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Intrinsic Circuits in the Lateral Central Amygdala

Circuits in the lateral central amygdala

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Abstract

Network activity in the lateral central amygdala (CeL) plays a crucial role in fear learning and emotional processing. However, the local circuits of the CeL are not fully understood and have only recently begun to be explored in detail. Here, we characterised the intrinsic circuits in the CeL using paired whole-call patch-clamp recordings, immunohistochemistry and optogenetics in C57/BLJ6 wildtype and somatostatin-cre (SOM-Cre) mice. Our results revealed that throughout the rostro-caudal extent of the CeL, neurons form inhibitory connections at a rate of ~29% with an average amplitude of 20 ± 3 pA (at -40 mV). Inhibitory input from a single neuron is sufficient to halt firing in the postsynaptic neuron. Post-hoc immunostaining for protein kinase C δ (PKCδ) in wildtype mice and paired recordings in SOM-Cre mice demonstrated that the most common local connections were PKCδ(-)→PKCδ(-), and SOM(+)→SOM(+). Finally, by optogenetically activating either SOM(+) or SOM(-) neurons, we found that almost all neurons in the CeL were innervated by these neuronal populations, and that connections between like-neurons were stronger than those between different neuronal types. These findings reveal a complex network of connection within the CeL, and provide the foundations for future behaviour-specific circuit analysis of this complex network.

Significance

Local inhibition in the lateral central amygdala (CeL) plays a crucial role in the processing of emotions, yet a complete understanding of these connections is still in its infancy. In this study, we show that CeL neurons are highly interconnected and that inhibition from a single neuron is sufficient to silence the postsynaptic neuron. Focusing on two well-known CeL neuronal subtypes: protein kinase C δ (PKCδ)- and somatostatin (SOM)-expressing neurons, we show that the most common local connections are PKCδ(-)→PKCδ(-) and SOM(+)→SOM(+). Optogenetic activation of either SOM(+) or SOM(-) neuronal populations revealed that inhibition was larger between like-neurons. These findings show that within the CeL there is a complex network, and provide the foundations for future behaviour-specific circuit studies.

Introduction

The amygdala has long been known to play a crucial role in processing innate emotions, particularly fear (Kluver and Bucy, 1939; Weiskrantz, 1956; Sah et al., 2003). In Pavlovian fear conditioning, an associate learning paradigm widely used to study amygdala

function, subjects learn to associate a neutral sensory stimulus (the conditioned stimulus, CS), with an aversive one (the unconditioned stimulus, US) (LeDoux, 2000). Following learning, the previously neutral CS now evokes a defensive response, freezing of movement or flight (Gross and Canteras, 2012). A converging body of evidence has established the amygdala as a central player in fear conditioning where the basolateral amygdala (BLA) and the central amygdala (CeA) are the key sites involved in the acquisition and expression of fear (LeDoux, 2000; Sah et al., 2003; Duvarci and Pare, 2014). The BLA has been extensively studied with respect to its cell types, intrinsic circuits, and extrinsic connections (LeDoux, 2000; Sah et al., 2003; Duvarci and Pare, 2014) while the CeA has received considerably less attention, and the intrinsic circuits within this nucleus are less well understood.

The CeA is a GABAergic nucleus (McDonald and Augustine, 1993; Sun and Cassell, 1993) that is anatomically divided into lateral (CeL) and medial (CeM) sectors, with substantial unidirectional connections between the CeL and the CeM (McDonald, 1982; Grove, 1988; Jolkkonen and Pitkanen, 1998). Neurons in both regions also make extensive local connections (McDonald, 1982; Sun and Cassell, 1993; Jolkkonen and Pitkanen, 1998), with local glutamate excitation of CeL neurons evoking inhibitory postsynaptic currents (IPSCs) in neighbouring neurons (Lopez de Armentia and Sah, 2004). Recent studies have divided CeL neurons into distinct populations based on the expression of immunohistochemical markers, electrophysiological properties, and synaptic connections (Ciocchi et al., 2010; Haubensak et al., 2010; Li et al., 2013). Of these, one population expresses protein kinase Cδ (PKCδ(+)), and these neurons are predominantly described as late-firing (LF) neurons, exhibiting a substantial delay to action potential (AP) initiation in response to depolarising somatic current injections. Following fear conditioning, these neurons respond to the CS with a reduction in activity, and have therefore been called CeL_{OFF} cells (Ciocchi et al., 2010; Haubensak et al., 2010). A second population of CeL neurons, which is largely separate from the PKCδ(+) population, expresses somatostatin (SOM+) (Li et al., 2013). These neurons receive direct synaptic input from the lateral amygdala that is potentiated following auditory fear conditioning (Li et al., 2013). Electrophysiologically, PKCδ(-) neurons which are predominantly SOM(+), have been described as either LF or regular spiking (RS). Following fear conditioning, PKCδ(-) neurons respond to the CS with an increase in activity, and have therefore been called CeL_{ON} neurons (Ciocchi et al., 2010; Haubensak et al., 2010), which likely also correspond to SOM(+) neurons (Yu et al., 2016).

PKCδ(-) neurons inhibit PKCδ(+) neurons, which in turn project to the CeM (Haubensak et al., 2010).

This organisation has led to a model in which fear expression is mediated by CS-related information driving PKCδ(-) neurons, presumably SOM(+) neurons, in the CeL via excitatory input from the BLA and thalamus. These neurons in turn inhibit PKCδ(+) neurons, resulting in disinhibition of the CeM and the expression of fear (Ciocchi et al., 2010; Haubensak et al., 2010). However, some SOM(+) neurons in the CeL also project to the periaqueductal gray (PAG) (Penzo et al., 2014), and CS driven activity of these neurons also contributes to fear expression (Tovote et al., 2016). Moreover, recent studies have reported that neurons in the CeL are also involved in feeding (Cai et al., 2014), and pain (Han et al., 2015). Neurons engaged during feeding and pain responses are also part of the PKCδ and SOM population, indicating that the intrinsic circuitry of the CeL is complex, and the strength, identity and physiological role of individual local connections are not fully understood. In this study, we provide a detailed investigation of local circuits in the CeL.

Materials and methods

Animals

All studies were approved by the University of Queensland Animal Ethics Committee and experiments were carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific purposes. Adult (6 to 15 weeks old) male wildtype C57/BL6J mice were used for electrophysiology experiments. Where stated, we also used both male and female mice (8 to 12 weeks old) from a somatostatin-IRES-cre mouse line (SOM-Cre; C57BL/6J background; $Sst^{tm2.1(cre)Zjh}$) that was acquired from the Jackson Laboratory. These mice express cre recombinase under the SOM promoter, thereby allowing selective targeting of SOM(+) neurons using cre-dependent viral constructs (described below). Mice were genotyped by the Australian Equine Genetics Research Centre.

Brain slice preparation

Mice were anaesthetised using isoflurane and decapitated, after which brains were quickly removed while submerged in an oxygenated ice-cold N-methyl-d-glucamine-based (NMDG) solution (NMDG 93 mM, KCl 2.5 mM, NaH_2PO_4 1.2 mM, $NaHCO_3$ 30 mM, HEPES 20 mM, glucose 25 mM, sodium ascorbate 5 mM, thiourea 2 mM, sodium pyruvate 3 mM, $MgSO_4$ 10 mM, $CaCl_2$ 0.5 mM, pH 7.2, 290-300 mOsm). This NMDG-based solution is particularly suited for dissections of adult mice (Zhao et al., 2011). Coronal brain slices (300

μm thick) were then prepared using a vibratome (Leica VT1000S) and placed to recover in oxygenated artificial cerebrospinal fluid (aCSF; NaCl 118 mM, $NaHCO_3$ 25 mM, glucose 10 mM, KCl 2.5 mM, $NaHPO_4$ 1.2 mM, $MgCl_2$ 1.3 mM, $CaCl_2$ 2.5 mM, pH 7.2, 290-300 mOsm) for 30 min at 34°C, and then at room temperature until required.

Electrophysiological recordings

Slices were visualised on an upright microscope (Olympus BX51WI), and whole-cell patch-clamp recordings were made using a Multiclamp 700B (Molecular Devices). The CeL was easily distinguishable *in vitro* based on the fire bundles that surround and clearly delineate this area (Fig. 4A). These landmarks are readily visible under the microscope and ensured that cells chosen for recordings were situated within the CeL. In addition, for electrophysiological recordings, cells in the CeL are typically smaller than those in the BLA and their cell density is higher than both the BLA and CeM. Data were filtered at 4 kHz and sampled at 20 kHz using an ITC-18 (Instrutech). Data were acquired and analysed using AxoGraph (AxoGraphX). Brain slices were continuously perfused with oxygenated aCSF (34°C; 3-4 ml/min) and recording electrodes (4-6 MΩ, Harvard Apparatus glass capillaries, Narishige PC-10 electrode puller) were filled with a $KMeSO_4$-based internal solution ($KMeSO_4$ 135 mM, NaCl 8 mM, HEPES 10 mM, MgATP 2 mM, GTP 0.3 mM, phosphocreatine 7 mM, EGTA 0.2 mM, biocytin 0.2%, pH 7.2 with KOH, osmolarity 295 mOsm/kg) unless otherwise stated, in which case a $CsMeSO_4$-based internal solution was used ($CsMeSO_4$ 135 mM, NaCl 8 mM, HEPES 10 mM, MgATP 2 mM, GTP 0.3 mM, phosphocreatine 7 mM, spermine 0.1, pH 7.2 with CsOH, osmolarity 300 mOsm/kg). In some experiments GABA (10 mM) was added to the $KMeSO_4$-based internal solution to avoid any run down of responses due to wash out during whole-cell recordings (Apostolides and Trussell, 2013), although no difference in response was observed when using GABA internal solutions. No corrections were made for junction potentials. The pairs of neurons chosen for recordings were located within 50 – 100 μm of each other in the coronal plane and 10 – 40 μm in the rostro-caudal plane. To probe for connections during paired recordings, one cell was held in current-clamp mode and injected with a 5 ms, 600-700 pA current pulse to evoke an AP. Meanwhile, the second (postsynaptic) neuron was held in voltage-clamp mode at -40 mV, well away from the chloride reversal potential (~ -73 mV) given that neurons in the CeL are known to be GABAergic, forming inhibitory synapses (Sun and Cassell, 1993; Pitkanen and Amaral, 1994; Lopez de Armentia and Sah, 2004; Haubensak et

al., 2010; Li et al., 2013). This protocol was repeated for at least 20 (but no more than 50) episodes and sweeps were averaged for analysis. The same was then done in the opposite direction. Only connections with an amplitude > 5 pA were considered to be connected. Finally, in pharmacology experiments, bicuculline (10 μM; Sigma) or CNQX (10 μM; Tocris) were bath applied to the slice.

Firing properties

APs were evoked using current injections applied in increments of 20 pA from -60 pA to 240 pA. AP threshold, amplitude, delay, half-width, rise time and spike accommodation were analysed offline (described below). Spike accommodation was measured as the difference in AP frequency over at least 8 APs at twice threshold. Although the two main firing types we observed ultimately had significantly different AP onsets, we used the absence or presence of spike accommodation to classify these firing types, as AP onset varied with small changes in holding membrane potential.

Data analysis

Electrophysiological properties. Resting membrane potential (R_m) was recorded online immediately after break-in, whereas input resistance (R_i) was measured offline as $R_i = {dVm}/{I}$ where dVm is the change in membrane potential in response to a -20 pA (800 ms) current injection (I). For connections, decay was measured by fitting the average IPSC by a sum of two exponentials (simplex sum of squared errors) in order to calculate a weighted time constant: $\tau_w = (t_1.a_1 + t_2.a_2)/(a_1 + a_2)$. Onset delay was calculated as the difference between the time of the presynaptic AP peak and the time of IPSC onset (time at 5% of peak). For firing properties, AP threshold was measured as the membrane potential at the start of the fast rising phase. AP amplitude was measured from the threshold to peak, and delay was measured as the duration from the start of the current injection to the start of the fast rising phase of the first AP.

Statistical tests. Data sets were tested for normality using the Shapiro-Wilks test. In the cases where a subset of the population was tested (e.g. drug application), we based our choice of statistical test on whether or not the overall data set was normally distributed. We used parametric tests (*t* tests) when the data followed a normal distribution, whereas non-parametric tests (Wilcoxon and Mann-Whitney tests) were used for data sets that were too small to reliably test for or did not follow a normal distribution. Two-tailed tests were used unless otherwise stated and differences were considered significant for $p < 0.05$.

Immunohistochemistry

Labelling for immunohistochemical characterisation. For characterisation of CeA neurons, mice were anaesthetised by intraperitoneal injection of pentobarbitone sodium (3250 mg/kg; Virbac) and transcardially perfused with 40 ml of a 1% sodium nitrite solution (phosphate buffer 0.1 M), followed by 40 ml of 4% paraformaldehyde (PFA in 0.1 M phosphate buffer). Brains were then removed and left in 4% PFA at room temperature overnight and washed (3 x 15 min, PBS 0.1M) before sectioning (50-60 µm sections). Brains were placed in 30% sucrose for 48 h and sectioned using a sliding microtome (Leica SM200R). Coronal subsections (50 µm) were then stained for PKCδ using a mouse anti-PKCδ antibody (1:500, BD Biosciences), for somatostatin (SOM) using a rabbit anti-SOM antibody (1:1000, Chemicon/Millipore) and for NeuN using a chicken anti-NeuN antibody (1:1000, Millipore; 72 h at room temperature). In the case of virus-injected animals, fluorescence was amplified using either a rabbit anti-red fluorescent protein antibody (1:1000, Abcam) or chicken anti-green fluorescent protein (1:1000, Life Technologies). Sections were then washed and incubated with mouse-fluorophore 647 (for PKCδ; 1:2000, Invitrogen), rabbit-fluorophore 488 (for SOM; 1:2000, Molecular Probes), rabbit-fluorophore 568 or chicken-fluorophore 488 (for fluorescence-enhanced sections; 1:2000, Molecular Probes). Brain sections used for counts were immunolabelled for NeuN to allow reliable identification of mature neurons and only NeuN(+) neurons were counted. Cell counts were made in both the right and left hemispheres but as these were not significantly different, the data were pooled for each Bregma location.

Post-hoc labelling of recorded neurons. Alexa-568 (1 ng/ml of internal solution) was added to the internal recording solution and images of dendritic morphology were taken during recordings in order to correctly identify the pre- and postsynaptic cells after recovery of recorded neurons. Following electrophysiological recordings, slices were fixed in 4% PFA (in 0.1M phosphate buffer) for either 1 h at room temperature or overnight at 4°C, and then washed for 3 x 15 min in 0.1 M PBS. Slices were then placed in blocking solution (1% BSA, 0.05% saponin, 0.05% sodium azide) for 1-2 h at room temperature before incubation with an Alexa555-bound streptavidin (overnight at room temperature; 1:2000 in blocking solution, Life Technologies). Slices were then washed (3 x 15 min, 0.1 M PBS), mounted (DABCO) and imaged using either an upright fluorescent microscope (5x and 20x, Zeiss, Zen software) or spinning disk confocal microscope (20x and 40x water immersion objective, CSU-W1 Yokogawa, Slidebook software). All images were analysed using FIJI (Image J). For protein PKCδ staining, slices were subsequently embedded in 4% agarose and subsectioned (50 µm

sections; Leica VT1000S vibratome) before being incubated with the PKCδ mouse-antibody (72 h at room temperature; 1:500; BD Biosciences). Sections were then washed and incubated with mouse-fluorophore 647 (1:2000, Invitrogen) and the nuclei of the cells stained with DAPI, prior to being mounted and imaged as described above. Although PKCδ clearly labelled somas, the somatostatin antibody did not deliver reliable post-hoc staining, as a result of which we focused on PKCδ for post-recording labelling experiments.

Morphology. Biocytin-recovered neurons that were used for morphological reconstruction were imaged using a spinning disk confocal microscope (40x 1.2 NA water immersion objective, 0.156x0.156x0.33 μm^3/pixel resolution, CSU-W1 Yokogawa, Slidebook software). Neurons were manually traced using Neurolucida (MBF Bioscience) and analysed using Neurolucida Explorer. For spine counts, dendrites were reimaged using a 63x 1.4 NA oil objective (0.099x0.099x0.15 μm^3/pixel resolution) and underwent deconvolution. Spines were counted automatically and manually verified (Neurolucida 360, MBF Bioscience, including the z-plane) over 60 µm of secondary dendrites. Three segments (each from a different secondary dendrite) were counted and averaged for each cell.

Viral injections and optical stimulation

Mice (21 to 28 days old) were anaesthetised (100 mg/kg ketamine, 10 mg/kg xylazil in saline) and placed in a stereotaxic frame. Bilateral injections were made into CeL using the following coordinates (Paxinos & Watson, 2001): -1.6 mm (anterio-posterior), +/- 2.8 mm (medio-lateral) and -4.8 mm (dorso-ventral from skull).

A small hole was drilled in the skull and virus was injected using a glass needle (pressure injection Picospritzer; 10-20 ms, 10-30 psi). Animals were injected stereotaxically with an AAV (adeno-associated virus; 0.1 to 0.3 µl, 0.1 µl/min Vector Core) containing one of the following constructs: AAV2/5- EF1α-DIO-tdTomato (titre: 1.0 x 10^{11}), AAV2/5-EF1α.dflox.hChR2(H134R)-mCherry (titre: 1.31 x 10^{13}) or AAV2/5-EF1α-DIO-Fwd.hChR2(H134R)-EYFP (titre: 1.0 x 10^{11}).

Animals were quarantined for 48 h then allowed to recover for at least 4 weeks post-injection. Brain slices were prepared as described above for electrophysiological experiments and cells were only recorded well within the spread of the virus to ensure that non-fluorescent neurons were indeed SOM(-), rather than simply not infected. To verify expression of channelrhodopsin (ChR2) and to activate ChR2 in infected cells, an LED system (470 nm, 1.4 mW, CoolLED pE-2) attached to the microscope (via the rear C-mount port) was used. A prolonged light pulse (100 ms) was used to verify that cells expressed functional ChR2. In

the case of AAV2/5- EF1α.dflox.hChR2(H134R)-mCherry experiments, for example, neurons were considered SOM(+) if they were both fluorescent and displayed a prolonged depolarisation in response to prolonged light stimulation (470nm, 100 ms), whereas a SOM(-) neuron was not fluorescent and showed no excitation to the light pulse. A light pulse of 2 ms (n = 57 neurons) or 1 ms (n = 10 neurons) was used to evoke responses in the CeL.

Results

Characterisation of neurons in the central lateral amygdala

Immunohistochemical characterisation. Neurons in the CeL have been separated based on the expression of a range of neuropeptides and markers that include PKCδ, SOM, corticotropin-releasing factor, oxytocin receptors, enkephalin, and others (Cassell and Gray, 1989; Haubensak et al., 2010). Of these, the two most highly expressed, and clearly distinct neuropeptides are PKCδ and SOM (Haubensak et al., 2010; Li et al., 2013). Immunostaining of brain sections from four locations posterior to bregma (-1.20 mm, -1.40 mm, -1.60 mm and -1.80 mm; ± 0.05 mm; top diagrams in Fig. 1A) shows that PKCδ labelling within the amygdala was specific to the CeL, whereas SOM expression was also present outside the central amygdala. In the CeL, 48 ± 5% of neurons expressed PKCδ, and 38 ± 3% SOM (Fig. 1A) with the two populations largely non-overlapping, and dual labelled (PKCδ(+)/SOM(+)) neurons accounting for only 1.5 ± 0.5% of neurons. The remaining neurons (13 ± 2%) were negative for both markers. It was notable that whereas the proportions of PKCδ(+)/SOM(-) and PKCδ(-)/SOM(+) neurons were similar between Bregma -1.40 mm and -1.60 mm, the difference between the total numbers of the two cell types changed at Bregma -1.20 mm and -1.80 mm, the rostral and caudal limits of the CeL (Fig. 1B).

Electrophysiological properties. Based on their response to somatic current injections, three general types of CeL neuron have previously been described. The two major types being late-firing (LF) neurons which show a significant delay before onset of the first AP (~ 100 – 200 ms), and early-spiking neurons (ES, also described as regular-spiking (AP onset: ~ 50 ms). A third smaller population of low-threshold bursting neurons has also been described (Dumont et al., 2002; Lopez de Armentia and Sah, 2004; Haubensak et al., 2010; Li et al., 2013; Hou et al., 2016). We characterised the firing properties of 151 CeL neurons. However, while classifying neurons we found that AP onset varied with changes in holding potential, whereas the presence of spike frequency accommodation was more reliable. Using this measure, neurons were classified either as non-accommodating where AP frequency

remained relatively consistent (~17 Hz), or accommodating neurons where there was clear spike frequency adaptation (AP_{1-2} ~32 Hz vs AP_{7-8} 13 Hz, $p < 0.001$ Wilcoxon matched-pairs test; Fig. 2C). The large majority of our neurons were non-accommodating (n = 80 neurons, Fig. 2A) or accommodating (n = 59 neurons, Fig. 2B). Non-accommodating neurons also had a significantly longer mean onset compared to that of accommodating neurons (Table 1), and these neurons generally corresponded to the LF and ES types (Haubensak et al., 2010; Amano et al., 2012). Thus, for consistency we have termed these late-firing non-accommodating (LF-NA) and early-spiking accommodating (ES-Ac) neurons. Apart from resting membrane potential, which was significantly more depolarised in ES-Ac neurons, other membrane properties such as input resistance, threshold potential, AP amplitude, rise time and half-width did not differ significantly between LF-NA and ES-Ac neurons (Table 1).

In the remaining 12 neurons (8%; Fig. 3) we found a distinct stuttering firing type that resembled that of some interneurons in the BLA (Woodruff and Sah, 2007; Sosulina et al., 2010; Spampanato et al., 2011). These neurons were easily distinguishable due to their distinctive firing pattern, with bursts of high frequency APs (~ 60 Hz; Fig. 3A). Moreover, these neurons had significantly briefer APs with a half width of 0.6 ± 0.04 ms compared to 1.1 ± 0.03 ms in ES-Ac neurons and 1.2 ± 0.03 ms in LF-NA (Table 1; Fig. 3B). Stuttering neurons also displayed a higher frequency of spontaneous synaptic events compared to LF-NA and ES-Ac neurons (Fig. 3C). For stuttering neurons, we were unable to recover the entire cell, however, dendrites were filled, and visible, and showed that unlike LF-NA and Es-Ac neuron, stuttering neurons were aspiny.

Twenty-five recorded neurons were successfully recovered with biocytin and labelled for PKCδ. Of these, PKCδ(+) neurons (n = 8) were either LF-NA or ES-Ac at equal incidence (50%), whereas PKCδ(-) neurons (n = 17) were more likely to be LF-NA (~59%) than ES-Ac (~23%). As previously described using Golgi methods (McDonald, 1982; Cassell and Gray, 1989), the majority of CeL neurons resembled medium-spiny neurons, (Fig. 5). Stuttering neurons that were successfully recovered and stained (n=3), were all PKCδ(-) (Fig. 3D, E). These results show that PKCδ (48%), and SOM (38%) expressing neurons are the major cell types in the CeL, with very few neurons expressing both markers (1.5%). These neurons have one of two firing properties, LF-NA or ES-Ac. We also identified a previously unrecognised population of stuttering neurons (8%) that express neither PKCδ or SOM (see below).

306

307 ***Local inhibitory connections.***

308 To determine the nature of local connections between neurons in the CeL, paired
309 whole-cell recordings were made in acute coronal slices of wildtype mice (Fig. 4A). A total
310 of 152 pairs were tested, of which 45 (29%) were connected. This was a monosynaptic
311 connection with an onset latency of 0.85 ± 0.06 ms after the AP peak, and a high release
312 probability (failure rate 23 ± 3%), consistent with a monosynaptic connection (Fig. 4B, C).
313 At a holding potential of -40 mV the inhibitory postsynaptic current (IPSC) had a mean
314 amplitude of 20 ± 3 pA, a 10-90% rise time of 1.7 ± 0.1 ms and a decay time constant of 19.2
315 ± 1.5 ms. Connections were predominantly unidirectional (n = 42 of 45 connected pairs;
316 Fig. 4B), with only 3 connected pairs displaying bidirectional connectivity (Fig. 4C, D).
317 Apart from the stuttering cells, these neurons resembled medium-spiny neurons, (Fig. 5A-C),
318 and spine density did not differ significantly between pre- and postsynaptic neurons (Fig.
319 5B), nor were differences observed in soma diameter, soma volume, number of primary
320 dendrites, number of nodes or total dendrite length (Table 2). Recordings were made
321 throughout the rostro-caudal extent of the CeL and the resulting map of connected and
322 unconnected pairs revealed no obvious location preference (Fig. 5D).

323 Neurons in the CeL are predominantly GABAergic, and in our connected pairs, the
324 IPSC reversal potential was -72 mV, which corresponds to the calculated chloride reversal
325 potential (~ -73 mV; Fig. 6A). Application of the $GABA_A$ receptor ($GABA_A$-R) antagonist,
326 bicuculline (10 μM) blocked these IPSCs (Fig. 6B; n = 5 paired recordings), confirming that
327 they were $GABA_A$-R-mediated chloride currents. In current clamp, these connections were
328 hyperpolarising, with a mean amplitude of -1.1 ± 0.3 mV (n = 17) sufficient to halt firing in
329 the postsynaptic cell (Fig. 6C; n = 5 paired recordings), and in some cases this inhibition was
330 followed by a rebound increase in spike probability (Fig. 6D). These results demonstrate that
331 neurons throughout the CeL form local inhibitory connections at a relatively high rate, which
332 are capable of shaping the activity of the postsynaptic cell.

333

334 ***Distinct connection patterns exist between local CeL neurons***

335 To determine the identity of recorded pairs, recovered neurons were processed using
336 immunohistochemistry. As expected (Ciocchi et al., 2010; Haubensak et al., 2010), we found
337 local connections between presynaptic PKCδ(-) and postsynaptic PKCδ(+) neurons PKCδ(-
338)→PKCδ(+) in 27% of successfully recovered pairs (Fig. 7A, D-E). However, the most

common connection type was between two PKCδ(-) neurons PKCδ(-)→PKCδ(-); ~55%; Fig 7B, D-E). In two cases, both the pre- and postsynaptic neurons were PKCδ(+) (18%; Fig 7C, D-E). No PKCδ(+)→PKCδ(-) connections were found. Connected cells displayed a variety of discharge properties (Fig. 7F), with the most common connections being either LF-NA→LF-NA (~26%; n = 5 of 19 paired recordings) or ES-Ac→LF-NA connections (~21%; n = 4 of 19 paired recordings). Although less common, we also found ES-Ac→ES-Ac (~10%; n = 2 of 19 paired recordings;). Stuttering neurons were always presynaptic (n=3) with two connections to LF-NA neurons, and one to an ES-Ac neuron.

These results show that local CeL connections occur between a variety of immunohistochemically and electrophysiologically distinct neuronal types with the most common connection between PKCδ(-) neurons. Given that ~75% of PKCδ(-) neurons are SOM(+) (Fig. 1), we turned to a SOM-Cre mouse line to reliably identify and selectively activate SOM(+) neurons *in vitro*. It was important to confirm that neurons considered to be PKCδ(-) were not false negatives due to protein washout during whole-cell recordings. To label SOM(+) neurons we injected an adeno-associated virus containing a DIO-td-tomato vector (AAV-DIO-tdTom) into the CeL of SOM-Cre mice (Fig. 8). SOM-tdTom and PKCδ labelling in the CeL revealed similar proportions of these markers to those in wildtype mice (Fig. 8A-B, n = 3 mice, at Bregma -1.40 mm to -1.60 mm). We also determined the firing properties of SOM(+) and SOM(-) neurons (Fig. 8C). In agreement with recordings in wild type mice, SOM(+) neurons were mostly LF-NA (~81% n = 13 of 16 neurons; ES-Ac: ~19% n = 3 of 16 neurons), whereas the SOM(-) neurons were mostly ES-Ac (~65% n = 11 of 17 neurons; LF-NA: ~29% n = 5 of 17 neurons). Notably, the one stuttering neuron found in these recordings was SOM(-). Given that the stuttering neurons observed in wildtype mice were PKCδ(-), it is possible these neurons are a major contributor to the population of PKCδ(-)/SOM(-) neurons.

Next, paired whole-cell recordings were obtained using identified SOM(+) neurons (Fig. 8D-F). Thirty one pairs of neurons were recorded: eight pairs between SOM(+) neurons, 16 pairs between a SOM(+) neuron and a SOM(-) neuron, and seven pairs between SOM(-) neurons (Fig. 8D-G). Nine of the 31 pairs were connected (~29%), which included eight unidirectional connections and one bidirectional connection (Fig. 8D). In these connections, the mean IPSC amplitude (at -40 mV) was 21 ± 5 pA (n = 9), and had an onset latency of 0.76 ± 0.11 ms, not significantly different from the results obtained in wildtype mice (wildtype mean IPSC: 20 ± 3 pA; p = 0.7, Mann-Whitney test). The IPSC 10-90% rise

time was 1.3 ± 0.1 ms, and had a decay time constant of 13.2 ± 1.9 ms. The most common connection (~56%) was between SOM(+) neurons (Fig. 8E), with the remaining connections being SOM(-)→SOM(-) (~22%) and SOM(-)→SOM(+) (~22%; Fig. 8E-G). When we compared the number of connected pairs to the total number of recordings for each combination, the least likely connection was between SOM(+) and SOM(-) neurons; only ~12% (n = 2 of 16 recordings) of these pairs being connected. In contrast, ~62% (n = 5 of 8 pairs) of SOM(+)/SOM(+) recordings and ~28% (n = 2 of 7 pairs) of SOM(-)/SOM(-) recordings were connected (Fig. 8H). No SOM(+)→SOM(-) connections were found.

Population-driven inhibition is greater between like-neurons

Somatostatin-positive neurons. As described above, paired recordings in coronal brain slices from both wild type, and SOM-Cre mice show that connections were most frequent between somatostatin expressing, PKCδ(-) neurons. However, previous studies indicate that inhibition of SOM(-) neurons by SOM(+) cells not only exists, but plays a key role in fear expression (Li et al., 2013; Hou et al., 2016). Such a motif is also suggested by inhibition of PKCδ(+) neurons by PKCδ(-) neurons (ON neuron→OFF neuron) (Ciocchi et al., 2010; Haubensak et al., 2010). One possibility for our low incidence of SOM(+)→SOM(-) connections is that we are sampling local connections (~50 µm to 100 µm apart) in the coronal plane, and SOM(+)→SOM(-) connections may be more common amongst "distal" (i.e. >100µm) connections. To address this, we injected an AAV containing DIO-channelrhodopsin-mCherry into the CeL of SOM-Cre mice (Fig. 9A-B) to directly activate SOM(+) terminals.

Whole-cell recordings were made from SOM (+) and SOM(-) neurons and terminals from SOM(+) neurons activated optically. All SOM(-) cells received input from SOM(+) neurons with a mean IPSC of 162 ± 24 pA (n = 15; holding voltage -40 mV; Fig. 9C). Next, paired recordings were made using a Cs-based internal solution, allowing voltage-clamping of cells at the ChR2 reversal potential (~0 mV) to test for SOM(+) to SOM(+) connections. In this configuration, all SOM(-) and SOM(+) neurons received large IPSCs when SOM(+) terminals were activated (SOM(-) = 22 neurons; SOM(+) = 10 neurons; Fig. 9D-F). IPSCs in response to SOM(+) terminal activation were fully blocked by bicuculline (10 µm, n = 5, Fig. 9G), and reversed at ~ -67 mV (n = 4) and were able to halt firing in the postsynaptic cell. From this cohort, 10 SOM(-) neurons were recovered of which five were PKCδ(+), showing direct SOM(+)→PKCδ(+) and SOM(+)→PKCδ(-) connections (Fig. 9H). While all

neurons received input from SOM neurons in the CeL, overall input to SOM(+) neurons was significantly larger than to SOM (-) neurons (Fig. 9I). This difference is consistent with our paired recordings where 5 of 8 SOM(+)→SOM(+) pairs were connected but none of the SOM(+)/SOM(-) pairs were (n=16 pairs). In the course of these recordings it was clear that, using SOM as a neuronal marker, a wide variety of connections are present in the CeL. Thus, for example, in one SOM(-)→SOM(-) single connected pair (illustrated in Figure 9J), both cells also received input from local SOM(+) neurons.

Somatostatin-negative neurons. Our paired recordings also showed that SOM(-)→SOM(-) and SOM(-)→SOM(+) local connections, while not frequent, were present (Fig. 8E, F). However, with the technique we used (Fig. 8) there was a risk that non-infected (and therefore non-fluorescent) SOM(+) neurons could be misidentified as SOM(-). Although the number of SOM(+) neurons in SOM-Cre mice (Fig. 8A-B) was consistent with that of wildtype mice (Fig. 1), and despite the fact that we made sure to restrict recordings to well within the spread of infection, we used an alternative approach to confirm the existence of these connections. We again used an optogenetic approach to target SOM(-) neurons of the CeL in SOM-Cre mice with an AAV containing a DIO-Fwd-hChR2(H134R)-EYFP construct (Fig. 10A). With this construct, the ChR2-EYFP sequence is "cut out" in the presence of Cre recombinase, thereby ensuring that only Cre-negative (in this case SOM(-)) neurons express ChR2-EYFP. Combining these injections with a DIO-tdTom-containing AAV (1:1 ratio) allowed simultaneous identification of SOM(+) neurons (tdTom fluorescent) and SOM(-) neurons (eYFP fluorescent and ChR2-expressing). We could therefore selectively activate SOM(-) neurons all while avoiding misidentification of neurons due to lack of fluorescence. These injections typically covered the majority of the width of the CeL (Fig. 10B). However, although a small volume of virus (~100-200 nl) was injected to minimise spread outside the CeL, we did observe eYFP(+) somas in the basal amygdala and the amygdalostriatal area, located dorsally to the CeL. Within the CeL, ~62% of all fluorescently labelled neurons were eYFP(+)/tdTom(-), whereas tdTom(+)/eYFP(-) neurons accounted for ~36%. Processing slices for PKCδ, revealed that the majority of eYFP(+) neurons were PKCδ(+) (~77%, Fig. 10C, D).

Using a Cs-based internal solution, whole-cell recordings were obtained from either SOM(+) (Fig. 10E) or SOM(-) neurons (Fig. 10F). As eYFP(+) neurons were present in the basal amygdala (Fig. 10B), we bath applied CNQX (10 μM) during these recordings to ensure that the recorded IPSCs were monosynaptic. Under these conditions, in ~91% of

SOM(+) neurons (10 of 11 neurons) and all SOM(-) neurons (n = 9 neurons), stimulation of SOM(-) terminals evoked an IPSC (Fig. 10G), and these responses were $GABA_A$-R-mediated (Fig. 10H). Moreover, SOM(-)→SOM(-) IPSCs were significantly larger than SOM(-)→SOM(+) IPSCs (Fig. 10I).

Together with our connected paired recordings, these results are consistent with the presence of SOM(-)→SOM(+) and SOM(-)→SOM(-) connections within the CeL. Furthermore, they suggest that, as with SOM(+) neurons, a high proportion of CeL neurons receive inhibitory local connections from SOM(-) neurons, and with inhibition within the population being stronger than that between populations.

Discussion

The CeA is generally considered to be the main output nucleus of the amygdalar complex, and is divided into the lateral and medial sectors. It contains GABAergic neurons that have been divided into several distinct populations using immunohistochemical and electrophysiological markers. These cells form local, as well as long-range connections, and different cell types have been associated with distinct functional roles (McDonald, 1982; Sun and Cassell, 1993; Jolkkonen and Pitkanen, 1998; Ciocchi et al., 2010; Haubensak et al., 2010; Li et al., 2013). Here, using whole-cell paired recordings, and optogenetics, we characterised neurons of the CeL, and their intrinsic connections. We find that neurons in the CeL are extensively interconnected, with local connections apparent between all types of neuron, but strongest between like neurons. Moreover, we describe a new type of neuron in the CeL with distinct firing properties. These results highlight the complex intrinsic circuits within the CeL, and suggest that particular cell groups identified using current methods, rather than mediating specific behaviours, participate in a range of different ones.

Local networks in the CeL

Consistent with previous studies, we found that PKCδ and SOM labelled two separate populations of neurons in the CeL (~48% and ~38% respectively), with very little overlap (~1 – 2%), that account for 88% of the total cell population. In response to current injection, these neurons show two types of discharge patterns, late firing (LF-NA) and early spiking (ES-Ac), and their overall incidences (~52% and ~39% respectively) were comparable to those previously described in mouse (Haubensak et al., 2010; Hou et al., 2016). While SOM(+) neurons were mostly LF-NA (~81%) and SOM(-) neurons (largely PKCδ

expressing) were more likely to be ES-Ac (~65%), these electrophysiological properties could not be used to separate the two populations. A smaller number of neurons (~12%) were PKCδ(-) and SOM(-). These neurons may express CRF or one of the other peptides that are known to be present in CeL neurons (Cassell and Gray 1989; Haubensak et al. 2010).

A small number of neurons (~8%), had faster action potentials, and a stuttering phenotype, with bursts of high frequency AP discharge. This type of neuron has not been previously reported in the mouse CeL, although a similar 'fast-spiking' neuron has been described in rare cases in the CeL and CeM of the guinea pig and cat (Martina et al., 1999; Dumont et al., 2002). These neurons were PKCδ(-) in wildtype mice, and the one stuttering neuron in SOM-Cre mice was SOM(-), suggesting that they may reflect a distinct PKCδ(-)/SOM(-) population. Although the role of this particular type of neuron is not clear, paired recordings showed that stuttering neurons were always presynaptic, and in cases where we had successful recovery of dendrites they had an aspiny morphology, different from that of the typically recovered CeL neuron. This, together with its fast-spiking properties suggests the presence in the CeL of a local interneuron-like cell as opposed to the principal-type neurons typically found in the CeL.

Paired recordings demonstrated that neurons in the CeL were connected with an incidence of ~29%. In these recordings, we find that at the local level (~50 to 100 µm in coronal slices), the most common connection was unidirectional and between two PKCδ(-) or two SOM(+) cells. In agreement with a recent report (Hou et al., 2016), connections between other pairs, as well as bidirectional connections were present but were much less prevalent. We did not, however, find cells that showed clear evidence of autapses which were reported in ~15% of neurons in the Hou (2016) study. In contrast, when SOM(+) or SOM(-) neurons were transduced with ChR2, we found that nearly all cells received a large input from both cell types. This difference in connectivity indicates that neurons make long range connections within the CeL, perhaps in the rostrocaudal plane.

For the SOM neurons, using paired recordings, the monosynaptic connection had a mean amplitude of ~20 pA (at -40 mV), whereas when SOM neurons were transduced with ChR2, the optically driven IPSC had a mean amplitude of ~160 pA, showing that on average ~8 SOM(+) neurons innervate each SOM(-) neuron. In paired recordings, the IPSC had rapid rise times suggesting that these contacts were likely to be somatic, or close to the soma (Delaney and Sah, 2001), consistent with the ability of these connections to halt spiking.

The CeL and behaviour

The role of the CeL in cued fear expression is clear: a large of body of data supports a model whereby conditioned stimulus-mediated disinhibition of CeM output drives conditioned fear (Ciocchi et al., 2010; Haubensak et al., 2010; Li et al., 2013). However, it remains unclear how the high level of CeL connectivity (both intra- and extra-CeL afferents) can be reconciled with the increasing number of important behaviours in which CeL activity has been implicated. For example, fear expression has also been suggested to require activation of the parabrachial nucleus (PB) input to the CeL (Han et al., 2015; Sato et al., 2015), and yet this PB→CeL circuit has also been implicated in appetite suppression (Carter et al., 2013; Cai et al., 2014). Meanwhile, other CeL circuits have been shown to underlie the switch between innate and conditioned fear (Isosaka et al., 2015), and anxiety generalisation (Botta et al., 2015). Lastly, as well as forming local inhibitory connections (Li et al., 2013), SOM(+) neurons are also projection neurons that target the PAG (Penzo et al., 2014) and this CeA→PAG projection is engaged in mediating defensive behaviours (Tovote et al., 2016). We have shown that these neurons are also highly interconnected both within and between distinct neuronal populations. Our results show that within the CeL, neither cytosolic markers (PKCδ and SOM), or their electrophysiological properties, identify cells engaged in particular behavioural roles.

The physiological role, if any, of SOM and PKCδ are not known, however they clearly label separate populations of neurons in the CeL. Developmentally, the CeL has a striatal origin (Medina et al., 2011), and SOM and PKCδ, rather than specifying different populations that mediated different functional roles, should be thought of as lineage markers. We suggest that PKCδ and SOM expressing neurons form heterogeneous populations of neurons, with different populations contributing to different behavioural outcomes. Understanding the flow of information through the CeA and its outputs, in a behaviourally specific and relevant manner, will be a challenge for future experiments. Similarly, it will be important to take these additional local circuits into account in further investigations of the CeL circuitry, particularly when judging the effects of pharmacological treatments during *in vivo* studies.

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Figure 1: Protein kinase C δ (PKCδ) and somatostatin (SOM) label distinct populations of neurons in the central lateral amygdala (CeL) of wildtype C57/BLJ6 mice. A: Top panel shows diagrams of coronal CeL slices of C57/BLJ6 mouse at -1.20 mm, -1.40mm, -1.60mm, -1.80mm from Bregma (± 0.05 mm, based on Paxinos & Watson, 2001). LA: lateral amygdala, BA: basal amygdala, CeA: central amygdala, which is divided into the CeL (orange) and the central medial amygdala (CeM, in white). Arrows show dorsal and medial orientation, scale bar: 1 mm. Bottom panels show close-ups of the CeL in 50 µm sections that were stained for NeuN (to stain somas of neurons, white fluorescence), PKCδ (green fluorescence) and SOM (red fluorescence), scale bar in bottom left square: 100 µm. For clarity, the merge panels represent the merge of PKCδ and SOM only. The CeL is outlined in the bottom panel and this outline was defined both by landmarks visible in brightfield (not shown) and the presence of PKCδ(+) somas. PKCδ(+) fibres can typically be seen in the CeM. The locations of both the BA and the CeM are also labelled in the merge panels, note that by 1.80 mm the CeM is no longer present. The inset in the lower right corner of the far right merge panel shows a close up of the most common cells types: PKCδ(+)/SOM(-) (white arrowhead) and SOM(+)/PKCδ(-) neurons (yellow arrowhead; scale bar: 10 µm; PKCδ green fluorescence, SOM red fluorescence, NeuN blue fluorescence). B: Only NeuN(+) neurons were counted to ensure that only mature neuronal cells were taken into account. Of these, 48 ± 5% were PKCδ(+)/SOM(-) (mean n = 83 ± 19 neurons/ $1.0x10^{-3}$ mm^3) and 38 ± 3% were SOM(+)/PKCδ(-) (mean n = 66 ± 14 neurons/ $1.0x10^{-3}$ mm^3). These two populations were largely distinct as only 1 ± 0.5% of neurons were PKCδ(+)/SOM(+) (mean n = 2 ± 0.3 neurons/ $1.0x10^{-3}$ mm^3) and 12 ± 2% NeuN(+) cells were PKC δ(-)/SOM(-) (mean n = 20 ± 4 neurons/ $1.0x10^{-3}$ mm^3). The dotted line on the graph indicates 50% and bregma specific percentages were as follows: PKCδ(+)/SOM(-) 34 ± 6% (-1.20 mm), 50 ± 2% (-1.40 mm), 52 ± 1% (-1.60 mm), 57 ± 4% (-1.80 mm). PKCδ(-)/SOM(+) 45 ± 3% (-1.20 mm), 38 ± 1% (-1.40 mm), 39 ± 3% (-1.60 mm), 30 ± 5% (-1.80 mm). PKCδ(-)/SOM(-) 20 ± 6% (-1.20 mm), 11 ± 3% (-1.40 mm), 8 ± 3% (-1.60 mm), 10 ± 1% (-1.80 mm). PKCδ(+)/SOM(+) 1 ± 0.3% (-1.20 mm), 1 ± 0.2% (-1.40 mm), 1 ± 0.05% (-1.60 mm), 3 ± 0.5% (-1.80 mm).

Figure 2: Firing types of neurons in the central lateral amygdala: late-firing non-accommodating and early-spiking accommodating. Example traces of the two main firing types recorded in the central lateral amygdala (CeL): (A) late-firing non-accommodating (LF-NA) and (B) early-spiking accommodating (ES-Ac) with example traces of current injections below. Scale bars: 20 mV, 500 ms, 80 pA. The top two current injections shown are at threshold and twice threshold (2T). On average LF-NA neurons displayed significantly longer onset to firing of the first action potential (AP, onset indicated by black arrowheads) when compared to ES-Ac neurons (LF-NA 330 ± 25 ms, n = 80 neurons, ES-Ac 209 ± 23, n = 59 neurons, $p < 0.001$, Mann-Whitney test) and little to no accommodation at 2T. To demonstrate accommodation, early (green lines) and late (red lines) interspike intervals are indicated. C: Whereas AP frequency over 8 action potentials remained consistent for LF-NA neurons (n = 20, AP_{1-2} frequency: 17 ± 1 Hz, AP_{7-8} frequency: 16 ± 1 Hz, $p = 0.6$, Wilcoxon matched-pairs test), ES-Ac AP frequency gradually decreased (AP_{1-2} frequency: 32 ± 4 Hz, AP_{7-8} frequency: 13 ± 1 Hz, $p < 0.001$, Wilcoxon matched-pairs test).

Figure 3: Stuttering neurons in the central lateral amygdala (CeL). A: Example trace of firing of a stuttering (S) neuron at threshold, and twice and three times threshold. In addition to its fast action potential (AP) kinetics (Table 1) and distinct firing pattern, large fast afterhyperpolarisations (as indicated by the red arrowhead) are also typical of this firing type. Inset shows a close-up of a spontaneous excitatory postsynaptic potential (sEPSP) in green. B: Overlay of the first AP of a stuttering (red), LF-NA (black) and ES-Ac (blue) neurons. The AP rise time and half width of S neurons were significantly faster than those of LF-NA and ES-Ac neurons (Table 1). C: sEPSPs in S neurons were significantly more numerous than in LF-NA and ES-Ac neurons during the hyperpolarising steps of this protocol. Numbers shown are the total counted over the -60, -40 and -20 pA current injections (B; S vs LF-NA: $p = 0.001$, unpaired t-test; S vs ES-Ac: $p < 0.0001$, unpaired t-test). D: Example biocytin recovery of an S neuron, which was PKCδ(-) (top inset, yellow arrowhead indicates the soma of the S neuron), scale bars: 20 µm, 10 µm (top inset) and 5 µm (bottom inset). This neuron displayed an extensive axon with inset showing a close-up of the axon in the dotted white square. E: Percentage of firing types for recovered neurons that were PKCδ(+) (n = 8) or PKCδ(-) (n = 17). F: Shows total percentage of each firing type.

Figure 4: Neurons in the central lateral amygdala form local connections. A: Paired recordings were performed in the central lateral amygdala (CeL), the location of which is shown in a diagram of a coronal slice (left panel). The middle panel shows a brightfield image (300 μm slice) of the area within the orange rectangle: the border of the CeL is clearly defined by visible fibre bundles and the right panel shows the approximate outline of the three main amygdala regions: basolateral amygdala (BLA), CeL and central medial amygdala (CeM). In reality the CeL extends slightly more ventrally than outlined here, however we aimed to keep recordings within the outlined area to ensure we did not mistakenly record from CeM neurons. B-C: Example traces of inhibitory postsynaptic currents (IPSCs), which were on average 20 ± 3 pA, from a unidirectional connection (B) and a bidirectional connection (C). In each case, 'cell 1' was current-clamped and given a short current injection (5 ms, 600-700 pA, illustrated in black directly under each current trace) to elicit one action potential (AP), while 'cell 2' was voltage clamped at -40 mV. The protocol was then repeated in the opposite direction: from 'cell 2' to 'cell 1'. Example average traces (black) and representative traces from single episodes (grey) are shown. D: ~29% of paired recordings (n = 45 of 152) were connected, with the large majority of connected pairs being unidirectional (42 of 45) the remainder being bidirectional connections. E: Biocytin recovery of the connected recorded pair in (B), where a yellow arrowhead indicates the presynaptic cell and a white arrowhead indicates the postsynaptic cell.

Figure 5: Morphology and anatomical location of local connections within the central lateral amygdala. A: Example morphological reconstruction (spines not depicted) of a connected pair with the presynaptic neuron in black and the postsynaptic neuron in grey. Blue arrowheads indicate where the presynaptic axon (red) crossed over a postsynaptic dendrite in the same z-plane, representing putative synapse locations. Inset shows average traces of this connection, with the presynaptic trace in black and the postsynaptic trace in grey (postsynaptic cell voltage clamped at -40 mV). B: Recovered neurons typically had a medium spiny morphology; spine counts of recovered connected neurons showed that the postsynaptic neuron was not significantly more spiny than its presynaptic neuron. Example images show close ups of secondary dendrites from a presynaptic ('Pre') neuron and corresponding postsynaptic ('Post') neuron from the pair shown in (A). Scale bar: 5 µm. Bar graph shows mean spine densities (number of spines per µm) for pre- and postsynaptic neurons, with connected neurons joined by a dotted line (n = 3 connected pairs). Data points with red borders correspond to the 'pre' and 'post' close ups depicted in (B). C: Image of biocytin recovery of the connected pair of neurons shown in (A) to show the location within the central lateral amygdala (CeL). BA: basal amygdala, D: dorsal, M: medial, scale bar: 100 µm. D: Locations within the CeL (yellow; central medial amygdala is in white) of 35 recorded pairs that could be reliably located at different rostro-caudal locations (Bregma -1.22 mm to -1.70 mm; i-iv). Presynaptic cells are represented by black circles and postsynaptic cells are solid grey circles. White circles indicate pairs where a connection was not detected.

Figure 6: Local connections in the central lateral amygdala (CeL) are inhibitory. A: Example traces of change in current to voltage in 10 mV steps (left, from -40 mV to -90 mV) and average current-voltage (I-V) curve of local IPSCs (right, n = 5 paired recordings). This I-V curve is typical of a chloride current: a linear I-V relationship (r^2 = 0.98) that reverses here at -72 mV, close to the theoretical reversal potential (~73 mV). B: Local IPSCs were also blocked by the $GABA_A$ receptor antagonist bicuculline (10 μM); example traces with aCSF in black and bicuculline in red (left). IPSCs were completely blocked by bicuculline (right, mean IPSC aCSF: 24.7 ± 5.4 pA; mean IPSC bicuculline: 1.7 ± 0.5 pA; n = 5 paired recordings; p = 0.03, one-tailed Wilcoxon test; dotted line joins data points from the same neuron). C: Overlay of 10 example traces from a connected pair where a short positive current injection (5 ms, 600-700 pA) was applied to the presynaptic cell to fire one action potential (AP) at t = 0 s (top trace). Meanwhile the postsynaptic cell was also in current-clamp mode and current was injected such that the cell fired continuously (bottom trace). A single AP in the presynaptic cell evoked an inhibitory postsynaptic potential (IPSP) that was sufficient to stop the postsynaptic cell from firing. Bottom histogram shows the number of APs fired in the above trace over time, in 50 ms bins. D: The spike probability was significantly lower in the 200 ms following inhibition onset compared to pre-inhibition (mean spike probability before inhibition: 0.14 ± 0.02; during inhibition: 0.02 ± 0.01; p = 0.02, paired t-test), and in most cases increased when the postsynaptic cells recommenced firing (mean spike probability before inhibition: 0.14 ± 0.02; post-inhibition: 0.2 ± 0.02; p = 0.01, paired t-test). Each colour represents data points from the same neuron (n = 5 pairs).

Figure 7: Protein kinase C δ-positive (PKCδ(+)) and PKCδ(-) neurons form local connections in the central lateral amygdala (CeL). A-C: Example images (left-hand panels, scale bars: 50 μm) of connected cells that were biocytin-filled and recovered with a fluorescent streptavidin (red). Insets show close ups of each cell with PKCδ staining (green fluorescence; DAPI is shown in blue in panel (B) to help locate the postsynaptic neuron). Yellow arrowheads indicate the presynaptic neuron and white arrowheads indicate the postsynaptic neuron. Example average traces for each recovered pair are shown in the right-hand panels (scale bars: 50 mV, 10 pA, 20 ms). A: PKCδ(-)→PKCδ(-) connection; B: PKCδ(-)→PKCδ(+) connection; C: PKCδ(+)→PKCδ(+) connection. D: Approximate locations of each successfully identified pair. E: Connected paired recordings were predominantly between PKCδ(-) cells (~ 55%; 6 of 11 successfully recovered and stained connected paired recordings), whereas ~27% of connections were PKCδ(-)→PKCδ(+) (3 of 11) and ~18% (2 of 11) were PKCδ(+)→PKCδ(+). No PKCδ(+)→PKCδ(-) connections were observed in these experiments. Inhibitory postsynaptic current (IPSC) amplitudes of each type were as follows: PKCδ(-)→PKCδ(-) 20.23 ± 5.6 pA, PKCδ(-)→PKCδ(+) 16.8 ± 8.7 pA, PKCδ(+)→PKCδ(+) 28.75 ± 0.7 pA. F: In terms of firing properties, the majority of connections occurred between LF-NA→LF-NA (~26%, n = 5 of 19), LF-NA→ES-Ac (~26%, n = 5 of 19) and ES-Ac→LF-NA (~21%, 4 of 19) neurons. ES-Ac→ES-Ac connections were less common (~11%, 2 of 19) and in all connections that involved a stuttering (S) neuron (~16%, n = 3 of 19), the S neuron was the presynaptic cell.

Figure 8: Somatostatin-positive neurons form local connections in the central lateral amygdala of somatostatin-cre mice. The CeLof somatostatin (SOM)-Cre C57/BJL6 mice was injected with an AAV-DIO-tdtomato to fluorescently label SOM(+) cells. A: Subsections (50 µm-thick) of injected CeL were stained with a NeuN antibody and a PKCδ antibody. Representative sections at Bregma -1.60 mm are shown. B: NeuN-positive cells were counted for PKCδ and SOM labelling. 47 ± 3% (mean n = 122 ± 27 neurons/1.3x10^{-3} mm^3) of total counted neurons were PKCδ(+) but SOM(-), whereas 39 ± 1% (mean n = 100 ± 16 neurons/1.3x10^{-3} mm^3) of total neurons were SOM(+)/ PKCδ(-), with very little overlap; i.e. SOM(+) and PKCδ(+): 2 ± 1% (mean n = 3 ± 1 neurons/1.3x10^{-3} mm^3) and 12 ± 1% negative for both (mean n = 32 ± 6 neurons/1.3x10^{-3} mm^3). C: Whole-cell recordings were performed and complete firing properties for 33 neurons were recorded from SOM(+) and SOM(-) neurons. As with wildtype mice LF-NA (~55%), ES-Ac (~42%) and stuttering (S) (3%) neurons were observed. SOM(-) neurons were mostly ES-Ac (~65%, LF-NA 29%, S 6%, n = 17 neurons) whereas SOM(+) neurons were mostly LF-NA (~81%, ES-Ac 19%, n = 16 neurons). D: ~29% of paired recordings showed either a unidirectional (n = 8 paired recordings) or bidirectional (n = 1 paired recording) connection, whereas in 71% of recordings no connection was detected. E: Unidirectional connections were observed between different combinations of SOM(-) and SOM(+) neurons: SOM(-)→SOM(+) (n = 2), SOM(+)→SOM(+) (n = 4), SOM(-)→SOM(-) (n = 2), and one bidirectional connection was recorded that occurred between two SOM(+) neurons. Scale bars: 50 mV, 20 pA, 20 ms. Current injection applied to the presynaptic cell is illustrated in black under each trace. F: Shows IPSC amplitudes for each connection type: SOM(-)→SOM(+) mean amplitude: 23.5 pA (n = 2 pairs), SOM(+)→SOM(+) mean amplitude: 24.9 ± 7.3 pA (n = 5 pairs – 4 unidirectional IPSCs, 2 bidirectional IPSCs), SOM(-)→SOM(-) mean amplitude: 6.6 pA (n = 2 pairs). Grey dots represent IPSCs from the bidirectional connection. G: Diagram showing the approximate location of connected paired recordings within the CeL. H: Shows number of paired recordings where either a connection was or was not detected for each SOM(+) and SOM(-) combination. A connection was more likely to be observed when recording from two SOM(+) neurons (~62% connection success rate) as opposed to a SOM(-)→SOM(+) (~12% connection success rate) or a SOM(-)→SOM(-) combination (~28% connection success rate).

Figure 9: Channelrhodopsin activation of somatostatin (SOM) terminals in the central lateral amygdala. AAV-DIO-channelrhodopsin-mCherry was injected into the CeL of SOM-Cre C57/BLJ6 mice (A). B: Example image of fluorescence of injection site in the CeL (BA: basal amygdala, CeM: central medial amygdala). C: Using a $KMeSO_4$ internal solution (K-Me), we recorded responses from SOM(-) cells in response to a short light pulse (2 ms, 470 nm; blue rectangle; example voltage-clamp traces at -40 mV and -70 mV), resulting in an inhibitory postsynaptic current (IPSC) (mean amplitude: 162 ± 24 pA, n = 15 cells). D-F: To determine whether all cell types received inhibition from SOM(+) CeL neurons, we also used a cesium-based internal solution (Cs), allowing voltage-clamping at 0 mV (ChR reversal potential), average traces are shown in black, and example individual traces are shown in grey. SOM(+) neurons responded with large IPSCs in response to light activation (D), as did SOM(-) cells (E). F: Light-activated IPSCs were detected in 100% of SOM(+) cells (n = 10 neurons) and 100% of SOM(-) cells (n = 22). The overall mean amplitude in SOM(+) neurons was 1358 ± 231 pA (n = 10 neurons, light pulse: 2 ms, 470 nm) and the mean amplitude in SOM(-) neurons was 609 ± 202 pA (n = 12, light pulse 2 ms, 470 ms; the remaining 10 neurons were tested with a 1 ms light pulse: mean amplitude 294 ± 70 pA). G: Bicuculline (10 μM) blocked SOM(+)-driven IPSCs (aCSF mean amplitude: 450 ± 206 pA, bicuculline mean amplitude: 11 ± 4 pA, p = 0.04, one-tailed paired t-test). H: SOM(-) neurons that received SOM(+)-driven inhibition were recovered and stained for PKCδ (n = 10 neurons). Five of these neurons were PKCδ(+) while the remainder were PKCδ(-). Example images are shown with biocytin recovery shown in cyan (left panel), PKCδ staining shown in purple (middle panel) and merge shown in right-hand panel. The white arrowhead indicates one PKCδ(-) neuron and the yellow arrowhead indicates one PKCδ(+) neuron across all three panels. I: To exclude variation in ChR2 infection and light intensity, and therefore allow direct comparison of light-evoked IPSC amplitudes, we performed simultaneous recordings from one SOM(+) neuron and one neighbouring SOM(-) neuron within the same slice (top diagram). SOM(-) cells typically had smaller IPSCs than their neighbouring SOM(+) cell (SOM(+) mean amplitude: 1206 ± 188 pA, SOM(-) mean amplitude: 399 ± 64.8 pA, p = 0.01 unpaired t-test, Welch's correction; bottom graph; dotted lines join cells that were recorded at the same time, n = 5 paired recordings). J: In two cases, light stimulation of SOM(+) terminals during connected paired recordings was possible. Here, a connected SOM(-)→SOM(-) paired recording is shown with example traces of the connection (i). Both the SOM(-) pre- and postsynaptic cells of this pair also received SOM(+) inputs (ii). These recordings were conducted using a $KMeSO_4$ internal solution.

Figure 10: Channelrhodopsin activation of somatostatin-negative (SOM(-)) terminals in the central lateral amygdala (CeL) of SOM-cre mice. A: In order to confirm whether SOM(-) neurons in the CeL also form local connections, we injected an AAV-forward-channelrhodopsin-enhanced yellow fluorescent protein (eYFP) mixed with an AAV-DIO-tdTomato into the CeL of SOM-cre mice; infected SOM(-) neurons express ChR2-eYFP but not tdTomato (tdTom), whereas SOM(+) neurons express tdTom but not ChR2-EYFP. B: Example image of maximal spread of ChR2-YFP expression at the injection site; the area shown corresponds to the orange square in (A). Although the injection covered the majority of the CeL (outlined in white), eYFP(+) somas can still be seen above the CeL and in the BA. Scale bar: 200 μm, dorsal (D) and medial (M) orientation is shown in bottom left corner. C: Shows close ups of the CeL in slices that were also stained for PKCδ. ChR2-eYFP (green), tdTom (red), PKCδ (purple) and merge panels are shown (BA: basal amygdala, CeM: central medial amygdala, scale bar: 100 μm). Insets in the merge panel show close ups of two neurons from a merge of eYFP and tdTom stainings (top) and a merge of eYFP and PKCδ staining (bottom). Arrowheads indicate the same neurons in both insets: a tdTom(+)/eYFP(-) neuron that was PKCδ(-) (white arrowhead), and a tdTom(-)/eYFP(+) neuron that was PKCδ(+) (yellow arrowhead). D: Neurons were counted; 62% were eYFP(+)/tdTom(-) (mean n = 67 ± 5 neurons/0.9x10^{-3} mm^3), and 36% were eYFP(-)/tdTom(+) (mean n = 39 ± 4 neurons/0.9x10^{-3} mm^3). Theoretically, there should be no overlap of eYFP(+) and tdTom(+) as the presence of Cre recombinase should either allow the expression of tdTom or prevent the expression of ChR2-eYFP. In reality, however, we did observe an overlap between eYFP(+) and SOM(+) neurons although this was only ~2% of fluorescently labelled neurons, which represented 1-3 neurons per 0.9x10^{-3} mm^3 of CeL. The majority of eYFP(+) neurons were also PKCδ(+) (77%, mean n = 51 ± 2 neurons/0.9x10^{-3} mm^3) whereas 23% (mean n = 16 ± 5 neurons/0.9x10^{-3} mm^3) were PKCδ(-). E-F: Whole-cell recordings ($CsMeSO_4$ internal solution) of SOM(+) (E) and SOM(-) neurons (F) revealed that both neuronal types displayed light-activated IPSCs from SOM(-) neurons (SOM(+) mean amplitude: 73.0 ± 19.7 pA; SOM(-) mean amplitude: 427.2 ± 77.8 pA). Example traces are shown with average traces in black and example individual traces in grey. G: 10 of 11 (91%) of recorded SOM(+) neurons showed a response to light activation of SOM(-) terminals, whereas 9 of 9 of SOM(-) neurons received inhibitory terminals. H: Bicuculline (10 μM) blocked SOM(-)-driven IPSCs (aCSF mean amplitude: 375 ± 137 pA, bicuculline mean amplitude: 16 ± 7 pA, p = 0.03 one-tail paired t-test). I: As with our previous experiments, paired recordings between a SOM(+) neuron and a neighbouring SOM(-) neuron allowed us to compare IPSC amplitudes from these two cell types (left diagram). These recordings showed that the amplitude of ChR2-driven SOM(-)→SOM(-) IPSCs was significantly greater than that of ChR2-driven SOM(-)→SOM(+) IPSCs (mean SOM(+) amplitude: 68 ± 18 pA, mean SOM(-) amplitude: 603 ± 81 pA, p = 0.002 unpaired t-test, Welch's correction).

Firing type	Non-accommodating (n = 80)	Accommodating (n = 59)	Stuttering (n = 12)
Incidence	53%	39%	8%
Input resistance (mΩ)	416 ± 17	419 ± 28	387 ± 64
Resting potential (mV)	-64 ± 1	-59 ± 1 [a]	-62 ± 2
Threshold (mV)	-33 ± 0.5	-34 ± 0.5	-34 ± 1.8
Onset (ms) at T	330 ± 25	209 ± 23 [b]	122 ± 54
Onset (ms) at 2T	77 ± 5	59 ± 7 [c]	28 ± 19
Amplitude (mV)	66 ± 1	69 ± 1	53 ± 4 [d, e]
Rise time (ms)	0.4 ± 0.02	0.4 ± 0.02	0.2 ± 0.02 [f, g]
Half-width (ms)	1.2 ± 0.03	1.1 ± 0.03	0.6 ± 0.04 [f, h]

Table 1: Membrane properties of neurons in the central lateral amygdala.
Values are means ± SEM. Low-threshold bursting neuron properties are not represented in this table since n = 1 for this firing type. T: threshold, 2T: twice threshold, NA: non-accommodating, Ac: accommodating.

[a]: $p < 0.001$ vs NA (two-tailed t-test)
[b]: $p < 0.001$ vs NA (Mann-Whitney test)
[c]: $p < 0.01$ vs NA (Mann-Whitney test)
[d]: $p < 0.001$ vs NA (two-tailed t-test)
[e]: $p < 0.0001$ vs Ac (two-tailed t-test)
[f]: $p < 0.0001$ vs NA (Mann-Whitney test)
[g]: $p < 0.001$ vs Ac (Mann-Whitney test)
[h]: $p < 0.0001$ vs Ac (Mann-Whitney test)

	Soma length (µm)	Soma volume (µm^3)	Number of primary dendrites	Number of nodes	Total dendrite length (µm)
Total (n=8)	15.6 ± 0.8	1117 ± 232	5.5 ± 0.4	13.2 ± 1.0	1389 ± 88
Presynaptic (n=4)	14.4 ± 0.9	929 ± 354	5.2 ± 0.6	14.7 ± 0.6	1309 ± 152
Postsynaptic (n=4)	16.9 ± 1.2	1304 ± 322	5.7 ± 0.5	11.7 ± 1.7	1469 ± 93

Table 2: Morphological properties of neurons in the central lateral amygdala.
Values are means ± SEM. Four connected pairs (total of 8 neurons) were recovered and their morphology analysed. When these properties were compared between pre- and postsynaptic neurons, no significant differences were observed (Mann-Whitney test).