first and third quartiles in parentheses. Significant differences, indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$), were determined by Kruskal–Wallis ANOVA followed by post hoc dunn's test (AP: action potential, AHP: after hyperpolarization).

Next, we compared the distribution of axonal collaterals of the reconstructed PNs in different amygdalar nuclei as well as the surrounding regions. An example PN shown in Fig. 7F demonstrates that its axons could be traced from the fundus of striatum (Bregma -0.5 mm) to the subiculum (Bregma -3.4 mm). Our analysis revealed characteristic axonal distributions for PNs located in the four subnuclei studied (Fig. 8A). The 2 LAa PNs had axons locally in the LA and BA and projected to the CEA and ventral part of the caudoputamen (CP, often referred to as the amygdalo-striatal area, AStria) (Fig. 8B, C). LAp PNs had more complex axonal arbors (Fig. 8D) (Supplementary Fig. S8 online). They densely arborized locally within the LA, innervated the CP and projected either to the BAa or to the BAP. Anterior to the cell body, their axons reached the posterior insular cortex (AIP), whereas the posterior branches arborized in the amygdalo-piriform transition area (TR) (Fig. 8D). The axons of BAa PNs primarily ramified locally and in the BAP (Fig. 8E). They were observed in the medial, but not in the lateral nucleus of the CEA, consistent with our retrograde tracing results (Fig. 6, Supplementary Fig. S8 online) and revealing that BAa PNs avoid the CEAL. In contrast, the axon collaterals of BAP PNs were typically present in the CEAL as well as other CEA subdivisions in addition to the neighboring areas, including the BAa and TR (Figs. 7F and 8F and G). Interestingly, we observed axon collaterals of both BAa and BAP PNs in the LA, too (Fig. 8) (Supplementary Fig. S8 online). Furthermore, 2 PNs were labeled in the BAP, the axons of which ramified extensively locally, and reached the different parts of the hippocampal formation (see Supplementary Fig. S9 online). These observations collectively show that, in addition to the subnucleus-restricted dendritic arbors of PNs in the LA and BA, their axons also arborize mostly in a stereotypical manner, which may enable a well-organized information flow within the BLA and the surrounding areas.

Discussion

In this study, we revealed that DsRed expression driven by the CCK promoter in BAC-CCK-DsRed mice^{33,36} divides the LA and BA into two subnuclei. The borders of these DsRed expression-defined areas do not completely align with the recently defined borders of LA and BA subnuclei: DsRed + BAa defined in our study corresponds mainly to the BLA.al and BLA.am, while the DsRed- BAP matches the BLA.ac and BLAp subdomains defined by Hintiryan et al., 2021¹¹. Furthermore, the DsRed + BAa significantly but not completely overlaps with the BLA, but the DsRed- BAP corresponds well to the BLP in the Mouse Brain Atlas⁴⁸. The DsRed + LAa does not resemble any previous subdivisions in the mouse LA.

Based on in situ hybridization results published by the Allen Brain Institute (exp. 77869074)⁴⁹, Cck mRNA expression is not homogenous across BLA neuronal populations. For instance, the expression is more pronounced in the BAa region compared to the BAP. We propose that variations in CCK levels among different BLA PNs are represented by the DsRed signal in BAC-CCK-DsRed mouse line, which highlights, albeit weakly,

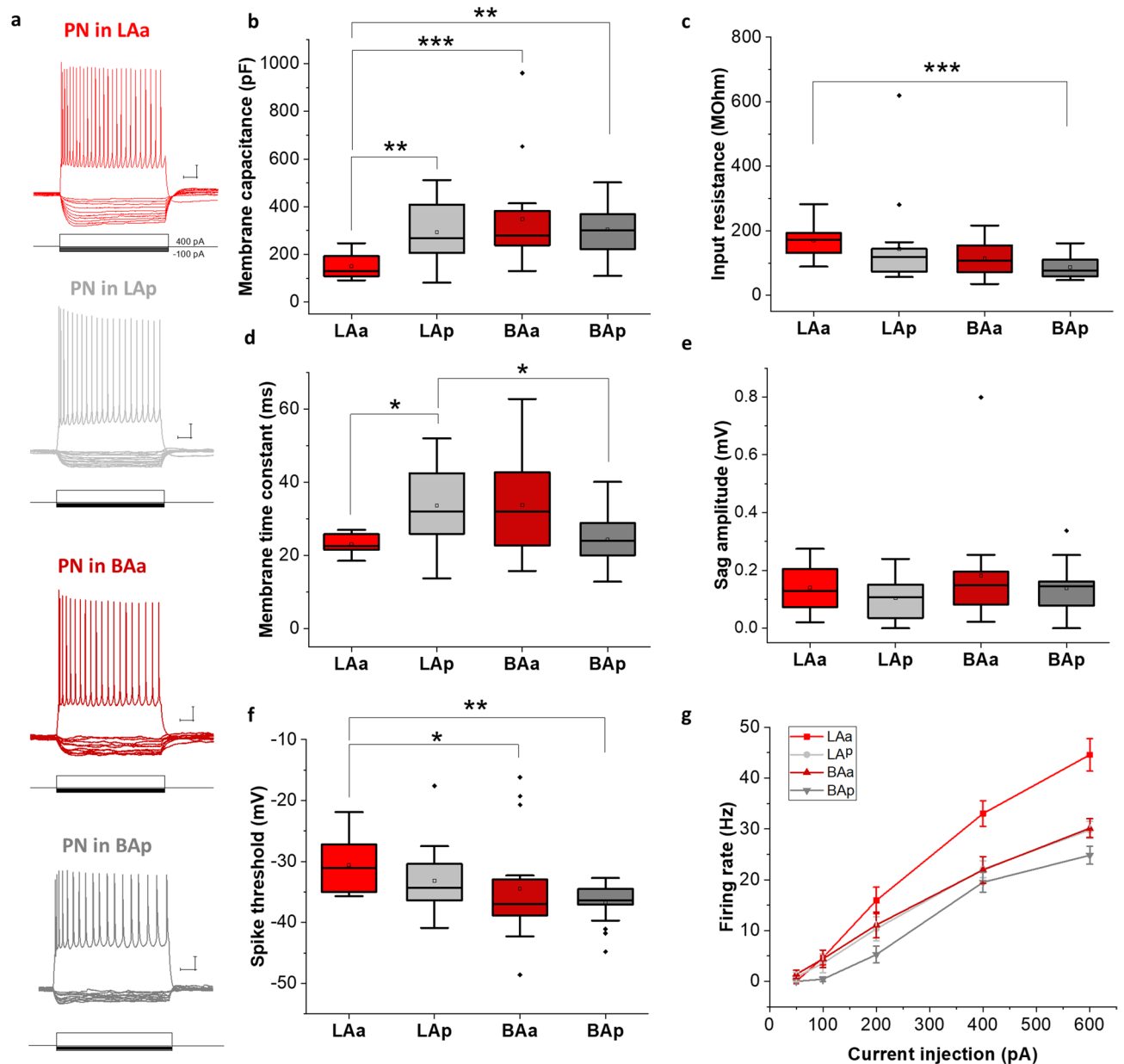


Fig. 5. The active and passive membrane properties of in vitro recorded principal neurons in LA and BA subnuclei. (a) Example firing traces of principal neurons from each LA and BA subnuclei obtained in BAC-CCK-DsRed animals. Scale bar: y: 10 mV and x: 100 ms. Box chart comparison of membrane capacitance (b), input resistance (c), membrane time constant (d), sag amplitude (e) and spike threshold (f) of the PNs in different LA and BA subnuclei (LAa: $n = 12$; LAP: $n = 19$; BAa: $n = 16$; BAp: $n = 18-20$, 67 PNs from 39 mice). Statistical comparisons were performed with Kruskal-Wallis ANOVA followed by *post hoc* Dunn's test. (g) Firing rate as a function of injected current (LAa: $n = 6$; LAP: $n = 3$; BAa: $n = 6$; BAp: $n = 15$). Data are presented as mean $\pm$ SEM. See Table 1 for further electrophysiological data and significance levels.

the PNs with elevated CCK levels. Consequently, the DsRed signal in this mouse model does not merely indicate the presence or absence of CCK in BLA PNs in this mouse line but rather shows relative CCK expression levels³⁶.

To assess whether these DsRed expression-defined subnuclei can be considered as structural subnuclei, a comprehensive approach was employed: (1) the location of dendritic trees of LA and BA PNs was determined, (2) examples for selective cortical innervation of LA and BA subnuclei were provided and (3) finally, distribution of PN populations with different projection areas were mapped relative to the DsRed expression. We observed that the vast majority of dendritic trees (71–99%) were restricted to the subnucleus where the soma was located, which suggests independent information-receiving capabilities for PNs in DsRed-defined subnuclei. In line with this conclusion, we found that prefrontal and insular cortices innervate LA and BA in a complementary manner; furthermore, in the medial portion along the anteroposterior axis of the LA and BA, the projections from the mPFC selectively terminate in the BAa, while the IC targets the LAP and BAp. Thus, DsRed+ and

	LAA (n = 17)	LAP (n = 21)	BAA (n = 18)	BAP (n = 32)	Kruskal-Wallis ANOVA	LAA-LAP Dunn's test	LAA-BAA Dunn's test	LAA-BAP Dunn's test	LA-BAA Dunn's test	LAP-BAP Dunn's test	BAA- BAP Dunn's test
Total dendritic length (mm)	4.24 (3.47, 5.47)	6.57 (5.88, 7.29)	6.29 (5.76, 6.83)	5.75 (4.24, 6.70)	<0.001 ***	<0.001 ***	0.008 **	0.11	1	0.16	1
Node number	26.00 (22.50, 33.50)	40.00 (32.00, 48.50)	36.00 (33.00, 42.75)	32.50 (22.25, 44.00)	0.002 **	0.002 **	0.037 *	0.44	1	0.12	1
The length of dendrites of different dendritic orders (mm)	1st 0.18 (0.07, 0.25)	0.15 (0.05, 0.41)	0.19 (0.07, 0.41)	0.10 (0.06, 0.19)	0.534						
	2nd 0.51 (0.25, 0.97)	0.69 (0.45, 0.90)	0.74 (0.45, 0.99)	0.56 (0.40, 0.82)	0.448						
	3rd 0.90 (0.66, 1.23)	1.37 (0.91, 2.01)	1.16 (0.99, 1.54)	1.18 (0.76, 1.43)	0.108						
	4th 0.96 (0.65, 1.49)	1.70 (1.13, 2.11)	1.61 (1.08, 2.01)	1.25 (0.97, 1.59)	0.009 **	0.015 *	0.042 *	0.622	1	0.435	0.897
	5th 0.68 (0.57, 0.87)	1.09 (0.95, 1.60)	0.92 (0.56, 1.31)	1.12 (0.66, 1.64)	0.016 *	0.021 *	1	0.055	0.594	1	1
	6th 0.77 (0.25, 0.98)	0.73 (0.47, 1.06)	0.54 (0.25, 0.97)	0.72 (0.41, 1.01)	0.480						
	7th 0.19 (0.13, 0.64)	0.49 (0.24, 0.73)	0.44 (0.19, 0.68)	0.34 (0.20, 0.54)	0.321						
Sholl-analysis	0-50 µm 0.55 (0.46, 0.72)	0.97 (0.80, 1.12)	0.91 (0.78, 1.09)	0.79 (0.60, 1.09)	<0.001 ***	<0.001 ***	<0.001 ***	0.021 *	1	0.208	0.740
	50-100 µm 1.20 (1.06, 1.51)	1.95 (1.70, 2.13)	1.94 (1.62, 2.01)	1.73 (1.30, 2.25)	<0.001 ***	<0.001 ***	0.007 **	0.016 *	1	1	1
	100-150 µm 1.25 (1.00, 1.68)	1.65 (1.48, 1.86)	1.68 (1.45, 1.84)	1.46 (1.15, 1.78)	0.040 *	0.078	0.122	1	1	0.681	0.930
	150-200 µm 0.78 (0.54, 1.02)	1.02 (0.76, 1.23)	0.89 (0.71, 1.06)	0.74 (0.55, 0.93)	0.049 *	0.266	1	1	1	0.045	1

Table 2. Morphological properties of in vitro recorded principal neurons in the different LA and BA subnuclei. Data presented as the median with the first and third quartiles in parentheses. Significant differences, indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$), were determined by Kruskal–Wallis ANOVA followed by post hoc Dunn’s test.

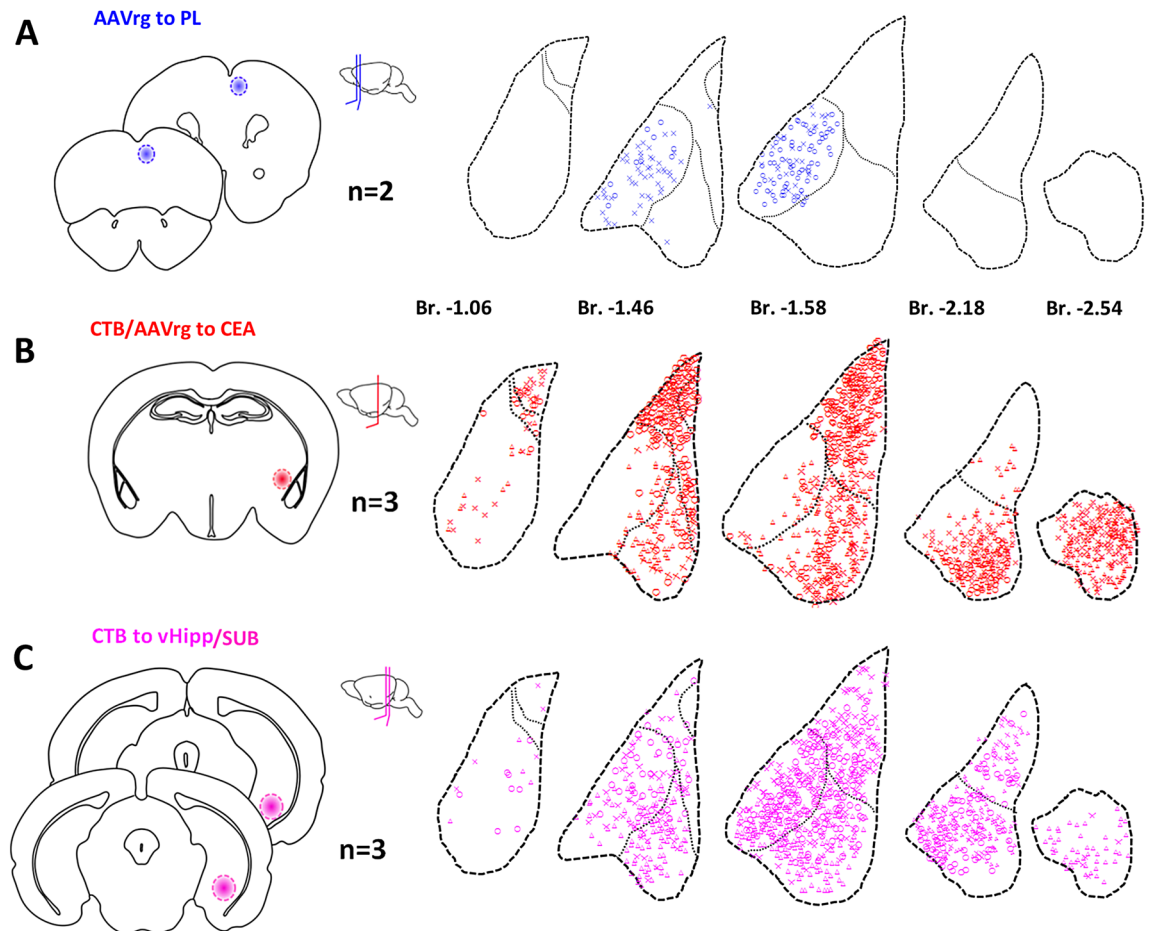


Fig. 6. The distribution of the somata of principal neurons with different projection sites within the BA corresponds to the DsRed-defined functional subnuclei. (a) Left panels show the schematic representation of injection sites in the prelimbic cortex (PL). The insert displays the sections of the mouse brain containing the core of the injection sites (2 injections/animal). On the right, retrogradely labeled neurons are presented in five LA and BA sections ($n=2$ mice). (b,c) Left panels show the schematic representations of Cholera toxin B subunit (CTB)/retrograde AAV injection sites to the lateral nucleus of the central amygdala (CEA), (b) or ventral hippocampus (vHipp)/subiculum (SUB), (c). Left schematics indicate the injection sites, while right panels show the retrogradely labeled neurons in five sections of the LA and BA ($n=3$ mice/target site). Thinner black dashed lines show the borders of the LA and BA subnuclei, different markers (O, Δ , X) represent the results from different animals. The distance from Bregma (Br.) is shown in mm.

DsRed- BA subnuclei independently receive information from the two pathways examined. Based on this logic, DsRed + LAa PNs may receive different projections than DsRed- LA PNs, a hypothesis that needs to be tested in future studies. Our anatomical studies highlight the existence of structural units within both the LA and BA. This finding implies that these DsRed-marked subnuclei, characterized by unique input-output properties should be regarded as functional subdivisions within the BLA, each contributing to specific operations associated with the amygdala-linked operation (further details provided below).

When amygdalar PN populations projecting to different areas were labeled by retrograde tracing, we found that PL-projecting neurons were exclusively located in the BAa, corroborating the earlier findings^{11,18,20,26,50,51}. In contrast to the PNs projecting to the PL, the location of the PN population innervating the CEA is limited to the BA_p, which is also consistent with recent findings^{19,29}. In the case of PN population projecting to the vHipp/SUB, no subnucleus-specificity was found, in accord with previous data²⁰. Although the rostral BAa $\rightarrow$ vHipp/SUB projection seems to be minimal based on our experiments, a recent study has provided support for the presence of CCK + BA afferents in the vHipp/SUB, originating from the anterior part of the BA⁵². We also observed a substantial number of LA PNs that were retrogradely labeled from the vHipp/SUB, which is in line with a previous tracing study⁵³. However, we cannot rule out the possibility that the spread of the retrograde tracers under our circumstances has reached layer VI in the lateral entorhinal cortex, where LA PNs heavily project¹¹, causing visualization of both LA PN populations projecting to the vHipp/SUB and/or lateral entorhinal cortex.

Our results show that while PL-projecting PNs are predominantly in the DsRed + area, vHipp/SUB-projecting PNs may be composed of DsRed + and DsRed- subpopulations, and CEA-projecting neurons are primarily in the DsRed- subregions. The BA $\rightarrow$ PL pathway has a prominent role in the regulation of reward-seeking and fear-related

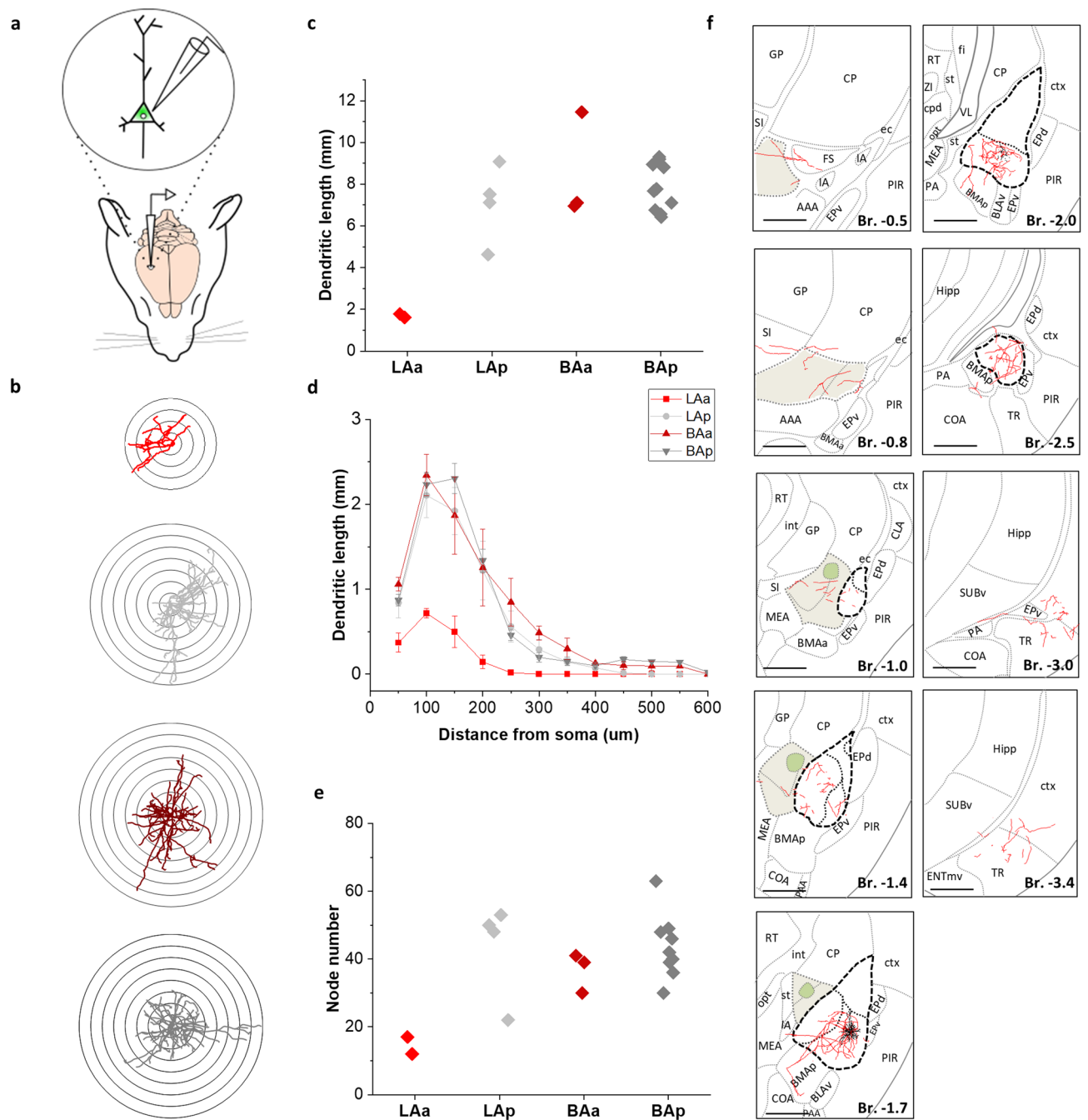


Fig. 7. In vivo juxtacellular labeling of principal neurons in the LA and BA. **(a)** Experimental setup. Juxtacellular labeling of principal neurons in the LA and BA of anesthetized mice (LAa: $n = 2$; LAP: $n = 4$; BAa: $n = 3$; BAp: $n = 12$; 21 PNs from 19 mice). **(b)** Dendritic reconstruction and Sholl-analysis of in vivo filled example neurons from each subnucleus. Scale: 50 μm difference between the radii of concentric circles (Cell IDs: LAa-1, LAP-1, BAa-2 and BAp-3). **(c)** Scatter plot showing the total dendritic length of the in vivo filled principal neurons. **(d)** The dendritic length as a function of distance from the soma. **(e)** Number of dendritic branching points of 21 in vivo reconstructed neurons from each subnucleus. **(f)** A juxtacellularly filled example neuron with a reconstructed axonal and dendritic arborization. The dendrites are shown in black, and the axons in red innervating brain areas across different sections from the fundus of striatum (Br. -0.5 mm) to the subiculum (Br. -3.4 mm). The lateral subnucleus of the CEA (CEAl) is shown in green, while the medial and capsular subnuclei (CEAm + c) are shown in beige. Scale bar: 500 μm . The distance from Bregma (Br.) is shown in mm. See the List of Abbreviations for the identification of brain areas.

Brain region	Cell ID	Data type	Reconstruction	Figure
LAa	1	Anatomy	Full	Figures 7B and 8B
	2	Anatomy	Full	Figure 8 C
LAp	1	Anatomy	Full	Figure 7B
	2	Anatomy	Full	
	3	Anatomy	Full	
	4	Anatomy	Full	Figure 8D
	5	Anatomy	Not suitable	
	6	Electrophysiology	Not suitable	
	7	Electrophysiology	Not suitable	
	8	Electrophysiology.	Not suitable	
	9	Electrophysiology.	Not suitable	
BAa	1	Anatomy and electrophys.	Full	Figure 8E
	2	Anatomy and electrophys.	Full	Suppl. Fig S7B
	3	Anatomy	Full	Suppl. Figure S7B; Fig. 7B
	4	Anatomy and electrophys.	Partial	
	5	Anatomy	Partial	
	6	Electrophysiology	Not suitable	
	7	Electrophysiology	Not suitable	
	8	Electrophysiology	Not suitable	
	9	Electrophysiology	Not suitable	
	10	Electrophysiology	Not suitable	
	11	Electrophysiology	Not suitable	
BAp	1	Anatomy and electrophys.	Full	
	2	Anatomy and electrophys.	Full	
	3	Anatomy and electrophys.	Full	Figure 7F and B
	4	Anatomy and electrophys.	Full	
	5	Anatomy and electrophys.	Full	
	6	Anatomy and electrophys.	Full	Figure 8F
	7	Anatomy and electrophys.	Full	
	8	Anatomy	Full	Suppl. Figure S8C
	9	Anatomy	Full	Suppl. Figure S8B
	10	Anatomy	Full	Figure 8G
	11	Anatomy and electrophys.	Partial	
	12	Anatomy	Full	
	13	Electrophysiology	Not suitable	
	14	Electrophysiology	Not suitable	
	15	Electrophysiology	Not suitable	
	16	Electrophysiology	Not suitable	
	17	Anatomy and electrophys.	Full	
	18	Electrophysiology	Not suitable	
	19	Electrophysiology	Not suitable	

Table 3. Details for in vivo juxtacellularly labeled PNs. In the ‘Data type’ column, ‘Electrophysiology’ refers to PNs with reliable spike detection and soma recovery, ‘Anatomy’ indicates PNs, processes of which were partially or completely labeled, and ‘Anatomy and electrophysiology’ denotes well-labeled PNs, spiking of which could also be analyzed.

behaviors^{23,54,55}, as well as in social behaviors^{56,57}. The BLA Δ vHipp/SUB projection plays a role in coordinating social⁵⁸ and anxiety-related behaviors⁵⁹. As the DsRed signal varies in vHipp/SUB-projecting BLA PNs, it may be worthwhile to separately examine the roles of DsRed+ and DsRed- PN populations in amygdala-controlled processes in future studies. The BAp Δ CEA innervation also contributes to the control of anxiety-like behavior²⁷ as well as to learning and expression of conditioned fear²⁹. These previous findings collectively underscore the hub-like nature of the BLA that channels the information flow to distinct downstream regions depending on the behavioral challenges and internal states and align with the hypothesis that the structural units defined by DsRed expression can be considered as functional subdivisions. Importantly, the DsRed-defined subnuclei appear to correspond to the anterior and posterior parts of the BA, as defined by Rspo2 and Ppp1r1b gene expression¹⁷. In that study, PNs within these two BA subnuclei were found to play distinct roles in appetitive and aversive behaviors and memories, supporting the existence of functional subregions within the BA.

	Cell ID	#Spikes	Mean firing rate (Hz)	Mean ISI (s)	ISI SD	ISI CV	Recording time (s)
LAp	6	270	1.38	0.73	1.69	2.32	195.06
	7	282	0.50	2.00	3.64	1.82	562.3
	8	2714	8.22	0.12	0.24	2.00	330.1
	9	755	2.89	0.35	0.82	2.34	261.46
BAa	1	555	1.04	0.96	1.39	1.45	532.63
	2	498	1.04	0.96	2.27	2.36	478.5
	4	24	0.12	8.93	9.01	1.01	205.49
	6	388	1.61	0.62	0.81	1.31	240.41
	7	2135	5.80	0.17	0.28	1.65	368.34
	8	260	0.96	1.04	3.47	3.34	270.63
	9	560	1.26	0.80	0.86	1.08	444.8
	10	4814	7.66	0.13	0.14	1.08	628.73
	11	154	1.45	0.69	0.84	1.22	106.01
BAp	1	1224	2.50	0.40	0.92	2.30	488.67
	2	1479	4.95	0.20	0.32	1.60	298.98
	3	133	0.38	2.66	3.97	1.49	350.82
	4	30	0.17	6.20	7.00	1.13	179.89
	5	54	0.28	3.66	5.61	1.53	193.75
	6	13	0.09	12.52	14.08	1.12	150.19
	7	114	0.35	2.90	4.63	1.60	328.12
	11	37	0.22	4.67	6.93	1.48	168.05
	13	59	0.33	3.07	3.53	1.15	178.12
	14	57	0.34	2.95	4.50	1.53	165.34
	15	149	0.50	2.03	2.42	1.19	300.81
	16	979	2.18	0.46	0.59	1.28	448.21
	17	463	1.19	0.84	1.37	1.63	389.07
	18	121	0.31	3.23	5.11	1.58	387.28
	19	54	0.15	6.86	7.12	1.04	363.56

Table 4. Spiking features of juxtacellularly recorded PN. Cell IDs are the same as in Table 3. #Spikes indicates the total number of analysed spikes. Mean firing rate (Hz) was calculated based on spike count and recording time. Mean inter-spike interval (ISI) and its standard deviation (SD) are also shown. The coefficient of variation of ISI (ISI CV) is calculated as ISI SD divided by mean ISI. None of these parameters differed among the groups (ANOVA, $p > 0.1$).

In vitro and in vivo cell reconstructions were conducted to morphologically characterize and compare the properties of PN of the different LA and BA subnuclei and reveal their local axonal arbors. Based on the properties of dendritic arborizations, the LAa PN were significantly smaller and had less ramified dendrites than the LA PN, which was also confirmed by their electrophysiological properties and supported by in vivo single-cell labeling. The presence of such small PN in the LA has not been reported earlier^{60–62}. Although previous studies obtained in rats and monkeys suggest that BA subdivisions can be distinguished based on cell size into parvo-, intermediate-, and magnocellular parts^{14,63}, we found no obvious indication for cell size differences in the mouse BA.

Based on previous results^{4,10,11,19}, both LA and BA subnuclei receive distinct sets of inputs that, after processing, transmit to separate as well as overlapping output areas. LA combines cortical inputs primarily from higher-order auditory cortices, temporal association cortex, and the IC with inputs from the posterior thalamic nuclei and sends collaterals to the subnuclei of the BA and TR in addition to the ventral part of the CP (i.e., to the AStria) and CEA. In contrast, the main input-output cortical structure of the BAa is the mPFC. Importantly, BAa PN send axons remotely to the mPFC and locally to BAp circuits and the medial part of the CEA, but not to its lateral part. BAp neurons receiving afferents from the IC heavily project to both parts of the CEA in addition to the BAa and TR. Thus, both the CEAl and TR receive inputs from LA and BAp, but not from the BAa, whereas LA, BAa and BAp innervate the medial nucleus of the CEA. Besides cortical inputs, BA receives excitatory innervation from the midline thalamic nuclei (MT)^{39,40,64}, which also parcel the BA. BAa receives inputs primarily from the central medial nucleus of thalamus, which does not target the CEA, whereas the paraventricular nucleus of thalamus innervates both the BAp and CEA^{39,64}. Interestingly, we observed many axon collaterals of in vivo labeled BA PN in the LA (Fig. 8E–G), an observation that suggests that bidirectional communication between the LA and BA may exist that has not been recognized earlier. Future studies should clarify the role of the BA to LA connectivity.

In summary, the subnuclei in the LA and BA – defined by DsRed expression and restricted dendritic arbors of PN – receive a specific combination of afferents and project to well-defined intra- and extra-amygdalar areas.

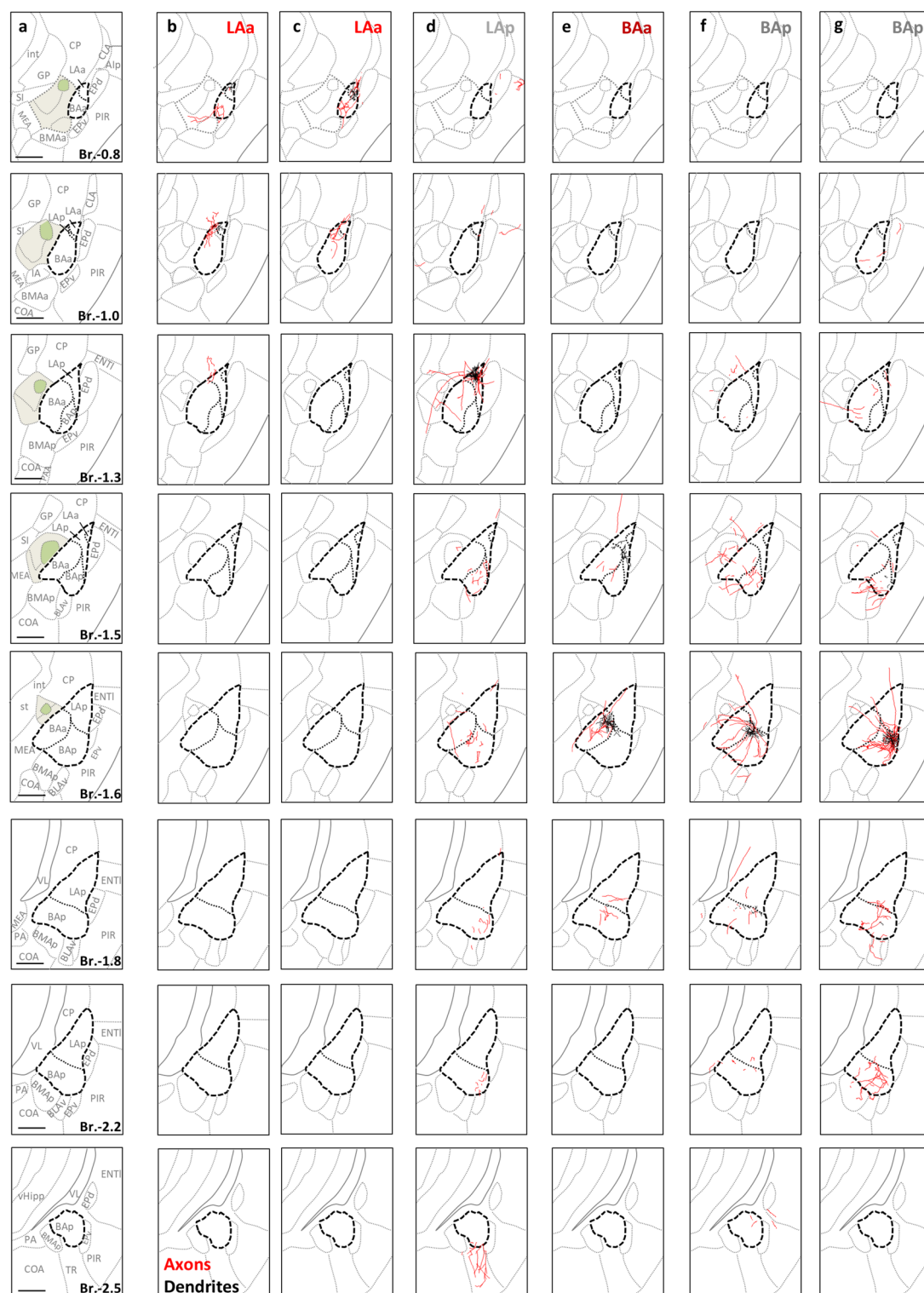


Fig. 8. In vivo filled principal neurons in different LA and BA subnuclei with typical projection target sites. (a) Maps showing the LA and BA and the surrounding brain areas along 8 anteroposterior sections based on Allen Brain Mouse Brain Atlas (2011). The lateral subnucleus of the CEA (CEAL) is shown in green, while the medial and capsular subnuclei (CEAM + c) are shown in beige. (b,c). In vivo reconstructed small principal neurons in the LAa innervating the central amygdala (CEA) and the caudoputamen (CP) (Cell IDs: LAa-1 and LAa-2). (d) A reconstructed large principal neuron located in the LAP (Cell ID: LAP-4). (e) A CEA-avoiding principal neuron with soma location in the BAa (Cell ID: BAa-1). (f,g) CEA-projecting principal neurons with soma location in the BAp (Cell IDs: BAp-6 and BAp-10). In each column, a single in vivo filled and reconstructed principal neuron is shown along the anteroposterior axis of the amygdala. Axons are shown in red and dendrites in black. Scale bar: 500 μ m. Distance from Bregma (Br) is shown in mm. See the List of Abbreviations for the identification of brain areas. All Cell IDs correspond to those listed in (Table 3).

The complex organization of inputs to the subnuclei and their outputs may ensure the critical combination and coding of information, necessary for predictive processing that is a key cortical function supporting adaptive behavior^{65,66}.

Methods

Experimental design

Subjects

Adult mice (median age: 130 days, interquartile range: 142.5 days) of both sexes were used for the experiments. BAC-CCK-DsRed transgenic mouse line expressing DsRed fluorescent protein under cholecystokinin (CCK) promoter generated in the HUN-REN Institute of Experimental Medicine³³ was used for in vitro measurements ($n=25$ males, 12 females) and to label midline thalamic afferents reaching the amygdala ($n=2$ males). Vglut1-Cre::BAC-CCK-DsRed animals expressing Cre driven by vesicular-glutamate transporter type 1 (Vglut1) promoter (Vglut1-IRES2-Cre-D, B6;129 S-Slc17a7tm1.1(cre)Hze/J, Stock #: 023527; Jax.org) and DsRed fluorescent protein under CCK promoter were used for anterograde tracing experiments labeling cortical afferents ($n=3$ males). C57Bl/6 (Charles River) wild type mice bred in the animal core facility of the HUN-REN Institute of Experimental Medicine were used for retrograde tracing ($n=7$ males and 1 female) and in vivo juxtacellular ($n=20$ males, 35 females) experiments. Animals were kept in groups of 2–4 mice in transparent Plexiglas cages, under 12-hour light/dark cycle. The constant temperature and humidity of the environment were controlled. Mice had *ad libitum* access to food and water. All cages were enriched with nesting material and shelter to support species-specific behaviors and improve animal welfare.

All experimental procedures were approved by the Committee of the Scientific Ethics of Animal Research (22.1/360/3/2011). All procedures were conducted in accordance with the relevant national legislation (1998 XXVIII. Section 243/1998, renewed in 40/2013.) and the institutional ethical guidelines. Experiments complied with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Directive 86/609/EEC, as revised by Directive 2010/63/EU). All efforts were made to minimize animal suffering and to reduce the number of animals used. The study also adhered to the ARRIVE guidelines.

Animal numbers and sampling strategy

The sample size was determined based on standard practice in neuroanatomical and electrophysiological studies. For anatomical experiments, data were collected from at least 2–3 animals per injection type, which is generally considered sufficient for reproducibility. To account for variability and technical loss, 4–6 animals were injected per condition using well-established coordinates. For in vitro electrophysiological recordings, a total of 80 principal neurons were recorded from 38 mice. The number of recorded cells was guided by technical feasibility and the need to capture the diversity of electrophysiological properties across identified neuron populations. For in vivo juxtacellular labeling, 100 principal neurons were successfully filled in 55 mice. From these, 21 neurons were selected for full reconstruction based on strict morphological criteria: sufficient staining intensity, visible and complete dendritic and axonal arbors, and clear somatic localization. These criteria were essential to ensure the interpretability of single-cell projection patterns.

Antero- and retrograde tracing

Anesthesia was induced and maintained by isoflurane or an intraperitoneal injection of a mixture of 10% ketamine and 2% xylazine solutions (Medicus Partner) at a 5:1 ratio, diluted threefold, administered at a dose corresponding to 125 mg/kg ketamine and 5 mg/kg xylazine, and then secured in a stereotaxic frame. Anteroposterior (AP) and mediolateral (ML) coordinates were measured from the Bregma, while the dorsoventral (DV) coordinate was measured from the surface of the dura mater. Small holes over the injection sites were created using a dental drill (Foredom).

Anterograde tracing experiments

Vglut1-Cre::BAC-CCK-DsRed animals were injected with AAV9-Ef1a-DIO-eYFP (Addgene, 27056-AAV9; gift from Karl Deisseroth; titer: 100 μ L at titer $\geq 1 \times 10^{13}$ vg/mL) virus bilaterally, targeting the: medial prefrontal cortex (mPFC; AP: 1.5 and 2.0 mm; ML: 0.5 mm and DV: 2.0 and 1.5 mm) and insular cortex (IC; AP: 2.0 mm; ML: 2.5 mm; DV: 2.0 mm). AAV5-CaMKII-eGFP (Addgene, 50469-AAV5; gift from Bryan Roth 100 μ L at titer $\geq 3 \times 10^{12}$ vg/mL) was used to inject the midline thalamus (MT; AP: -1.8 mm; ML: 0.4 mm and DV: 3.2 mm) using Nanoject III Programmable Nanoliter Injector (Drummond Scientific Company).

Retrograde tracing experiments

AAVrg-Ef1a-IRES-Cre-mCherry (Addgene, 55632-AAVrg, gift from Karl Deisseroth; titer: $\geq 7 \times 10^{12}$ vg/mL; 100 nl/injection site)⁶⁷, AAVrg-CAG-GFP (Addgene, 37825-AAVrg; gift from Edward Boyden; titer: $\geq 7 \times 10^{12}$ vg/mL; 100 nl/injection site) or 0.5% Cholera toxin B subunit (CTB; List Labs, #104; 25 nl/injection site) were used aiming one of the following target sites uni- or bilaterally: prelimbic cortex (PL; AP: 1.78 and 2.1 mm; ML: 0.3 mm; DV: 1.0 mm; $n=2$), central amygdala (CEA; AP: -1.6 mm; ML: 2.6 mm; DV: 4.1 mm) and ventral hippocampus/subiculum (vHipp/SUB; AP: -3.5 and -3.8 mm; ML: 3.4 mm; DV: 4.1 mm). Tracers were applied to the brain through a glass pipette (ID = 0.530 mm $\pm$ 25 μ m, OD 1.14 mm, World Precision Instruments). The flow rate of the injections was 1 nl/sec. Due to its small size, the CEA was injected iontophoretically (2 or 5 μ A pulses with 2/2 s on/off duty cycle for 7–10 min, respectively) with CTB using a Drummond Recording Nanoject II (Drummond Scientific).

Processing brain tissue

After 4–5 days (in case of the CTB iontophoreses) or approximately 4 weeks (in case of AAV injections) of survival, mice were deeply anesthetized with an intraperitoneal injection of 125 mg/kg ketamine and 5 mg/kg xylazine, supplemented with isoflurane inhalation until complete loss of nociceptive reflexes. Transcardial perfusion was then performed using 4% paraformaldehyde (PFA; Sigma-Aldrich) in 0.1 M phosphate buffer (PB; pH 7.4) for 40–50 min at a flow rate of 3 ml/min. On the same day, the brains were cut into 100 μ m-thick slices using a Vibratome (Leica VT1000S), and sections were stored in 0.1 M PB containing 0.05% sodium azide at 4 °C until further processing.

In vitro recording and labeling

For preparing acute brain slices, mice were deeply anesthetized with isoflurane and decapitated. The brain was quickly removed and placed into an ice-cold solution, containing (in mM): 252 sucrose, 2.5 KCl, 26 NaHCO₃, 0.5 CaCl₂, 5 MgCl₂, 1.25 NaH₂PO₄, 10 glucose, bubbled with 95% O₂/5% CO₂ (carbogen gas). Coronal or horizontal slices of 200–350 μ m thickness containing the LA or BA were prepared with a Leica VT1000S or VT1200S vibratome and kept in an interface-type holding chamber containing ACSF at 36 °C that gradually cooled down to room temperature. ACSF contained the following (in mM): 126 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 2 MgCl₂, 2 CaCl₂, 26 NaHCO₃, and 10 glucose, bubbled with carbogen gas.

After at least 1 h incubation, slices were transferred to a submerged type recording chamber perfused with 32 °C ACSF with approximately 2–2.5 ml/min flow rate. Recordings were performed under visual guidance using differential interference contrast microscopy (Olympus BX61W or Nikon FN-1) using a 40x water dipping objective. Neurons expressing DsRed were visualized with the aid of a mercury arc lamp or a monochromator (Till Photonics) and detected with a CCD camera (Hamamatsu Photonics or Andor Zyla). Patch pipettes (4–7 M Ω) for whole-cell recordings were pulled from borosilicate capillaries with an inner filament (thin-walled, OD 1.5) using a DMZ-Universal Puller (Zeitz Instruments) or using a P1000 pipette puller (Sutter Instruments). In whole-cell recordings the patch pipette contained a K-gluconate-based intrapipette solution containing the following (in mM): 110 K-gluconate, 4 NaCl, 2 Mg-ATP, 20 HEPES, 0.1 EGTA, 0.3 GTP (sodium salt), and 10 phosphocreatine adjusted to pH 7.3 using KOH, with an osmolarity of 290 mOsm/L and additional 0.2% biocytin.

Recordings were performed with a Multiclamp 700B amplifier (Molecular Devices), low-pass filtered at 3 kHz, digitized at 10 kHz, recorded with an in-house data acquisition and stimulus software (Stimulog, courtesy of Zoltán Nusser, Institute of Experimental Medicine)⁶⁸ or Clampex 10.4 (Molecular Devices), and were analyzed with EVAN 1.3 (courtesy of Istvan Mody, Department of Neurology and Physiology, University of California, Los Angeles)⁶⁹, Clampfit 10.4 (Molecular Devices), and OriginPro 2018 (OriginLab). Recordings were not corrected for junction potential. To test the firing characteristics, neurons were injected with 800-ms-long hyperpolarizing and depolarizing square current pulses with increasing amplitudes from –100 to 600 pA. In the present study, 80 PNs were filled in 38 animals. PNs were identified based on their broad action potential waveform, accommodating firing pattern, and slow after-hyperpolarization, in addition to their morphological appearance identified *post hoc*.

In vivo recording and labeling

The anesthesia was induced with isoflurane and maintained with an intraperitoneal injection of 125 mg/kg ketamine and 5 mg/kg xylazine. Glass electrodes were pulled from borosilicate capillaries (Sutter Instruments BF150-86-10) to achieve an impedance of approximately 20 M Ω and filled with 3% neurobiotin (Vector Laboratories) in 0.5 M NaCl solution. Thirty min before the experiment, a small hole was drilled in the skull above the target region to create a craniotomy, allowing access for the micropipette. The pipette was inserted into the brain through the craniotomy and lowered to the target depth at a speed of 1 μ m/sec using a micromanipulator.

Upon reaching the target region, neurons were located under electrophysiological guidance, recorded for 5–10 min and then labeled. Current pulses (0.5–10.0 nA, 200–500 ms duration) were applied to fill the recorded neuron with Neurobiotin (Supplementary Fig. S7 online), enabling subsequent histological visualization⁴⁶. Recordings were conducted using a MultiClamp 700B amplifier (Molecular Devices) and digitized with a CED Power1401 data acquisition interface (Cambridge Electronic Design). Data were recorded using Spike2 software (Cambridge Electronic Design) and analyzed with custom-written MATLAB scripts for further processing and visualization.

Experiments lasted for 2–4 h. Animals were sacrificed immediately after the experiment concluded (see the steps of the perfusion procedure above). Brains were sectioned into 80–150 μ m thick slices and stored in 0.1 M phosphate buffer containing 0.05% sodium azide at 4 °C until further processing.

Analysis of spiking was carried out only on neurons whose soma was labeled and could be reliably localized within the amygdala. PNs were selected based on their firing properties and/or anatomical features (large, spiny dendritic tree; pyramidal-like soma; long, rarely branching axon). PNs were included in the morphological reconstruction only if both their axonal and dendritic arbors were visible and could be traced throughout the tissue.

Immunohistochemistry

Tracing experiments

Sections underwent an initial washing process in 0.1 M PB consisting of 3 washing steps lasting 10 min each. Then, the sections were blocked for 30 min using a solution of 10% Normal Donkey Serum (NDS, Vector Laboratories) and 0.5% Triton X-100 (Acros Organics) diluted in 0.1 M PB. This was followed by treatment with a solution containing 2% NDS, 0.5% Triton-X, 0.05% Na-azide and primary antibodies diluted in 0.1 M PB for 1 night at RT. In virus tracing experiments, mouse anti-GFP (mGFP; Molecular Probes #MAB1083, RRID: AB_1587098;

1:5000 overnight) and rat anti-RFP (rat RFP; Chromotek Clone ID: 5F8, RRID: AB_2336064; 1:1000 overnight) were used to enhance the signal of GFP and RFP, respectively. In CTB-tracing experiments, goat anti-CTB (gCTB; List Labs #703, RRID: AB_10013220; 1:20,000 overnight) was applied to detect the retrogradely labeled cells. Then, the sections were thoroughly washed (3–4 washing steps, 15 min/wash) and incubated for 3 h in a solution comprising of 1% NDS and the secondary antibodies diluted in 0.1 M PB. The secondary antibodies were the followings: Alexa 488 donkey anti-mouse (A488 DAM; Molecular Probes #A21202, RRID: AB_141607; 1:500 for 3 h) and Alexa 594 donkey anti-rat (A594 DARat; Molecular Probes #A21209, RRID: AB_2535795; 1:500 for 3 h) to enhance the visualization of mGFP and ratRFP, respectively; and Alexa 488 donkey anti-goat (A488 DAG; Jackson #705-545-147, RRID: AB_2336933; 1:500 for 3 h) to reveal gCTB. Vesicular acetylcholine transporters (VACHT) were visualized using rabbit anti-VACHT primary antibody (Frontier Institute #139 103, RRID: AB_887864, 1:1000 overnight), aiding the identification of borders between BA and LA^{8,37}. Finally, following several washes in PB, sections were mounted on glass slides in Vectashield (Vector Laboratories) or ProLong Diamond Antifade Mountant (Diagnosticum).

Visualization of in vitro and in vivo filled neurons

The visualization process of filled neurons, both in vitro and in vivo, involved the following steps: after the initial washing in tris-buffered saline (TBS), the slices were incubated for 12 h in a solution containing 0.5% Triton-X and fluorophore-conjugated streptavidin (Alexa488-SA, Molecular Probes S-11223; 1:10000 or Cy3-SA; Sigma-Aldrich S6402; 1:2000 for 12 h). For in vivo filled neurons VACHT was visualized to help the identification of borders between BA and LA. This step was followed by multiple washing in TBS and incubation in a solution containing Cy3 donkey anti rabbit (Cy3 DAR; Jackson; 1:500) secondary antibody diluted in TBS. Finally, the slices underwent thorough washing, 3 times in TBS and 3 more times in 0.1 M PB (10 min/wash) and then were mounted on glass slides in Vectashield.

Confocal microscopy

Multichannel fluorescent images at high resolution were acquired with Nikon C2 confocal laser scanning microscope (Nikon Europe, Amsterdam, the Netherlands) in channel series mode. Plan Apo VC 20x (NA = 0.75) objective was used to take images for 3D reconstructions (z step size: 1–2 μm ; xy: 0.61–0.63 $\mu\text{m}/\text{px}$) and to determine the location of retrogradely labeled neurons in the LA and BA (1 focal plane/section; xy: 0.63 $\mu\text{m}/\text{px}$). Coronal and horizontal amygdala-sections of BAC-CCK-DsRed animals, mPFC and IC inputs reaching the LA and BA were captured with Plan Fluor 10x (NA = 0.3) objective (1 focal plane/section; xy: 0.93–1.23 $\mu\text{m}/\text{px}$).

Reconstruction and analysis

Principal neuronal identity was confirmed *post hoc* by their characteristic spiny dendrites and morphological appearance. Reconstruction and analysis of in vitro and in vivo filled neurons were performed using Neurolucida 10.53 software (MBF Bioscience) (1 PN was reconstructed from 9 to 37 sections). Dendrites were defined as thick processes bearing spines and exhibiting gradual tapering. In contrast, axon collaterals were thinner, lacked spines and were frequently decorated with varicosities^{4,61}. The properties of axonal and dendritic arbors, including lengths, node numbers, and Sholl analysis, were determined using Neurolucida Explorer (MBF Bioscience). Tissue shrinkage along the x and y axes was quantified by comparing the distance between brain regions in the same sections before and after the mounting process. Shrinkage along the z-axis was calculated based on the difference in section thickness before and after mounting. Finally, correction values were the following: xy: 1 and z: 2.5 for in vitro slices and xyz: 1.185 for in vivo sections (using Plan Apo VC 20x objective). The shrinkage caused by the fixation processes of the slices was not considered. The borders of the amygdala and the surrounding brain areas were drawn based on VACHT staining and the Allen Mouse Brain Atlas (<https://mouse.brain-map.org/static/atlas>). In this study, the nomenclature of the Allen Brain Atlas was used.

The analysis of retrograde injections involved marking the location of retrogradely labeled neurons in a single focal plane in the LA and BA using Neurolucida. The boundaries of LA and BA subregions in wild-type animals injecting with retrograde tracers were determined based on those obtained in BAC-CCK-DsRed animals. The neuron populations projecting to the PL, CEAL or vHipp/SUB were shown in amygdalar sections from different planes along the AP axis.

Schematic diagrams illustrating brain areas, brain sections with injections sites, and experiments were created using Inkscape version 1.2.2 (732a01da63, 2022-12-09; <https://inkscape.org/>), an open-access program provided by the Free Software Foundation.

Statistical analysis

The Shapiro-Wilk test was used to assess whether the data were drawn from a normal distribution or not. Since the datasets typically did not exhibit a normal distribution, comparisons between LAa, LAP, BAa and BAp of anatomical and electrophysiological data were conducted using the Kruskal-Wallis non-parametric test. For *post hoc* analysis Dunn's test was used. For comparison of spiking parameters obtained in vivo recordings, one-way ANOVA was used. All statistics were performed using Origin 8.6 or 9.2 240 (Northampton, MA). Exact p-values were indicated when p was higher than 0.001 considering the rounding rules. Data are presented as mean $\pm$ SEM, unless indicated otherwise. On box charts the mean (small open square), the median (continuous line within the box), the interquartile range (box) and the 5–95% values (ends of whisker bars) are plotted. In the scatter plots, each symbol represents an individual data point. Due to the small number of elements, no statistical test was used for the representation of the in vivo filled principal neurons.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author on request.

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Author contributions

Z.R. performed retrograde tracing experiments and data analysis, analyzed in vitro electrophysiological recordings, reconstructed and analyzed both in vitro and in vivo labeled cells, and wrote the first draft of the manuscript. D.M. carried out the in vivo recordings, contributed to writing parts of the Methods section, and provided feedback on the initial draft. F.W., Z.F., A.V., Z.P. and A.T. performed and analyzed in vitro recordings and commented on the first draft; Z.F. also contributed to writing parts of the Methods section. K.M. contributed to retrograde tracing experiments, participated in the analysis of in vitro data, and reviewed the first draft. J.M.V. contributed to the conceptual analysis of the cell reconstructions and provided comments on the manuscript. N.H. initiated and supervised the project, reviewed, and revised the initial draft, and approved the final version of the manuscript. All authors reviewed the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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