

**Role of intercalated and NPY-expressing cells in
neuronal circuit of the amygdala.**

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Thesis submitted for the degree of

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at the

University of Oxford

Dla Babci Danusi

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Abstract

Local inhibitory microcircuit of amygdala is an active component in processing emotional information. Despite prominent evidence of its importance, our understanding of GABAergic cell types, their connectivity and role in amygdala network is limited. The aim of this thesis is to understand connectivity and physiology of two specific components of GABAergic microcircuit of amygdala: so-called intercalated cells and neuropeptide Y (NPY) expressing interneurons.

Intercalated cells (ITCs) of the amygdala are clusters of GABAergic neurons that surround the basolateral complex of amygdala (BLA). There is growing evidence suggesting that ITCs are required for the expression of fear extinction. The main intercalated nucleus (Im) is the largest of the ITC clusters and could be also important for emotional processing. Using patch-clamp whole-cell recordings paired with subsequent anatomical analysis I described basic physiology and anatomy of neurons within the Im. I found that these neurons share common characteristics to earlier described neurons within the medial ITC cluster, yet they can be divided into three distinct groups. Next, I provided anatomical and functional evidence that Im neurons project to central and basal nucleus of amygdala and that they are reciprocally connected with medial and lateral ITCs clusters. I found that Im neurons receive excitatory inputs from BLA as well as cortex; next I verified that heterogeneous inputs do not interact with each other. I have shown that the Im neurons express both AMPA and NMDA receptors, suggesting that they may undergo NMDA-dependent plasticity. I have reported that dopamine hyperpolarizes Im neurons via dopamine receptor 1, therefore providing a cellular substrate for disinhibition of the amygdala at the systemic level. Thus, the Im is likely to be an additional site of integration of the distributed network underlying acquisition, expression and extinction of conditioned fear.

In another project, I report novel interneuron type of the BLA and call it neurogliaform cell (NGFC) of amygdala. I used a mouse line expressing green fluorescent protein (GFP) under NPY promoter and patch clamp technique combined with pharmacology and electron microscope analysis. I performed paired recordings between presynaptic NPY-GFP positive (+) cells and postsynaptic principal neurons (PNs). Presynaptic NPY-GFP+ neurons display small soma and short dendrites embedded in a cloud of highly arborized axon. I showed that NPY-GFP+ cells are source of GABAA receptor-mediated slow inhibitory postsynaptic currents (IPSCs, decay time constant > 30 ms) evoked in PNs and in themselves (autapses). These slow IPSCs are known in literature as GABAA,slow. My results indicate that the slow kinetics of these IPSCs was likely caused by the low concentration and spillover of extracellular GABA. Physiologically-relevant in vivo firing re-played in NPY+-NGFCs in vitro evoked a transient depression of the IPSCs. Presynaptic GABAB receptors controlled the strength of this short-term plasticity. Interestingly, synaptic contacts made by NGFCs showed close appositions, without identifiable classical synaptic structures, between presynaptic boutons of the recorded cells and postsynaptic profiles. Thus, volume transmission of GABA is likely to be generated by this interneuron of the amygdala. NPY+-NGFC is a novel interneuron type of the BLA. The peculiar functional mode of NGFCs makes them unique amongst all GABAergic cell types of the amygdala identified so far.

Declaration

All the work in thesis was done by me, Mirosława Mańko, except for what is mentioned below.

I have benefited from collaborations with other scientists. Their contribution to the work presented in this thesis is outlined below.

Dr Raffaella Geracitano performed some experiments with DA presented in Figure 3.3., and recorded cell (r100207s1) presented in Figure 3.4.

Dr Daniela Busti produced the reconstruction of the ITC cell (r100207s1) shown in Figure 3.4., and performed the light and electron microscopy analysis presented in Figure 3.7A and B.

Dr Thomas Bienvenu recorded *in vivo* the firing pattern of the NPY+ cell of amygdala used in the experiments presented in Figure 4.16., and took the picture presented in Figure 4.4.

Prof Yannis Dalezios performed the electron microscopy analysis presented in Figure 4.17., and assisted me in the cluster analysis presented in Figure 3.2.

Dr David Elfant wrote algorithms implemented in IGOR Pro that measure rise time of the currents and half-width of action potential. Scripts of procedures are presented in relevant method section.

List of abbreviations

μOR	μ opioid receptor
AB	accessory basal nucleus of amygdala
ACSF	artificial cerebrospinal fluid
AHP	afterhyperpolarization
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AP	action potential
AStria	amygdala/striatum
ATP	Adenosine-5'-triphosphate
β ^{-/-}	β3 subunit-deficient mice
BA	basal nucleus of amygdala
BAC	bacterial artificial chromosome
BLA	basolateral complex of amygdala
BZ	benzodiazepine
Ca ²⁺	calcium
CB	calbindin
CCK	cholecystokinin
CE	central nucleus of amygdala
CEc	capsular division of the central nucleus of amygdala
CEl	lateral division of the central nucleus of amygdala
CEm	medial division of the central nucleus of amygdala
Cl ⁻	chloride
CNS	central nervous system
Cp-mITC	CE projecting mITC neurons
CR	calretinin
CS	conditioned stimulus
CTRL	control
D	dorsal
D1-R	dopamine receptor 1
D2-R	dopamine receptor 2
DA	dopamine
DAB	diaminobenzidine
D-AP5	(2R)-amino-5-phosphonovaleric acid
DIC	differential interference contrast
E	embryonic age
EC	external capsula

eEPSC	evoke excitatory postsynaptic current
EGTA	ethylene glycol tetraacetic acid
Eion	reversal potential
eIPSC	evoked inhibitory postsynaptic current
EM	electron microscopy
EPSP	excitatory post-synaptic potential
FS	fast spiking
γ	membrane conductance
GABA	γ -aminobutyric acid
GABA _A R	GABA _A receptor
GABA _B R	GABA _B receptor
GAD	glutamic acid decarboxylase
GAT	GABA transporter
GFP	green fluorescent protein
hrGFP	humanized renilla green fluorescent protein
I	current
IC	intermediate capsula
IL	infralimbic prefrontal cortex
IPSC	inhibitory post-synaptic potential
IR	immunoreactivity
ITCs	intercalated cells
K ⁺	potassium
L	lateral
LA	lateral nucleus of amygdala
LGE	lateral ganglionic eminence
lITC	lateral intercalated cell cluster
Im	main intercalated nucleus
LTD	long term depression
LTP	long term potentiation
M	medial
MCp-mITC	medial capsular projecting mITC neurons
mITC	medial intercalated cell cluster
mITCa	medial intercalated cell cluster anterior
mITCp	medial intercalated cell cluster posterior
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NGC	new born dentate gyrus granule cell

NGFC	neurogliaform cell
NMDA	N-Methyl-D-aspartic acid or N-Methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NPS	neuropeptide S
NPY	neuropeptide Y
OL-M	oriens lacunosum-moleculare
PB	phosphate buffer
PN	principal neuron
PV	parvalbumin
R	resistance
R _{in}	input resistance
RMP	resting membrane potential
R _s	series resistance
RT	room temperature
SC	Schaffer collateral
SEM	standard error to the mean
sIPSC	spontaneous inhibitory postsynaptic current
SL-M	stratum lacunosum-moleculare
SLp-mITC	sublenticular projecting mITC neurons
SO	stratum oriens
SOM	somatostatin
SP	stratum pyramidale
SR	stratum radiatum
τ	membrane time constant
TH	tyrosine hydroxylase
TPMPA	(1,2,5,6-Tetrahydropyridin-4-yl)methylphosphinic acid
uIPSC	unitary inhibitory postsynaptic current
US	unconditioned stimulus
V	voltage
V	ventral
VH	holding potential
VIP	vasoactive intestinal peptide
V _m	membrane potential

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1. General introduction

Charles Darwin in his book *The Expression of Emotions in Man and Animals* (1872) points that basic emotions are universal counterparts influencing animals and humans behaviour. In the XX century, the psychologist Paul Ekman, defined six basic emotions: anger, disgust, fear, happiness, sadness and surprise. Importantly, he showed that facial expressions of emotion are not culturally determined, but universal across human cultures and thus biological in origin. Emotions are highly associated with animal behaviour and therefore essential for survival. Paul Ekman noticed that *'Emotions can have a very fast onset, beginning so quickly that they can happen before one is aware that they have begun. Quick onset is central to the adaptive value of emotions, mobilizing us quickly to respond to important events.'*¹ In humans and animals, amygdala is a key brain area that is involved in processing of essential emotional information (LeDoux, 2000). Despite growing efforts, understanding of mechanisms underlying that process is very limited.

This thesis is focused on the role of synaptic inhibition in the rodent amygdala. Local inhibitory interneurons of the amygdala are an active component in processing emotional information (Ehrlich et al., 2009). Despite prominent evidence of its importance, our understanding of GABAergic cell types, their connectivity and role in the amygdala network is limited. This is a fundamental question given the potential behavioural and clinical relevance of the pharmacological modulation of the GABA receptors.

The aim of this thesis is to understand the connectivity and physiology of two specific GABAergic components of the amygdala. In the first part of my studies I

¹ From Ekman, P. (1999). Basic Emotions. Chapter 3. Handbook of Cognition and Emotions. John Wiley & Sons Ltd, Sussex, UK.

focused on so-called intercalated cells of the amygdala. In the second part I studied neuropeptide Y expressing interneurons of the amygdala.

1.1. The amygdala: function and neuronal organisation

1.1.1. Role of the amygdala in emotional regulation

The role of the amygdala in regulating emotions was established during the XX century. The first insight into its role in emotional information processing comes from lesion studies performed by Kluver and Bucy in the 1930s. Rhesus monkeys with a damaged temporal lobe show a variety of symptoms including inappropriate response to stimuli, loss of fear, agnosia, hyperorality, and hypersexuality. Afterwards, the role of the amygdala has been associated with many functions involved in emotional processing such as: decision making (Ghods-Sharifi et al., 2009); appetitive and stimulus-reward learning (Baxter and Murray, 2002; Adolphs, 2010), modulation of arousal and perception (Phelps and LeDoux, 2005; Pessoa, 2011). Finally, human patients that suffer from Urbach-Wiethe Syndrome, which is a bilateral amygdala calcification, suffer from impaired social and emotional behaviours, facial recognition of fear and fear conditioning (Adolphs et al., 1994).

The function of the amygdala is most commonly studied with the use of so-called fear conditioning protocol (LeDoux, 2000; Herry et al., 2008; Cioocchi et al., 2010). It is a form of simple, associative learning first described by the Russian psychologist and physiologist Ivan Pavlov at the beginning of the XX century. In the classical paradigm, animals are exposed to a neutral stimulus e.g. a tone or light (the conditioned stimulus; CS) during the habituation phase. Next, the presentation of CS is paired with a noxious stimulus e.g. mild electrical shock (the unconditioned stimulus; US) during the conditioning phase. As a result, after a small number of pairing, the initially neutral stimulus gains aversive properties. Afterward, the presentation of the CS alone evokes in animals a conditioned response, in that case fear. In other words, animals learn to associate the two stimuli. Fear is manifested in

animals by defensive behaviours (increased freezing), response from autonomic (increase in heart rate and blood pressure) and endocrine system (hormone release). Subsequently, repeated presentation of the CS alone gradually reduces the fear response, this process being known as extinction (Myers and Davis, 2007; Quirk and Mueller, 2008). Extinction is not simply 'erasing' the previously acquired memory trace, it is a form of new learning, when animals recognise that the CS is no longer fearful. Evidence supporting this hypothesis is that principal neurons of the BLA maintain their response to the presentation of the CS after extinction training, meaning that two engrams co-exist (Repa et al., 2001; Hobin et al., 2003). Spontaneous recovery of fear can also occur in response to stress or specific clues (Bouton, 1993; Myers and Davis, 2007). It is generally thought that information and coding of CS and US are computed within the amygdala and connected brain areas. The medial prefrontal cortex controls this process and context representation is encoded by the hippocampal-cortical network. Before describing with more details the flow of information within the amygdala during fear conditioning and extinction, I will first present anatomical aspects of the amygdala in rodents.

1.1.2. Neuronal organisation and connectivity of the amygdala

The anatomist Burdach suggested the term 'amygdala' (from Greek) to describe an almond-like structure located in the human temporal lobe. Today we know that the amygdala is anatomically a complex structure consisting of several nuclei (~13) that can be further subdivided (Fig. 1.1.). In primates and non-primates the amygdala is placed ventro-medial to the striatum and anterior to the ventral hippocampal formation (Paxinos and Franklin, 2007). Extensive anatomical studies resulted in few different nomenclature systems. In this thesis I will follow the taxonomy and abbreviations detailed below.

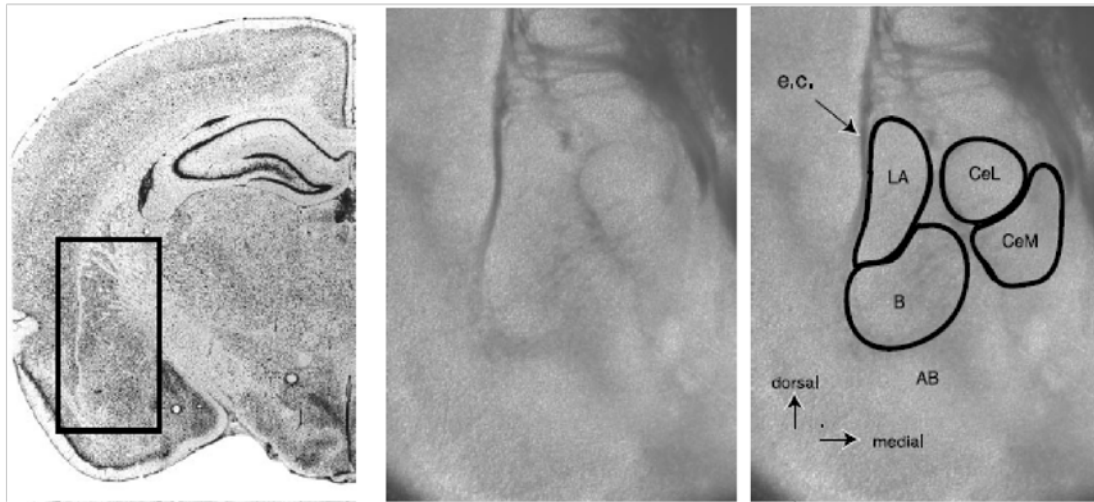


Figure 1.1. An example of the amygdala as it appears in acutely prepared coronal section. Left panel: a Nissl-stained hemisection of a rat brain, outline region are shown in an acutely prepared brain slice, middle and right panel. Middle panel: brain slice as it appears under bright illumination. Right panel: outline of approximate regions of the lateral (LA), basal (B), accessory basal (AB), central lateral (CeL) and medial (CeM) nuclei (Sah et al., 2003).

Based on the cytoarchitecture, connectivity and developmental origin, amygdala nuclei can be divided into three groups:

1. The basolateral complex (BLA): it consists of basal, lateral and accessory basal nuclei (BA, LA and AB respectively). It is a cortical-like structure composed by glutamatergic and GABAergic neurons (McDonald, 1982b). Most of the neurons (~80%) are spiny, glutamatergic projections cells, called principal neurons (McDonald, 1982b). The second population (~20%) consists of aspiny, local GABAergic interneurons (McDonald and Augustine, 1993). Afferent connections to the BLA arise at least from the thalamus, hippocampus, rhinal and prefrontal cortex (Canteras and Swanson, 1992; McDonald and Mascagni, 1996b; Pitkanen et al., 2000). Cortical and thalamic inputs convey information from sensory areas of all modalities: olfactory, gustatory, somatosensory, auditory and visual (LeDoux et al., 1991; Shi and Cassell, 1998; Shi and Davis, 2001). Hippocampus, rhinal and

prefrontal cortex are reciprocally connected to the BLA. Importantly, LA neurons are projecting to the BA and both divisions project to the central nucleus (Smith and Pare, 1994).

2. The centro-medial group consisting of the medial and central nucleus (Sah et al., 2003). The central nucleus (CE) can be further divided into the capsular, lateral and medial division (CEc, CEI and CEm respectively). It is a striatal-like structure; the majority of the cells are GABAergic with medium-spiny morphology. The CE nucleus is strongly interconnected, the CEI nucleus reciprocally projects to the CEc and unidirectionally to the CEm (Jolkkonen and Pitkanen, 1998). Importantly, neurons of the CEm subdivision are called the output of the amygdala as they project to the brainstem and the hypothalamus. The main input to the CE nucleus originates from the BLA, but also from the ventral entorhinal cortex (to the CEI), insular cortex (to the CEI), medial prefrontal cortex (to the CEm), paraventricular nucleus of thalamus (all divisions) and brainstem nuclei (parabrachial nucleus and nucleus of solitary tract to the CEm and CEI) (Sah et al., 2003).

3. The cortical-like group consisting of the cortical nuclei and nucleus of the lateral olfactory tract (Sah et al., 2003).

An additional striatal-like component is the intercalated cell masses. Since the anatomy and physiology of one intercalated nuclei is a subject of my thesis I will review the current knowledge about this structure in Chapter 1.2.

In a simplified model, the flow of information through the amygdala is processed by series of nuclei (see summary Figure 1.2.). Shortly, multimodal early sensory information from cortex and thalamus enters the LA and is further conveyed to the BA nuclei. In classical view, BA projects further to the CE nucleus, thought to be the major output of the amygdala. CEm neurons project to the brainstem and hypothalamus leading to the expression of autonomic and motor responses (Krettek and Price, 1978; Veening et al., 1984; Viviani et al., 2011). However it is important to

remember that CE and BLA receive extra-amygdaloid inputs and can process information in parallel (Sah et al., 2003).

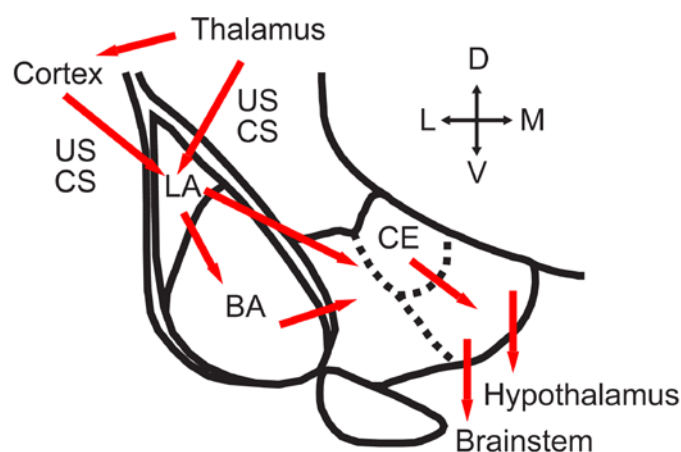


Figure 1.2. Basic neuronal circuits involved in fear conditioning.

During fear conditioning information about CS and US (from thalamus and cortex) is associated in LA. Next, LA project to the BA, both structures project onto CE nucleus and further to the brainstem and hypothalamus. D, dorsal; L, lateral; M, medial; V, ventral.

There are multiple evidence showing the role of GABAergic neurons of the amygdala in processing emotional information (Harris and Westbrook, 1998). Before consideration of the role of inhibitory transmission in the amygdala microcircuit, I will present our current understanding of BLA interneurons that is relevant for the work presented in this thesis.

1.1.3. Diversity of interneuron types within the basolateral complex

The interneurons of the BLA form about 15-20% of the neuronal population of this nucleus (McDonald, 1985; McDonald and Augustine, 1993; Sah et al., 2003) and have a subpallial origin (Stenman et al., 2003). They exhibit a powerful inhibition of PNs, stronger than the inhibition usually impinging onto cortical principal cells (Pare

and Gaudreau, 1996; Lang and Pare, 1997b). Up to date, several populations of interneurons are recognised in the BLA. Cell type classification is based on a combination of data regarding their morphology and postsynaptic target identity, expression of calcium binding proteins and neuropeptides, and physiology (Ehrlich et al., 2009; Spampanato et al., 2011). Based on immunocytochemistry, in general we can divide BLA GABAergic neurons into calbindin (CB+) or calretinin positive (CR+) populations. CB+ cells can be further divided into parvalbumin (PV+), somatostatin (SOM+) or cholecystokinin (CCK+) groups. Cells within the CR+ group are often co-expressing CCK+/VIP+ (vasoactive intestinal peptide). Recently, approach based on whole-cell patch-clamp and single-cell RT-PCR led to an alternative classification of five interneuron subtypes (Sosulina et al., 2010). Unfortunately, this approach did not lead to a clear separation of interneuron types. Clearly, unambiguous cell type definition performed in other brain structures, like in the hippocampus, is still largely missing (Klausberger and Somogyi, 2008). I review below the current knowledge about the four interneurons populations broadly recognised in the BLA.

1) PV+/ CB+ cells are the most numerous in the BLA (McDonald and Mascagni, 2001). These neurons show a variety of sizes and shapes (Kemppainen and Pitkanen, 2000; McDonald and Mascagni, 2001). The axon of these cells was found to establish synapses with somata, dendrites and axon initial segments of PNs (Kemppainen and Pitkanen, 2000; Muller et al., 2006; Rainnie et al., 2006). *In vitro*, PV+ cells show four types of firing pattern (fast-spiking, stuttering, non-adapting and adapting) (Woodruff and Sah, 2007b), which points to the possibility of diverse cell types within the PV+ population. They can be further characterised by short action potential (AP) width (~ 0.5 ms) and the ability to fire train of APs of high frequency, up to 100 Hz. They are therefore commonly referred as fast-spiking interneurons (Rainnie et al., 2006; Woodruff and Sah, 2007a). Recent studies performed in our group combining *in vivo* single-cell physiology with post-hoc anatomical investigation

established that within PV+ neurons of BLA, axo-axonic and PV+ basked cells are distinct cell types (Bienvenu et al., 2012). Similar to their cortical counterparts, they form electrical and chemical junctions within the PV+ cell population (Muller et al., 2006; Woodruff and Sah, 2007a). It was therefore assumed that they could synchronise cell assemblies and therefore influence BLA activity (Woodruff and Sah, 2007a). However in *in vivo* recordings both cell types fire similarly during dorsal CA1 hippocampal theta band oscillations (3-6 Hz under urethane anaesthesia). Remarkably, only axo-axonic cells strongly increase their activity in response to noxious stimuli (Bienvenu et al., 2012).

2) CB+/SOM+ neurons show fusiform somata and multi- or bi-polar dendritic tree (McDonald, 1985; McDonald and Mascagni, 2001). About half of these neurons also express the neuropeptide Y (Truitt et al., 2009). These are most likely dendrite-targeting cells, as SOM+ boutons make synapses with distal dendrites and spines (Muller et al., 2007).

Similar to the PV+ cell population, studies combining *in vivo* single-cell physiology with post-hoc anatomical investigation performed in our laboratory show two novel interneurons type expressing CB: CB+ dendrite-targeting and Amygdala/striatum transition area projection interneurons (AStria-projection interneurons). AStria projecting neurons are the first example of GABAergic projection cells in the BLA. Surprisingly, the firing of CB+ dendrite-targeting interneurons is locked to dorsal CA1 theta oscillation, but they do not respond to noxious stimuli. The firing of a population of AStria projecting neurons is more weakly phase locked to the hippocampal theta oscillation, but they are strongly inhibited in response to noxious stimuli (Bienvenu et al., 2012).

3) Cells that express CR+/VIP+/CCK+ are small bipolar or bitufted neurons (McDonald, 1989; McDonald and Pearson, 1989; Mascagni and McDonald, 2003).

VIP+ boutons form synapses with soma and dendrites of PNs and interneurons that are CB+/VIP+ (Muller et al., 2003)

4) Large multipolar CCK+ neurons that often express CB (McDonald, 1989; Mascagni and McDonald, 2003). This cell population often express the cannabinoid receptor type 1 (Katona et al., 2001; McDonald and Mascagni, 2001).

All together, these data suggest a large, not yet fully characterised diversity of interneurons in the BLA. Based on the number of different interneuron types in the CA1 hippocampus (at least 21) (Klausberger and Somogyi, 2008), it is likely that several novel GABAergic cell types will be identified in the BLA in the near future.

1.1.4. A tonic inhibition of the BLA suppresses anxiety

Increasing evidence show a prominent role of the GABAergic neurons of the amygdala in fear conditioning and extinction. A strong inhibitory input provides constant silencing of PNs (Gaudreau and Pare, 1996; Lang and Pare, 1997b, a). Local inhibitory cells are the most prominent source of such inhibition (Lang and Pare, 1998). Inhibitory currents are mediated by Cl^- channels (activation of GABA_A receptor) and a synaptically activated Ca^{2+} -dependent K^+ conductance (Lang and Pare, 1997b). Weak K^+ -mediated current (activated via GABA_B receptor) mediates the later phase of the response (Lang and Pare, 1997b). Lang and Pare tested *in vivo* the response of BLA neurons to the cortical stimulation (Lang and Pare, 1998). Low intensity of stimulation evoked excitatory postsynaptic potentials (EPSPs) followed by inhibitory postsynaptic potentials (IPSPs) in PNs and interneurons. However with increased stimulation intensities interneurons were more excited and PNs more inhibited. As a result only a small range of intensities could evoke APs in PNs, showing that information flow is tightly regulated by interneurons activity. In agreement with this study, increase in firing rate and synchronisation of PNs during

fear can be partially achieved by suppression of inhibition (Pare and Collins, 2000). Behavioural studies further demonstrated the importance of synaptic inhibition in anxiety related paradigms. Injection of GABA_A receptor (GABA_AR) antagonists into the BLA results in anxiogenic behaviour (Cole et al., 1995; Sanders and Shekhar, 1995). It is important to note that inhibitory neurons in the amygdala are the main source targets of neuromodulatory systems such as dopaminergic (Fuxe et al., 2003; Pinard et al., 2008) and serotonergic (Muller et al., 2007).

Along with the interneurons of the BLA, two other inhibitory parts of the amygdala, the CE (Ciocchi et al., 2010; Viviani et al., 2011) and ITCs, are active components of fear conditioning.

1.2. Intercalated cells

Intercalated cells masses are a constant feature of the mammalian amygdala, early recognised as clusters of densely packed cells. Initially they were considered as assembly of glia cells, however subsequent studies showed their GABAergic nature (Hall, 1972; Kamal and Tombol, 1975). Intercalated masses contain the highest density of cells in the amygdala, and are organized in clusters surrounding the BLA that are well preserved among mammals (Millhouse, 1986; Nitecka and Ben-Ari, 1987; Pare and Smith, 1993b, a; Alheid, 1994; Pitkanen and Amaral, 1994; Royer et al., 1999; Marowsky et al., 2005). Despite their early recognition (Sah et al., 2003), only in recent years data were collected on their basic anatomy and physiology showing an active role during fear conditioning and extinction (Likhtik et al., 2008; Amano et al., 2010; Busti et al., 2011).

1.2.1. Cellular components and anatomical organisation of ITC clusters

Based on morphology, two types of intercalated cells are distinguished. Small cells that form ~95% of the clusters with the remaining ~5% composed by large ITC cells (Millhouse, 1986). The majority of ITC cells have a small soma (9-18 μm in diameter), bipolar dendritic tree confined within the capsula and an axon that ramifies within the cluster or in intra- or extra-amygdaloid regions (Millhouse, 1986; Pare and Smith, 1994; Royer et al., 2000b; Geracitano et al., 2007; Busti et al., 2011). Large ITC are characterised by a big soma with thick and mostly aspiny dendrites (Millhouse, 1986). Current published data report that these neurons are cholinergic with unknown axonal projection. However, unpublished work performed by Dr Bienvenu in collaboration with Dr Busti had two unexpected results: their GABAergic nature and their large projections to the BLA and other brain regions (over 6 mm in the rat). A full reconstruction of the axonal arborisation of one of these

cells shows that it projects to a broad cortical areas spanning from the endopiriform nucleus to the piriform cortex. Due to the subject of my work, further part of introduction will concern only small ITCs.

Based on their location, the ITC clusters can be further divided into: medial and lateral intercalated cell clusters, and main intercalated nucleus (mITC, lITC, and Im, respectively). The Im nucleus, the biggest cluster among ITCs, is located ventro-medially to the BLA (Millhouse, 1986; Pare and Smith, 1994). Medial and lateral clusters are respectively located in the intermediate and in the external capsula (Millhouse, 1986; Marowsky et al., 2005). Some anatomical studies reported that these clusters are connected by scattered ITC neurons and form a continuous net that surround the BLA (Millhouse, 1986; Pare and Smith, 1994). Recently, a careful study of the mouse ITCs with the use of several markers led to the calculation of their volume and 3D position relative to the BLA (see Fig. 1.3A). All ITC clusters make ~17% of the BLA and ~45% of the CE nuclei volume. Moreover, based on observations that discontinuing cells form the medial and lateral clusters they were further divided. The medial cluster consists of the anterior and medial part, and the lateral cluster of the lateral and supralateral part (Fig. 1.3). As previously observed (Marowsky et al., 2005) an additional small cluster was identified within the LA nucleus, therefore named intralateral cluster (Fig. 1.3). Its function and anatomy has not been studied so far. Because the function of ITC clusters seems to be mostly related to its position I will further use the simple description of medial, lateral and main cluster.

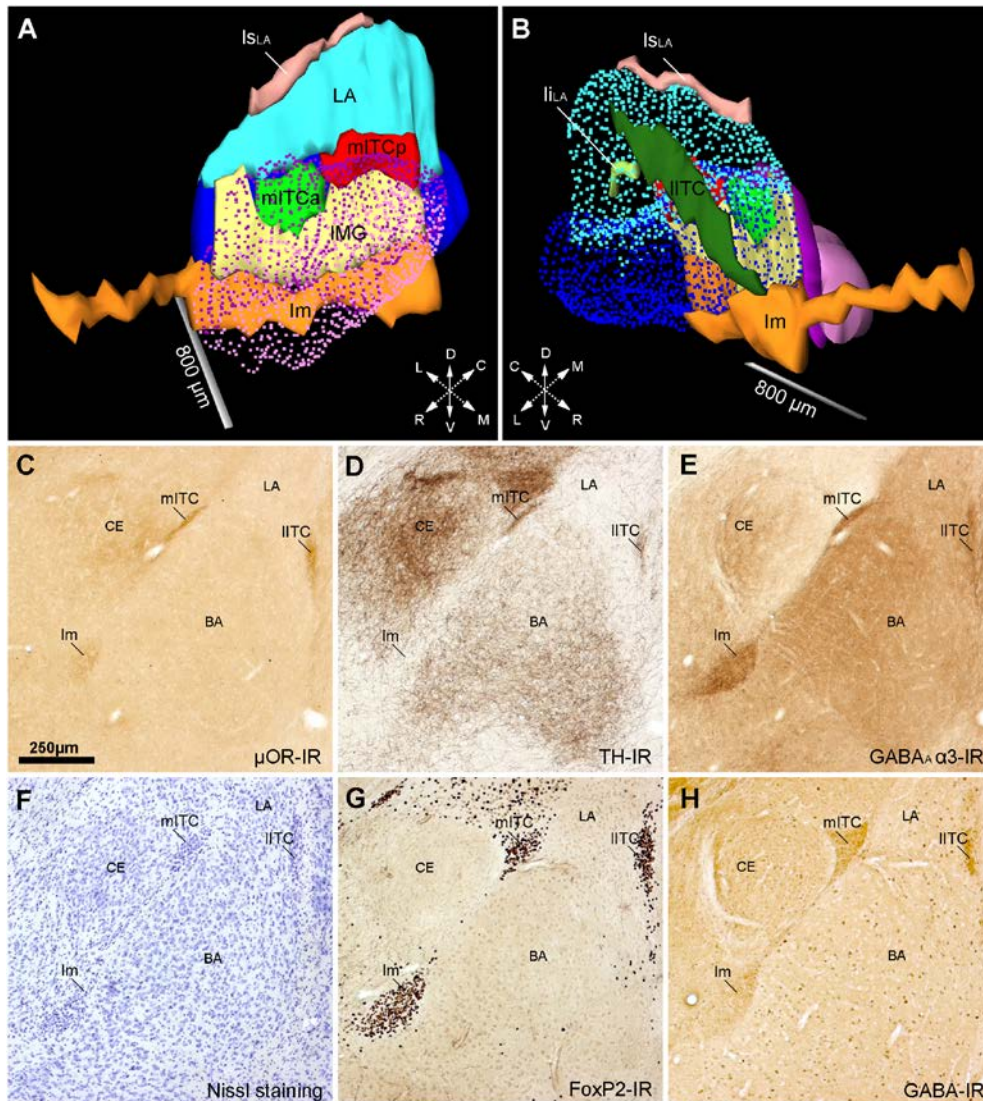


Figure 1.3. 3D reconstruction and immunochemical profile of the ITC clusters of the mouse amygdala. *A, B*, Medial (*A*) and lateral (*B*) views of ITC clusters, CE (CEc/l in purple; CEm in light purple) and BLA (LA in light blue; BA in blue) of the mouse amygdala. The Im (in orange), located ventro-medially to the BA, extend rostrally beneath the anterior commissure for considerable length. Rostro-caudally along the intermediate capsule, two discontinuous clusters are shown: mITC posterior (mITCp) in green and mITC anterior (mITCa) in red. The intermediate capsule interposed between the mITCp and Im is identified as intramedullary grey (in yellow; de Olmos, 1990). The clusters observed laterally and dorsally to the BLA, previously known as paralaminar nucleus (Amaral and Price, 1984) show no apparent cellular continuity in mouse and were named IITC (in green) and supralateral cluster (Is_{LA}, in pink), respectively. A small cluster inside LA was termed

intralateral cluster (li_{LA} , light green). *C-H*, representative micrographs of consecutive sections (bregma levels, -1.46 to -1.70) of the Im, IITC and mITC visualised through different immunohistochemical stains: μ OR-IR (*C*), TH-IR (*D*), GABA_A α 3-IR (*E*), cresyl violet (*F*), *FoxP2*-IR (*G*), and GABA (*H*). Scale bars: *A, B*, 800 μ m; *C-H*, 250 μ m. (Busti et al., 2011).

1.2.2. Striatal-like characteristic of intercalated cells

Intercalated cells have been classically identified based not only on their highest density compared to cells of other nuclei of amygdala. However, some specificity based on their histochemical profile recently started to emerge (Fig. 1.3.). These cells do not express classical calcium binding proteins and neuropeptides often used to identify interneurons (Marowsky et al., 2005). Nevertheless, it is possible to identify them based on their rather selective expression of receptors, channels and transcription factors (Fig. 1.3.).

One typical marker of the ITC is the μ opioid receptor (μ OR; fig. 1.3C), in contrast this receptor is weakly expressed in the BLA and CE (Mansour et al., 1995; Ding et al., 1996; Jacobsen et al., 2006; Kaoru et al., 2010; Busti et al., 2011). This receptor is exclusively expressed postsynaptically, making it an essential tool in selective cell manipulations (Likhtik et al., 2008). Another functional receptor heavily expressed by the ITCs is the dopamine receptor 1 (D1-R). Similar to the μ OR, this receptor is more weakly expressed in the BLA and CE (Scibilia et al., 1992; Fuxe et al., 2003; Jacobsen et al., 2006; Kaoru et al., 2010). Interestingly, only a minor dopaminergic innervation of the D1-R-immunoreactive cells is found in the rostromedial and caudal component of the main intercalated island, suggesting volume transmission as the main communication mode for dopamine in these regions. In contrast, the D1-R immunoreactive areas in the rostrolateral part of the main island and also the paracapsular intercalated islands show a high degree of dopaminergic innervation, suggesting that synaptic and perisynaptic dopamine transmission plays a dominant role in these regions (Fuxe et al., 2003).

The mapping of GABA_AR subunit composition of the amygdala showed a high expression of $\alpha 2$ and $\alpha 3$ subunits within the ITCs (Pirker et al., 2000; Marowsky et al., 2004; Busti et al., 2011; Geracitano et al., 2012) and moderate expression of the $\beta 2$ and $\beta 3$ subunits (McDonald and Mascagni, 1996b; Pirker et al., 2000). The ITCs

are also selectively enriched in the α subunit of Kv4.2 channel and the K⁺ channel interactive proteins 2, 3 and 4 (Dabrowska and Rainnie, 2010)

There are several lines of evidence pointing to the same developmental origin of the corpus striatum and ITCs. Millhouse made the first suggestion of a common developmental origin based on the similar cell anatomy regarding soma size and shape as well as characteristic branching and spine density (Millhouse, 1986). The next evidence to support the hypothesis that the ITCs are a ventral extension of the striatum is the similar expression pattern of the μ OR found in the rostral striatum, expression of either D1-R or dopamine receptor 2 (D2-R) and Kv4.2 by medium spiny neurons (Serodio and Rudy, 1998). To further investigate this question, a number of studies elucidated the gene expression profile of ITCs and striatum.

The *Fox* gene family encodes a large number of transcription factors highly express in several brain regions during development (Takahashi et al., 2008). The mammalian *FoxP2* gene, observed in brain regions involved in motor control and procedural memory is observed from E12.5 in developing and mature striatum (Takahashi et al., 2008). In adulthood, *FoxP2* mRNA can be also found in the amygdala, highly and exclusively expressed in the ITCs (Fig. 1.3G) (Campbell et al., 2009; Kaoru et al., 2010). Importantly, *FoxP2* strongly co-localises with the μ OR in both amygdala and striatum (Takahashi et al., 2003; Waclaw et al., 2010). Moreover, *FoxP2* in the ITCs is co-expressed with *Meis2* (Stenman et al., 2003; Kaoru et al., 2010; Waclaw et al., 2010). It is a member of *Meis* genes family group that serves as a co-transcription factor (Swift et al., 1998). This gene is used as preferential marker for striatal neurons that derive from lateral ganglionic eminence (LGE) and is present in the adult brain (Toresson et al., 1999; Ghanem et al., 2008). The third common gene for both structures belongs to the *Pbx* group that is involved in the regulation of developmental gene expression (Di Giacomo et al., 2006). The

primate *Pbx3* is expressed in developing caudate putamen and is present in the postnatal striatum as well as in ITCs (Takahashi et al., 2008). Next, NR4a1, member of the ligand binding transcription factor superfamily, is present in ITCs and in the basal ganglia. In the ITCs it is co-expressed with the μ OR, D1-R and tyrosine hydroxylase (TH) fibres (Davis and Puhl, 2011). Lastly, the ITCs express *Dlx5/6* gene, which are homeobox-containing genes, found in the precursors of GABAergic cells (Kaoru et al., 2010; Waclaw et al., 2010). Strong immunoreactivity for *Dlx5* is found in LGE where most striatal and ITCs originate (Medina et al., 2004; Cocas et al., 2009).

1.2.3. Connectivity and physiology of intercalated cells

A full understanding of the afferent and efferent connections of the ITCs clusters is essential to understand the flow of information in the amygdala. Since the mITC is strategically placed between the main input and output nuclei of the amygdala, it is the most extensively studied cluster. Therefore most of the data presented below consider only this particular cluster.

Anatomical and physiological studies were performed on cats, guinea pigs, rats and mice (Millhouse, 1986; Pare and Smith, 1993b, a; Collins and Pare, 1999; Royer et al., 1999, 2000b, a; Royer and Pare, 2002, 2003; Marowsky et al., 2005; Geracitano et al., 2007; Amir et al., 2011; Busti et al., 2011). Careful comparison of clusters position in all species shows that ITCs are a well-preserved structure with similar connectivity.

The basic electrophysiological characteristics of ITCs closely match across species and between *in vivo* or *in vitro* recordings (Royer et al., 1999, 2000b; Royer and Pare, 2002, 2003; Marowsky et al., 2005; Geracitano et al., 2007; Amir et al., 2011). This includes a negative membrane potential (below -70 mV), a high input resistance (> 400 M Ω), intermediate duration of AP (~ 1 ms) and spike frequency

adaptation evoked by depolarizing current pulses. The spontaneous activity of ITCs in anaesthetised cats is higher compared to the nearby cells of the BLA or CE nuclei (Collins and Pare, 1999). This is in line with EM observations that soma of intercalated cells have no GABAergic synaptic contacts therefore lack a powerful perisomatic inhibition and can display higher frequency rates (Pare and Smith, 1993a). The ITCs receive equal proportions of GABA-positive and negative synaptic boutons, mostly making synapses with small dendritic shafts (Pare and Smith, 1993a). Except from the frequency of tonic activity, ITCs are indistinguishable from the BLA and CE neurons based on AP shape (Amir et al., 2011).

Intercalated cells are reciprocally connected within the mITC cluster (Royer et al., 2000b; Geracitano et al., 2007; Geracitano et al., 2012). The synaptic network formed by mITC neurons exhibit distinct short-term plastic synaptic responses: depressing, facilitating and constant (Geracitano et al., 2007). These differences originate from heterogeneity in the presynaptic release probability and not due to target cell specificity (Geracitano et al., 2007). Moreover synaptic responses are not influenced by the activation of GABA_BR or cannabinoid 1-R (CB1) (Geracitano et al., 2007).

In addition to local inhibitory connection, ITC clusters receive a variety of inputs from distinct brain regions. Golgi studies in the rat show innervations from the BLA PNs targeting the mITC (Millhouse, 1986). This excitatory input is highly topographically organised, meaning that an ITC neuron will be excited by an input localised at the same latero-medial level in the BLA (Royer et al., 1999). Stimulation of BLA afferents results in an NMDA-dependent long-term potentiation or depression (LTP; LTD) of ITCs synaptic response (Royer and Pare, 2002).

Both, lITC and mITC are embedded in cortical fibres (EC and IC). Electrical stimulation of the cortical efferent elicits EPSPs in the cells located in the mITC and lITC cluster, large enough to fire APs, followed by IPSPs (Marowsky et al., 2005).

Furthermore, the combination of anatomical and physiological data shows that cells within the ITC cluster contact the distal parts of PNs within the BLA (Marowsky et al., 2005). Crucially, they convey the majority of cortical feedforward inhibition as compared to the interneurons of the BLA (Marowsky et al., 2005).

The infralimbic prefrontal cortex (IL) provides the strongest glutamatergic innervation of the ITCs, specifically onto dendritic spines (McDonald and Mascagni, 1996a; Vertes, 2004; Pinto and Sesack, 2008). The fibres density is highest in the mITC and less abundant in the lITC and Im (McDonald and Mascagni, 1996a; McDonald, 1998). This input appears to be particularly important based on the observations that activity within the IL cortex is required for the recall of fear extinction (Milad and Quirk, 2002; Quirk et al., 2003).

Contextual information during fear conditioning and extinction is processed in the hippocampus. The rostral ventral subiculum and the CA1 hippocampal region are the source of glutamatergic afferent projections to the ITCs (LeDoux et al., 1990; Canteras and Swanson, 1992; McDonald, 1998).

Intercalated cells also receive dense dopaminergic (DAergic) afferents from the ventral tegmental area and substantia nigra pars compacta (Cho and Fudge, 2010; de la Mora et al., 2010). Axon terminals form symmetrical synapses on dendrites and spines of mITCs (Asan, 1997). In contrast, to the interneurons of the BLA that express D2-R, ITCs express a high level of D1-R (Asan, 1998; Fuxe et al., 2003). This innervation is likely to have strong behavioural significance as DA levels are increased during stress (Inglis and Moghaddam, 1999; Yokoyama et al., 2005) and enhance amygdala related behaviour (Borowski and Kokkinidis, 1998). Application of D1-R agonist produces fear enhancement during Pavlovian conditioning (Borowski and Kokkinidis, 1998). Importantly, DA depolarises BLA interneurons, and on the other hand hyperpolarizes ITCs that can result in a disinhibition of the amygdala (Kroner et al., 2005; Marowsky et al., 2005). For that reason, ITC are

likely to mediate anxiety-like behaviours during elevated DA states (Marowsky et al., 2005).

Other brain regions known to target ITCs are: the rostral portion of the posterior thalamus (LeDoux et al., 1990), the paraventricular and paratenial thalamic nuclei (Vertes and Hoover, 2008), the ventral medulla (Roder and Ciriello, 1993), the periamygdaloid cortex, the anterior subdivision of the basomedial nucleus, gustatory and visceral portion of the dysgranular cortex (McDonald, 1998).

Unfortunately, considerably less information is available on the ITC efferent connectivity. The first established and most studied connection from the mITC projects to the CE nucleus (Pare and Smith, 1993b; Pitkanen and Amaral, 1994). This deep-rooted model that the mITC predominantly target the CE nucleus requires careful consideration in light of latest discoveries. Reconstruction of 51 single filled cells and analysis of their axonal projections led to the identification of three subclasses within mITC neurons: medial capsular-projecting (MCp-mITC); sublenticular-projecting (SLp-mITC) and CE-projecting neurons (Cp-mITC) (Busti et al., 2011). Similar pattern could be observed in other studies that reconstructed the anatomy of single mITC (Geracitano et al., 2007; Amir et al., 2011). Within the amygdala, ITC neurons are the source of GABAergic projections also to the medial part of the amygdala, however no studies established a physiological and/or behavioural importance of that connection (Pare and Smith, 1993b; Pitkanen and Amaral, 1994).

Furthermore, in the retrograde-tracing experiments injections were made in several brain areas known to receive projections from the amygdala. Combination of retrograde- and anterograde-tracing experiments shows a massive connectivity with the basal forebrain specifically with the entire extent of the substantia innominata and horizontal segment of the diagonal band of Broca (Smith and Pare, 1994). Importantly, EM observations point to the possibility that ITCs target cholinergic

neurons within the basal forebrain; however a direct proof is still missing (Smith and Pare, 1994).

No retrograde labelling was seen after injections in cortical areas, this includes: perirhinal, entorhinal, insular, infralimbic, prelimbic and retrosplenial cortex as well as hippocampal formation, subiculum and presubiculum (Smith and Pare, 1994). Similar lack of projections from ITCs was seen in the thalamus, caudate nucleus, nucleus accumbens, stria terminalis and the anterior commissure (Smith and Pare, 1994).

1.2.4. Intercalated cells as a key neuronal component of fear extinction

Selective lesion of the mITCs provided direct evidence that these cells are required for the expression of fear extinction (Likhtik et al., 2008). The high level of expression and the postsynaptic location of μ ORs allowed the chemical lesion of these neurons by using a peptide-toxin conjugate that target selectively mITCs (Likhtik et al., 2008). The injection of the toxin was performed after fear conditioning followed by an extinction training and resulted in impaired extinction recall (Likhtik et al., 2008). Importantly, the same treated group of mice showed normal subsequent fear conditioning in a novel context (Likhtik et al., 2008). As pointed before, neurons within the mITC receive the CS information from the BLA and send projections to the CE (Royer et al., 1999; Marowsky et al., 2005; Busti et al., 2011). The common interpretation of these results is that extinction training potentiates the BLA input to the mITCs that in turn results in more feedforward inhibition to the CEm leading to a reduction in the fear response (Amano et al., 2010).

Stages of extinction (acquisition, consolidation, retrieval) depend not only on the amygdala function but also on the activity of the medial prefrontal cortex and hippocampus (Quirk and Mueller, 2008). Sufficient depolarization of ITCs by IL activation (Milad and Quirk, 2002; Amano et al., 2010) can lead to a sustained firing in response to a suprathreshold stimulation. If the activity of the IL cortex strongly

depolarizes the mITC, in turn they can provide a strong inhibition to the CE nucleus and dampen fear response. In light of a latest report, it is possible, but technically challenging, to identify ITC *in vivo* and therefore to test the involvement of these neurons in behavioural experiments (Amir et al., 2011).

Several behavioural tests (open field, elevated plus maze and light-dark test) showed an anxiolytic effect of the endogenous neuropeptide S (NPS) in the amygdala (Xu et al., 2004; Jungling et al., 2008). Although, NPS-Rs are not present in the ITCs, this neuropeptide exclusively modulates synaptic transmission between BLA PNs and mITC, and not from the lITC or intra-BLA synapses (Jungling et al., 2008). Application of NPS increases monosynaptic glutamate synaptic transmission via presynaptic receptors located at the PNs of the BLA (Jungling et al., 2008). These data suggest the possibility of a target specific modulation of presynaptic release or of the existence of functional subtypes of PNs in the BLA that target the ITCs. It is reasonable to propose that NPS enhances feedforward inhibition on the neurons of the CE nucleus resulting in an inhibition of the fear response. In line with this interpretation and with studies detailed above, fear conditioning pre-application of NPS to the BLA decreases conditioned fear response but not fear learning (Jungling et al., 2008). However it is important to keep in mind that fear extinction has a faster time course upon NPS application (Jungling et al., 2008).

Latest studies evaluate the expression of immediate early gene expression in mice that were subject to the fear conditioning protocol (Busti et al., 2011). The results reveal the complexity of the ITC system activation during fear acquisition, extinction training and retrieval. After fear conditioning only the mITCs show an increased activation. Fear extinction training requires the activation of both mITC and Im. Furthermore, extinction retrieval selectively increases the activation of Im neurons (Busti et al., 2011).

Altogether, multidisciplinary studies provide information on the role of the ITCs in shaping amygdala functions. However details of those mechanisms, specifically their spatial segregation and temporal sequence remain to be investigated.

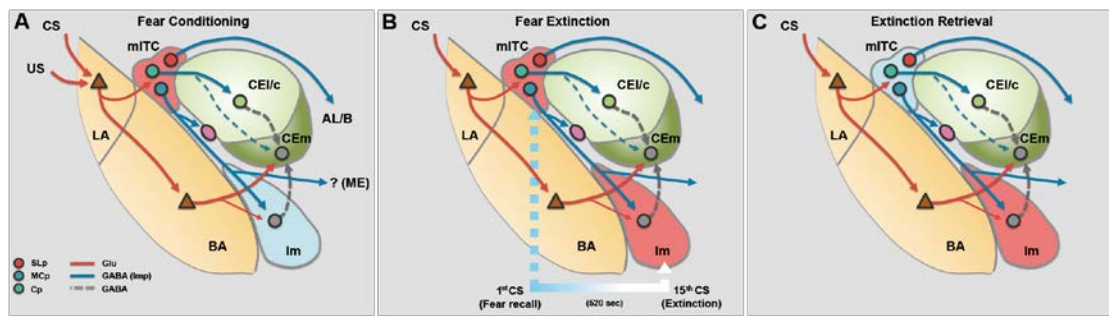


Figure 1.4. ITC neurons mediate multiple inhibitory circuits in the amygdala during fear conditioning.

A, three distinct classes of ITC neurons have been observed in the mITC: (1) MCP-mITC (blue circle); (2) Cp-mITC (green circle); (3) SLp-mITC (red circle). A, during fear conditioning, the selective activation (shown as the cluster coloured in red) of the mITC by the LA nucleus after US-CS pairing. B, during fear extinction training, the initial presentation of CS (light blue dashed arrow) produces fear recall and activation of mITC. Persistent presentation of CS alone (white arrow) leads to the activation of Im. C, during extinction retrieval only Im nucleus remains active. (Busti et al., 2011)

1.3. GABAergic synaptic transmission

The most abundant transmitter in the vertebrate nervous system is γ -aminobutyric acid (GABA) that activates GABA-R and leads to several classes of inhibitory postsynaptic currents (Pearce, 1993; Hajos and Mody, 1997). The complexity and variety of inhibitory transmission arise from multiple factors such as: the diversity of GABAergic cell types; the concentration and temporal presence of GABA at effective sites; the multiple GABA-R subtypes, their location, function and modulation.

For clarity, I will open this chapter with a brief summary of the knowledge on GABAergic transmission. In the following parts I aim to describe a form of a slow, phasic, GABA_AR-mediated synaptic transmission, its specific physiology, pharmacology, function and cellular components.

1.3.1. Basis of transmission at inhibitory synapses

Inhibitory transmission regulates neuronal excitability in the nervous system. In vertebrates GABA acts at inhibitory synapses by binding to specific receptors. This amino acid is synthesised by the decarboxylation of glutamate facilitated by one of two types of enzyme called glutamic acid decarboxylase (GAD65 or GAD67). It is released from vesicles after the arrival of the AP at the axon terminal, followed by an influx of Ca^{2+} that initiates exocytosis. The inactivation of the neurotransmitter is achieved by re-uptake mechanisms that involve four types of transporters (GAT1 to 4) located on neurons and glia cells (Schousboe, 2000). There are two classes of GABA receptors: ionotropic (termed GABA_A and GABA_C) and metabotropic (called GABA_B). They are both widely present in the CNS (Bormann and Feigenspan, 1995).

1.3.2. Physiology, location and pharmacology of GABA_A receptors

GABA_AR are pentameric proteins that form an ion channel permeable to Cl⁻ and bicarbonate ions (Farrant and Kaila, 2007). Currently, 16 isoforms of mammalian receptors subunits have been cloned: α_{1-6} , β_{1-3} , γ_{1-3} , δ , ϵ , π and θ (Farrant and Nusser, 2005). All known subunits are similar in size (450-550 amino acids), are strongly conserved among species and have a common structure. They are composed of extracellular, a N-terminal hydrophilic domain and four hydrophobic 20 amino acids segments crossing the plasma membrane (named M1-4). The diversity of subunits gives the possibility of many combinations, however, only several of them exist in the CNS. The common receptor consists of two α_{2-6} , two β_{1-3} and γ_2 subunit (sometimes replaced by δ , ϵ or π ; (Tretter et al., 1997)).

The physiological role of GABA_AR is mainly determined by its subunit composition, cellular localisation and spatio-temporal profile of GABA (Farrant and Nusser, 2005; Barberis et al., 2011). Below, I will address some examples that support this statement.

The molecular heterogeneity of GABA_AR determine cell surface distribution, gating properties and dynamic regulation of the receptors (Luscher and Keller, 2004). The receptors can be found on the synaptic and the extrasynaptic sites. The synaptic receptors usually contain α_1 , α_2 , α_3 , α_6 , $\beta_{2/3}$ and γ_2 subunits. The clustering of GABA_ARs at the postsynaptic site is dependent on the presence of the γ_2 subunit. Transgenic mice lacking the γ_2 subunit fails to accumulate GABA_ARs during development accompanied by a loss of gephyrin and normal synaptic function (Essrich et al., 1998). It is clear that some subunits are present predominantly outside synapses and that their number may be higher than the synaptic one

(Nusser, 1995). Two subunits, δ and α_5 , have been usually found at extrasynaptic sites (Nusser et al., 1998; Brunig et al., 2002; Wei et al., 2003).

Expression of GABA_AR containing specific subunits may be attributed to synaptic specificity. Such heterogeneity can be seen among synapses formed by various types of hippocampal interneurons. For example, axo-axonic cells express receptors containing α_1 and α_2 subunits (Somogyi et al., 1996), PV+ basket cells express receptors containing mostly the α_1 subunit (Klausberger et al., 2002), CCK+ basket cells express receptors containing mostly the α_2 subunit (Nyiri et al., 2001), and bistratified cells contain the α_5 subunit (Pawelzik et al., 1999).

It is crucial to study the subunit composition of GABA_AR as it affects affinity, efficacy and desensitisation kinetics upon exposure to GABA. The rank of sensitivity to GABA varies between the subunits: $\alpha_6 < \alpha_1 < \alpha_2 < \alpha_4 < \alpha_5 < \alpha_3$ (Bohme et al., 2004). Moreover replacing γ_2 with δ subunit results in 5-fold change of the mean opening of the receptors (Fisher and Macdonald, 1997). In addition, GABA_A receptors containing the δ subunit desensitise less than receptors expressing the γ_2 subunit (Haas and Macdonald, 1999; Bianchi et al., 2002).

The subunit composition of GABA_AR allows also selective drug modulation. The most studied class of modulatory agents are known as benzodiazepines (Sieghart and Sperk, 2002). One of them, diazepam has been shown to have several effects depending on the subunit composition of receptor. Consequently, this drug can be used as sedative (Rudolph and Mohler, 2004), anxiolytic (Low et al., 2000) or muscle relaxant (Crestani et al., 2001).

The activation of the postsynaptic receptors can have two effects on the postsynaptic membrane: a change in the membrane potential or the so-called 'shunting effect'. The direction of the current generated through the opening of the postsynaptic receptors (and thus hyper- or de-polarization of the membrane) depends on the difference between V_m and E (reversal potential of an ion, dependent on the concentration difference between intra- and extra-cellular space). In the adult brain, due to the decreased level of intracellular Cl^- compared to the extracellular compartment, maintained by K^+-Cl^- co-transporter, the opening of the channels results in a Cl^- influx and hyperpolarisation of the plasma membrane (Rivera et al., 1999). On the contrary, in the developing brain, the level of intracellular Cl^- is high as compared to the extracellular compartment, maintained by $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ co-transporter; therefore the opening of the channels results in a Cl^- efflux and depolarisation of the plasma membrane (Ben-Ari, 2002).

A shunting effect is a result of the increase in membrane conductance (or decrease in resistance as $R_m = 1/\gamma$; R_m - membrane resistance; γ - membrane conductance) as ions flow through open GABA_ARs . It can result in a smaller voltage change of the membrane potential, in agreement with Ohm's Law: $V = I \cdot R$ (V - voltage; I - current) (Staley and Mody, 1992).

Classically, there are two types of GABA_AR -mediated currents: phasic and tonic.

Phasic current is a result of the brief exposure to a high concentration of the neurotransmitter. After exocytosis of GABA from presynaptic vesicles, the neurotransmitter is transiently present (~ 0.5 ms) at the synaptic cleft and then reaches the postsynaptic receptors with a peak concentration of a millimolar range (Mody et al., 1994).

The tonic current is the result of the prolonged exposure to a low concentration of the neurotransmitter. The persistent activation of the GABA_AR results in an increase

in the cell conductance, therefore affecting the voltage change to a current injection (Farrant and Nusser, 2005).

Both types of currents produce the general inhibition of the neuronal network. However, differences between phasic and tonic inhibition reflects their specialised role in shaping network activity. Phasic inhibition is well suited to generate rhythmic brain activity (Klausberger and Somogyi, 2008) or synaptic integration (Pouille and Scanziani, 2001), whereas tonic inhibition decreases the time window of signal integration (Farrant and Nusser, 2005).

1.3.3. GABA_B receptors: diversity and function

The GABA_BR heteromeric assembly is formed by two subunits, B1 (isoform a or b) and B2. The co-expression of both subunits is required to activate slow postsynaptic currents *via* Kir3-type K⁺ channels (Kaupmann et al., 1998; Fritschy et al., 1999). The expression of GABA_{B1a} and GABA_{B1b} subunits is cell type-dependent and associated to different sub-cellular compartments and therefore may mediate different functions (Vigot et al., 2006; Ulrich and Bettler, 2007). The effect of GABA_BR activation is mediated through G_{βγ} subunit of the G-protein (Ulrich and Bettler, 2007). Moreover, GABA_BR inhibits adenylyl cyclase, but the details of this pathway activation are poorly understood (Ulrich and Bettler, 2007). The GABA_BR are dominantly expressed at extra-synaptic sites therefore their activation requires the spillover of GABA classically resulting from simultaneous activation of many inhibitory cells (Scanziani, 2000; Kulik et al., 2003). The neurogliaform cells (described in chapter 1.5.2.) are the only interneuron type that can activate GABA_BR-mediated conductance following unitary GABA release (Tamas et al., 2003; Price et al., 2005).

The activation of GABA_BR has extremely diverse effects on the network. The consequences of this receptor activity depend on its location (pre- or post- synaptic)

and the terminal type (inhibitory or excitatory) (Ulrich and Bettler, 2007). Postsynaptic GABA_BR are inducing slow inhibitory synaptic potentials by gating Kir3-type K⁺ channels (Luscher et al., 1997) that are located in their vicinity (Kulik et al., 2006), or, as recently shown, can inhibit Kir2 channels, leading to excitation rather than inhibition (Rossi et al., 2006). Presynaptic receptors control the release of GABA and other neurotransmitters, by inhibiting voltage-sensitive Ca²⁺ channels or through a direct modulation of synaptic vesicle priming (Scanziani et al., 1992; Sakaba and Neher, 2003). Presynaptic GABA_BR present on glutamatergic terminals are often referred as heteroreceptors, their activation having inhibitory effect on the postsynaptic neuron. Presynaptic GABA_BR present on GABAergic terminals are often referred as autoreceptors and their activation have a dis-inhibitory effect on the postsynaptic neurons (Ulrich and Bettler, 2007). The activation of auto- or heteroreceptors may therefore have opposite effect on LTP induction (Davies et al., 1991; Davies and Collingridge, 1996).

1.4. Volume transmission

There are two types of communication within the brain. Some suggest referring to them as 'wiring' and 'volume transmission' (Agnati et al., 2010). The wiring transmission occurs through well defined anatomical structures such as synapses, gap junctions and membrane juxtapositions. On the contrary, volume transmission (or extrasynaptic transmission) occurs through signal diffusion in the extracellular space of the brain tissue (Agnati et al., 2010). To categorise a signal as volume transmission, it must match several criteria: (1) be released by cells of the nervous system; (2) diffuse at efficient concentration at distances greater than 20 nm; (3) selectively activate receptors in a number of targets and trigger physiological responses (Agnati et al., 2010). There are two main findings confirming the possibility of volume transmission in the brain. First is the identification of three types of release sites: synaptic, non-synaptic and para-synaptic (Golding, 1994). Second is a phenomenon called 'transmitter-receptor mismatch', which describes that in many cases there is no tight spatial correlation between transmitter release and its receptor expression site (Agnati et al., 2010). Many receptors are found in extrasynaptic locations. Examples can be found among peptidergic (Liu et al., 1994) and monoaminergic receptors (Aoki et al., 1987). Classically, peptidergic systems are considered to operate *via* volume transmission. Vesicles with neuropeptides are located at parasynaptic or non-synaptic positions, they do not have specific re-uptake systems thus they can be present in extracellular space for a long time and bind to a high-affinity G-coupled receptors (Agnati et al., 2010). Furthermore, peptidergic transmission is characterised by a clear transmitter-receptor mismatch, demonstrated by many examples such as the NPY neuronal system (Zoli et al., 1989). Another example of volume transmission is represented by the nitric oxide signalling (Garthwaite and Boulton, 1995). An additional example is the 'synaptic

cross-talk' which requires the diffusion of the neurotransmitter from the synaptic cleft (called synaptic spillover) to extrasynaptic receptors at neighbouring synapses (Kullmann and Asztely, 1998). Volume transmission is thus a necessary and widespread element of the communication between elements of brain circuits.

1.5. GABA_{A,slow}: a form of slow phasic inhibition

1.5.1. The discovery of GABA_{A,slow}

Classically, two types of GABA_AR-mediated forms of inhibition are recognized, a fast phasic and a continuous tonic inhibition (Farrant and Nusser, 2005). Recently, a novel form of phasic inhibitory current has been described. It is characterised by very slow kinetics with a rise time longer than 3 ms and a decay time longer than 30 ms, hence it was termed GABA_{A,slow} (Pearce, 1993; Capogna and Pearce, 2011). It was first described in the hippocampus and since then it received increasing attention (Pearce, 1993; Banks et al., 1998; Price et al., 2005; Price et al., 2008; Karayannis et al., 2010; Armstrong et al., 2011; Markwardt et al., 2011). The first observation of a slow GABA_AR-mediated synaptic response was reported by Robert Pearce in hippocampal CA1 PNs (Pearce, 1993). Somatic recordings during electrical stimulation of the stratum radiatum (SR) revealed IPSCs with a bi-phasic component of the decay time, fast and slow (τ fast = 3-8 ms; τ slow = 30-100 ms) (Pearce, 1993). The presence of a slow component was correlated with the position of the stimulating electrode far from the recording site (in SR and stratum lacunosum-moleculare; SL-M). Both currents were abolished by GABA_AR antagonist and reversed at the same potential (Pearce, 1993). Subsequent experiments, pointed to the possibility that both components were mediated by synapses located in distinct anatomical sites, with GABA_{A,slow} occurring at dendrites and GABA_{A,fast} at/nearby the cell body (Pearce, 1993). The presence of the two types of IPSC kinetics can be also noticed during the recordings of spontaneous and miniature events (sIPSC and mIPSC, respectively) (Banks et al., 1998). Slow AP-dependent spontaneous events occur with much lower frequency than the fast ones (4 orders of magnitude), perhaps indicating that they are mediated by interneurons with low intrinsic firing rate (Banks et al., 1998). In addition, the occurrence of action

potential-independent slow mIPSCs argues against a role of presynaptic firing properties, such as bursting in interneurons, as a major determinant of GABA_{A,slow} time course (Banks et al., 1998). Instead, additional factors such as lower release probability, smaller synaptic vesicle releasable pool, less presynaptic calcium sensitivity could make the occurrence of slow mIPSCs less frequent than that of fast mIPSCs. A lower number of releasing sites in the cells eliciting slow mIPSCs in principle could be an additional factor but it is against experimental evidence (Olah et al., 2009). Over the last two decades other differences between slow and fast IPSCs were noticed: (1) different sensitivity to the GABA transporter antagonist (Banks et al., 1998; Szabadics et al., 2007) (2) and GABA_B receptor ligands (Banks et al., 1998), and (3) different paired pulse ratios.

Afterwards, slow IPSCs were found in other brain areas such as: neocortex (Tamas et al., 2003; Olah et al., 2007; Szabadics et al., 2007; Olah et al., 2009; Wozny and Williams, 2011; Suzuki and Bekkers, 2012); striatum (Ibanez-Sandoval et al., 2011; English et al., 2012); thalamus (Huntsman et al., 1999; Schofield and Huguenard, 2007); cerebellum (Crowley et al., 2009); and brainstem (Kuo et al., 2009).

1.5.2. Cellular source of GABA_{A,slow} events

Based on the characteristic of slow currents described above, later studies aimed to demonstrate their origin from specific cell types. A first and well-described type of neuron giving rise to slow IPSCs is the so-called neurogliaform cell (NGFC) of the neocortex (Tamas et al., 2003; Olah et al., 2007; Szabadics et al., 2007; Olah et al., 2009). Among the variety of hippocampal interneurons (Klausberger and Somogyi, 2008), two cell types were clearly shown to give rise to the GABA_{A,slow} (Capogna, 2011). The first discovered cell type was the NGFC, consistent with the data in the neocortex, with cell body and dendrites in SL-M and axon spanning over distal dendrites of hippocampal PNs (Price et al., 2005). The second type of interneuron found to mediate slow IPSCs is called Ivy cell, with the soma closer to the SP,

dendrites in the SO and SR and axon spanning over the dendrites of PNs (Fuentelba et al., 2008). Despite their different inputs, localisation and slightly different molecular markers, the two interneuron cell types share a broad range of similarities. Both NGFC and Ivy cell types display a main axon branching close to its origin and give rise to a very dense and fine axonal arbour; furthermore typically 4 to 6 primary dendrites are arranged around the cell body (Khazipov et al., 1995; Vida et al., 1998; Price et al., 2005; Fuentelba et al., 2008; Olah et al., 2009). The axons have small boutons containing a small vesicles and infrequent mitochondria and can be found away from synaptic specialisations (1-5 μm away from dendrites as compared to the 10-20 nm of synaptic cleft) (Tamas et al., 2003; Price et al., 2005; Fuentelba et al., 2008; Olah et al., 2009; Fuentelba et al., 2010). They target the dendrites of principal cells and other interneurons including members of NGFC/Ivy cell family (Tamas et al., 2003; Price et al., 2005; Fuentelba et al., 2008) and themselves through autaptic connections (Karayannis et al., 2010). Furthermore, cells recorded *in vitro* can be characterised by a delayed or stuttering firing pattern in response to the depolarising current steps, often with proceeding depolarisation ramp, broad AP and frequency adaptation (Price et al., 2005; Fuentelba et al., 2008; Fuentelba et al., 2010). In addition, only NGFCs recorded from monkey and human tissue exhibit a sag during hyperpolarizing current injections (Olah et al., 2007; Povysheva et al., 2007). Similar to other types of interneurons they can be identified by a set of specific neuronal markers (Freund and Buzsaki, 1996). Ivy and NGFC of hippocampus co-localise NPY with neuronal nitric oxide synthase (nNOS) along with α -actinin2 (a cytoskeleton protein involved in cell signalling, 75% co-localisation with NPY+nNOS+ cells), the transcription factor COUP-TFII, GABA_AR α 1 (Price et al., 2005; Fuentelba et al., 2008; Fuentelba et al., 2010) and δ subunits. In addition the extracellular matrix protein reelin can be found in NGFCs but not in Ivy cells (Fuentelba et al., 2008). Only a very weak co-localisation, less

than 5%, can be detected between nNOS+/NPY+ cells and CB, CR or SOM (Fuatealba et al., 2008). Nevertheless, there is some variability in the marker expression by some cells that can reflect their developmental origin. For example, nNOS+ NGFCs derived from the medial ganglionic eminence and represent the majority of the cells within this group, while nNOS+ NGFCs derive from the caudal ganglionic eminence (Tricoire et al., 2010). The differences in molecule expression within the NGFCs family (specifically reelin and nNOS) and the exact location in the hippocampal layers, appear to have no influence on cells inhibition by μ OR agonist (Krook-Magnuson et al., 2011), suggesting that this neuromodulator target all of them.

Importantly, cells co-expressing nNOS and NPY form the largest interneuron population in the hippocampus (37% of NPY+/nNOS+ vs. 26% of PV+ vs. 15% of PV+/NPY+ neurons) (Fuatealba et al., 2008). In addition, Ivy cells axon length is significantly bigger than bistratified cells (Fuatealba et al., 2008) and it has been estimated that the releasing site of one neocortical NGFC correspond to 6 basket cells (Olah et al., 2009). This result points out to the abundance and hence importance of slow inhibitory signalling within various brain areas.

As outlined in the previous chapters, their profuse presence within hippocampus is coupled with low frequency of in vitro recorded $\text{GABA}_{\text{A,slow}}$ spontaneous currents. Regarding sIPSCs, their low frequency could be mainly due to the low spontaneous firing activity of NGFCs and Ivy cells that generate these slow events. In vivo recordings of identified CA1 NGFCs and Ivy cells under anaesthesia show relatively low discharge rates (2-10Hz and 2.5Hz, respectively) (Fuatealba et al., 2008). This has been confirmed for Ivy cells in drug-free and freely-moving rats (Lapray et al., 2012). The role of $\text{GABA}_{\text{A,slow}}$ events within the neuronal circuit in relation to their discharge rate is discussed further (Chapter 1.5.4.). As explained above additional mechanisms are likely to cause the low frequency of occurrence of mIPSCs.

Interestingly, NGFCs have recently been observed to originate GABA_{A,slow} events in several other brain regions. They include: the dentate gyrus (Armstrong et al., 2011), the piriform cortex (Suzuki and Bekkers, 2012); the striatum (Ibanez-Sandoval et al., 2011) and the habenula of the thalamus (Weiss and Veh, 2011).

From functional point of view, paired recordings between NGFCs and their postsynaptic targets revealed that NGFCs evoke a biphasic current that consists of an early GABA_AR-mediated component followed by a GABA_BR-mediated one (Tamas et al., 2003; Price et al., 2005; Szabadics et al., 2007; Armstrong et al., 2011). Both components can be separated by distinct reversal potentials and pharmacology (Szabadics et al., 2007; Olah et al., 2009; Karayannis et al., 2010). Because of the slow kinetics, the charge transfer carried by IPSCs is four times higher than of fast spiking basket cells (Szabadics et al., 2007). In addition, the probability of connectivity between NGFCs and a postsynaptic cell is very high, a single action potential evokes a uIPSC with almost no failures and a small trial-to-trial peak amplitude variability (Szabadics et al., 2007; Karayannis et al., 2010). Moreover NGFCs form networks coupled also by electrical connections with other interneurons of the same and of other types (Simon et al., 2005; Olah et al., 2009; Karayannis et al., 2010; Armstrong et al., 2011). This widespread communication is likely to allow the cells to enter a state of persistent firing, described in chapter 1.5.4 (Krook-Magnuson et al., 2011).

1.5.3. The source of the slow kinetics

What are the mechanisms underlying the unusual slow kinetics of IPSCs? In theory, there could be several factors that influence the kinetics of IPSCs including the temporal lifetime and spatial distribution of GABA at the synaptic cleft and the properties of its target receptors. This issue was tested experimentally studying cortical and hippocampal NGFC-mediated IPSCs leading to the hypothesis that slow

kinetics of the IPSCs are the result of prolonged spatio-temporal profile of low-level GABA transient at non-classical synapses formed by axons of cells belonging to the NGFC family (Capogna and Pearce, 2011). Below, I will present several key observations supporting this hypothesis.

Due to the distal site of entry of slow IPSCs, the most straightforward explanation of its slow kinetics would be an imperfect space clamp. Since the site of the recordings was somatic, it seems likely that the signal with an initial fast kinetic was distorted by dendritic filtering. However a number of evidence show that this factor, although it exists, it is not the most important to account for the slowness of the IPSCs. Firstly, fast and slow currents can be distinguished by the voltage jump method. In this experiments, synaptic activation is performed when cells are initially held at the reversal potential of the tested current to avoid ion flow and following charge of the membrane that could disturb the recordings. After a range of time delays, the initial holding potential steps rapidly to a different potential. By subtracting the two resulting currents one can determine the effective synaptic current and its time course. The transient currents recorded in CA1 PNs at $V_h = -60$ mV and $V_h = -90$ mV with/without stimulation of SP/SL-M clearly show that $GABA_{A,slow}$ and $GABA_{A,fast}$ have distinct conductance courses (Pearce, 1993). Furthermore, NGFCs and Ivy cells belong to the dendrite-targeting subpopulation among others, such as bistratified or OLM cells, which do not give rise to $GABA_{A,slow}$ events (Buhl et al., 1994; Maccaferri et al., 2000). In addition, neocortical Martinotti cells form synapses on dendrites at a distance from soma greater than NGFCs, yet they evoke fast IPSCs (Szabadics et al., 2007). On the contrary, slow IPSCs are recorded also from other NGFCs that have short, therefore electrically compact dendrites that are minimally affected by dendritic filtering (Price et al., 2005). Together, it seems highly unlikely that dendritic filtering is a major determinant of $GABA_{A,slow}$.

Another possibility is that even a single action potential can initiate many asynchronous, fast events from a high number of releasing sites leading to apparent slow kinetics of IPSCs. Altering the extracellular Ca^{2+} , and thus release probability, have no effect on the kinetics of IPSCs and therefore cannot be the mechanism generating slow currents (Szabadics et al., 2007). An important observation from this experiment is that even small events (generated by one or few of release sites) have kinetics comparable to bigger event (presumably generated by multiple release sites) (Szabadics et al., 2007; Karayannis et al., 2010).

Furthermore, the slow kinetic of the IPSCs is not due to a lack of efficient GABA reuptake mechanism. Application of a blocker of GABA transporter 1 prolongs the decay time of slow currents (Banks and Pearce, 2000; Prenosil et al., 2006; Szabadics et al., 2007; Karayannis et al., 2010), showing that these plasma membrane proteins are present in physiological conditions. In addition, this class of drugs also enhances the GABA_B -mediated component of the current (Szabadics et al., 2007).

These data indicate that GABA released after one action potential evoked by a presynaptic NGFC, spillover to the extrasynaptic region and acts on GABA_B . Experimentally it was tested whether other high-affinity, extrasynaptic GABA_A Rs containing α_5 and δ subunits were also activated and therefore could shape the kinetics of the slow IPSCs (Nusser et al., 1998; Szabadics et al., 2007; Olah et al., 2009; Karayannis et al., 2010). Specific agonists and inverse agonists for these subunits had some moderate or minor effects on slow IPSCs, therefore excluding the activation of extrasynaptic receptors as the major factor shaping $\text{GABA}_{A,\text{slow}}$ events (Szabadics et al., 2007; Olah et al., 2009; Karayannis et al., 2010).

In addition, the contribution of several low-affinity GABA_A R subunits was tested (Szabadics et al., 2007; Karayannis et al., 2010). Zolpidem is a BZ-like compound

that preferentially acts on GABA_AR containing $\alpha 1$ subunit, but also on $\alpha 2/3$ subunits. It increases the receptor affinity for GABA and enhances the opening probability of channels in the presence of the transmitter (Perrais and Ropert, 1999). A similar drug to zolpidem, but with higher selectivity for the $\alpha 1$ subunit is called CI218872 (Korpi et al., 2002). Experimentally, $\alpha 1$ subunit agonists greatly enhance the amplitude of NGFC-slow IPSCs, indicating that GABA_AR receptors underlying this synaptic connection are not saturated (Szabadics et al., 2007; Karayannis et al., 2010). This result is therefore consistent with the low concentration of GABA suggested by experiments performed with the low affinity GABA_AR competitive antagonist TPMPA (Szabadics et al., 2007; Karayannis et al., 2010).

Furthermore, different contributions of α subunits to evoked and spontaneous synaptic inhibition in CA1 PNs was studied with pharmacological tools and knock-in mutant mice that carry diazepam-insensitive GABA_AR subtypes (mouse with single mutation in $\alpha 1$, $\alpha 2$, $\alpha 3$ gene, and double or triple mutants) (Prenosil et al., 2006). In the hippocampus slow synaptic inhibition is mediated by $\alpha 1$ - and $\alpha 2$ -GABA_ARs as well as by $\alpha 5$ -GABA_AR. In addition, the authors convincingly show that the same pool of $\alpha 5$ -containing GABA_AR are contributing to both slow sIPSCs and tonic current. Most likely $\alpha 5$ subunit acts as a 'detector' of low GABA concentration at extrasynaptic sites and therefore is not necessary to mediate slow sIPSCs. Indeed, slow sIPSCs are present in the subiculum, that do not express the $\alpha 5$ subunit, suggesting that GABA_AR subunit composition is not crucially involved in the generation of slow synaptic inhibitory events (Prenosil et al., 2006). Complementary studies with the use of knock-in mutant mice that carry diazepam-insensitive $\alpha 5$ -GABA_AR subtype, confirmed the contribution of that subunit in mediating slow but not fast GABA currents (Zarnowska et al., 2009). These data support the idea that the differences between 'fast' and 'slow' synapses are not a result of the GABA_AR composition. However, $\alpha 5$ -containing GABA_AR are involved in mediating fast phasic

IPSCs in the neocortex (Ali and Thomson, 2008). Authors examined the sensitivity of IPSCs arising from three types of interneurons to GABA_AR modulators. The current evoked by interneurons that predominantly target dendrites of PNs was mainly mediated by $\alpha 5$ -GABA_AR. On the contrary, IPSCs evoked by perisomatic targeting interneurons were mediated by $\alpha 1$ -GABA_AR or $\alpha 2/3$ -GABA_AR. It is an additional evidence that different α subunits are present at specific locations that are input dependent and correlate with the type of presynaptic interneuron (Nusser et al., 1996; Nyiri et al., 2001; Ali and Thomson, 2008). Moreover, activity pattern-dependent feed-forward activation of $\alpha 5$ -GABA_AR in CA1 can be detected following the stimulation of Schaffer collaterals (Vargas-Caballero et al., 2010).

Furthermore, since the β_3 subunit is present in dendritic regions of hippocampus it was of interest to test its contribution to GABA_{A,slow} events. To determine the role of these receptors, spontaneous and evoked inhibitory currents were recorded in wild type and receptor β_3 subunit-deficient mice ($\beta^{-/-}$) (Hentschke et al., 2009). The rate of occurrence of slow, but not fast spontaneous IPSCs in CA1 pyramidal neurons of $\beta^{-/-}$ mice was highly decreased as compared to control animals. In addition, evoked GABA_{A,slow} currents showed a decrease in amplitude indicating that GABA_AR-containing β_3 subunit significantly, but not completely mediate slow currents (Hentschke et al., 2009). Multi-site field potential recordings from CA1, of both genotype in freely-moving mice, showed slower and weaker theta oscillation, and weaker but faster gamma oscillation in mutant as compared to wild type animals (Hentschke et al., 2009). This result demonstrates the role of slow inhibition in the generation and coordination of hippocampal network rhythms. In agreement with the studies mentioned above, the lack of β_3 subunit appears to induce changes in the oscillatory synchrony and in the power in the olfactory bulb (Nusser et al., 2001b) and thalamus (Huntsman et al., 1999).

Nevertheless, specific kinetics of IPSCs have a unique role in the brain microcircuits, some of the current hypotheses are presented below.

1.5.4. Role of GABA_{A, slow} events in neuronal circuits

When discussing the function of cells of the NGFCs family, we have to consider their position within the circuit, especially the diversity of inputs they are likely to receive. Information is available on the circuit impinging on and targeted by hippocampal NGFCs and Ivy cells of the CA1 (Price et al., 2005; Fuentealba et al., 2008; Price et al., 2008; Fuentealba et al., 2010). The NGFCs of hippocampus receive inputs from CA3 Schaffer collaterals (CA3 SC) and the temporo-ammonic pathway (Price et al., 2005; Fuentealba et al., 2010), and are innervated by O-LM cells (Elfant et al., 2008). They provide feedforward inhibition to the distal dendrites of PNs (Price et al., 2005; Fuentealba et al., 2010). On the contrary, Ivy cells soma reside around SP and proximal dendrites of CA1 pyramidal cells (in SR), and their inputs consist primary of PNs of CA1 and some CA3 SC (Fuentealba et al., 2008; Somogyi et al., 2012). They therefore mediate feedforward and feedback inhibition onto distal and oblique dendrites of PNs (Fuentealba et al., 2008).

In vivo recordings of CA1 NGFCs under anaesthesia have shown their low frequency discharge rate (2-10 Hz), with a clear preference to fire close to the peak of the theta cycle (3-6 Hz, under anaesthesia) (Fuentealba et al., 2010). This coincides with the synaptic volley from entorhinal cortex (Buzsaki, 2002), and depolarisation of the distal dendrites of PNs. They are mostly silent during sharp wave associated ripple oscillations (90-140 Hz, under anaesthesia) and phase coupled to the local gamma oscillations (30-80 Hz) (Fuentealba et al., 2010). NGFCs are thought to be essential in the hippocampal theta generation (Capogna and Pearce, 2011). Similarly, slow inhibitory currents within the thalamus are essential for network oscillations (Huntsman et al., 1999).

On the contrary to NGFCs, *in vivo* recorded Ivy cells show a clear preference to fire after the trough of the theta cycle (Fuentelba et al., 2010) which coincides with the synaptic volley from CA3 (Buzsaki, 2002). Similar to NGFCs, they are mostly silent during sharp wave associated ripple oscillations and phase coupled to the local gamma oscillations (Fuentelba et al., 2010). Ivy cells are characterized as slow-spiking GABAergic interneurons. They differ from fast-spiking interneurons by their lack of Kv3.1b subunit expression, which most likely contributes to the firing patterns of PV+ cells (Martina et al., 1998; Fuentelba et al., 2008). In anaesthetised animals, the Ivy cells can be distinguished from pyramidal cells by lack of the complex spikes burst (Fuentelba et al., 2008). They discharge low frequency spikes (~2.5 Hz) across all brain states. This observation was recently confirmed by recording and labelling single Ivy cells in drug-free and freely-moving rats (Lapray et al., 2012). Importantly, the firing rate of Ivy cells showed a clear relationship with the frequency and the amplitude of the theta oscillations, an acceleration of theta together with an increase in the cells' firing (Lapray et al., 2012). It is therefore proposed that Ivy cells are well suited to maintain homeostasis in the network. It is important to mention that population of NGFCs are under the control of opioid modulation (Krook-Magnuson et al., 2011). Specifically, these cells are hyperpolarized by the activation of μ OR and such modulation can therefore influence all hippocampal network, for example facilitate long term potentiation (Krook-Magnuson et al., 2011).

Both Ivy cells and NGFCs can enter a state of continued firing in the absence of input, so-called persistent firing (Krook-Magnuson et al., 2011). The role of this recently described phenomenon is still under debate. However it is worth to mention that 80% of NGFCs family as compared to 20% of other interneurons can display persistent firing. In addition, μ ORs activation can prevent entering to a state of persistent activity (Krook-Magnuson et al., 2011). Partially, it may be a result of a high connectivity among NGFCs family members *via* gap junctions. In addition, gap-

junctions connectivity may be an important component allowing the synchronisation of the group of target network or act as low-pass filters of incoming inputs (Price et al., 2005; Zsiros et al., 2007; Karayannis et al., 2010; Krook-Magnuson et al., 2011). Recently described NGFCs in the dentate gyrus molecular layer receive prefrontal path input from EC, which is facilitating at high frequencies (Armstrong et al., 2011). It suggests that NGFCs may be the source of feedforward inhibition during high frequency activity (Armstrong et al., 2011).

An interesting microcircuit mechanism was recently described in neostriatum (English et al., 2012). Optogenetic dissection of the circuit revealed that cholinergic interneurons of the neostriatum activate NGFCs that in turn exert a powerful inhibition over spiny projection neurons; this phenomenon has been observed *in vivo* and *in vitro*. This allows cholinergic interneurons to control projection neurons and it may underlie reinforcement-related information (English et al., 2012).

During adult neurogenesis, NGFCs/Ivy cells provide the first source of input to new born dentate gyrus granule cells (NGCs) (Markwardt et al., 2011). The synaptic responses detected in NGCs have characteristic comparable to the slow IPSCs detected in mature neurons of hippocampus (Markwardt et al., 2009). Specifically, (1) GABA transporter inhibitor increased the amplitude and prolonged the decay time of slow IPSCs; (2) decrease in GABA mobility, by addition of dextran, increased the amplitude of slow currents; (3) slow, but not fast currents were partially blocked upon application of low-affinity competitive antagonist, and their decay time was prolonged, together indicating lower concentration and prolonged presence of GABA at the synaptic cleft of NGCs; (4) reducing release probability did not affect the kinetics of slow IPSCs (Markwardt et al., 2009). Moreover, NGFCs/Ivy cells are highly connected to other interneurons in adult hippocampal network, therefore their coordinated activation can lead to disinhibition of mature GCs and synchronise all elements of network (Markwardt et al., 2011).

In summary, the importance and variability of the functions attributed to the cells of the NGFCs family is clearly demonstrated by *in vivo* and *in vitro* studies.

1.6. Autapse

The term autapse was proposed by van der Loos and Glaser to describe a synapse formed by the axon of a neuron onto its own soma or dendrites (Van der Loos and Glaser, 1972). In mammals, the occurrence of autapses was proposed after light microscopy observations of apparent self-innervation of neurons in many brain regions such as neocortex, hippocampus, cerebellum (Van der Loos and Glaser, 1972). Later studies using EM confirmed the synaptic nature of autapses and showed their common appearance. Examples of autaptic innervation, confirmed by EM, can be found in hippocampal basket cell (Cobb et al., 1997), stellate cell (Peters and Proskauer, 1980), and cortical fast spiking interneuron (Thomson et al., 1996) or layer V pyramidal cells (Lubke et al., 1996). Detailed investigation of the cat visual cortex (layer II-V) showed very rare self-innervation of pyramidal cells and a high presence of autapses among interneurons (Tamas et al., 1997). Furthermore, the degree of self-innervation appear to be cell type specific. Examined basket cells and dendrite-targeting cells establish on average 12 and 22 autapses respectively, while only one double-bouquet cell was found to establish 3 autapses (Tamas et al., 1997). In addition, the location of the autaptic synapse was similar to the postsynaptic target domain of the innervated postsynaptic neuron (Tamas et al., 1997). Autaptic currents were also recorded in interneurons of rat cerebellum, and they were present in ~20% of the tested cells (Pouzat and Marty, 1998). Subsequently, autaptic currents were recorded in neocortical fast-spiking (FS) GABAergic neurons, they were common (in ~85% of tested cells), occurring after every action potential and providing a powerful inhibition (range of autaptic conductance: 6-10 nS) (Bacci et al., 2003). In both structures they were proved to be of synaptic origin based on the combination of the following evidences: (1) they are eliminated by pre- and post-synaptic blockers (blockage of presynaptic

transmission by Cd^{2+} , BAPTA and postsynaptic GABA_A R inhibitors), (2) they have a fixed latency overlapping with action potential afterhyperpolarisation, (3) they show Cl^- dependent reversal potential, (4) they display fast rise times, (5) they show peak amplitude fluctuations, hence synaptic origin and (6) they display some failures (Pouzat and Marty, 1998; Bacci et al., 2003; Connelly and Lees, 2010). Subsequently, dynamic-clamp experiments suggest that feedback inhibition provided by autaptic current is essential to set a high temporal precision of FS interneurons, likely involving a shunting inhibition mechanism (Bacci and Huguenard, 2006). Interestingly, the authors of this study observed that artificial autaptic activity can set a high spike timing precision also in PNs (Bacci and Huguenard, 2006). Unexpectedly, following high-frequency activation of FS neurons, autapses are the source of delayed asynchronous GABA release leading to a decrease of precision and reliability of APs in these neuron (Manseau et al., 2010). Another example of the presence of autaptic currents comes from hippocampal NGFCs (Karayannis et al., 2010). Like the autaptic currents recorded in FS neocortical interneurons they share features with postsynaptic IPSCs, namely similar kinetics, mid-term depression upon repetitive stimulation and comparable pharmacological profile (Karayannis et al., 2010). Analogous to autapses in FS interneurons, they decrease the excitability of NGFCs (Karayannis et al., 2010). Bacci and colleagues proposed that inhibitory self-innervation is a novel form of feed-back inhibition (Bacci et al., 2003). It appears to be a simple form of inhibition and does not need the development of a complex network. Moreover, because of its synaptic nature it is highly plastic and may have an important influence on the network function (Bekkers, 2003).

1.7. Neuropeptide Y: a prominent modulator of the amygdala microcircuit

Since one of the common marker in GABAergic cells including NGFCs is NPY (Karagiannis et al., 2009) I conclude this section of my thesis by briefly reviewing some information on this peptide. The NPY is the most widespread peptide in the mammalian brain found in the cerebral cortex including the hippocampal formation, the inner layer of the olfactory bulb, the basal ganglia, the septum, the basal forebrain, and the amygdala (Tatemoto et al., 1982). Not surprisingly it is involved in many physiological functions and pathophysiological conditions. A large variety of examples can be found in the literature, pointing to the action of NPY in food intake, synthesis of corticotrophin-releasing factors, immune regulation, learning and memory, neuroprotection, epilepsy, addiction, reproduction, alcohol dependence, depression and anxiety (Bowers et al., 2012). Currently, there are six known NPY receptors (Y1-Y6) which mediate their effect through G-protein signalling. Four types of NPY-R are expressed in the BLA (Y1, Y2, Y4, and Y5), but only Y1 and Y5 receptors are found in the CE nucleus (Parker and Herzog, 1999). Within the amygdala, NPY is expressed predominantly in local GABAergic interneurons (McDonald and Mascagni, 2002), but NPY mRNA can be found also in projection cells (Sosulina et al., 2006). The role of NPY within the amygdala has been tested in a variety of experiments. For example, NPY knockout mice display an anxiogenic-like phenotype (Bannon et al., 2000). On the contrary, the overexpression of NPY in the amygdala reduce the animal anxiety (Primeaux et al., 2005). Consistently, other anxieties-related behavioural tests demonstrate an anxiolytic action of NPY (Sajdyk et al., 1999). The application of NPY decreases the excitability of most PNs within the LA *via* the activation of Y1 receptors and subsequently G-protein coupled inwardly rectifying potassium channels, and therefore can mediate the anxiolytic

effect (Sosulina et al., 2008). A growing number of evidences shows that NPY inhibits fear acquisition and facilitate fear extinction (Bowers et al., 2012).

2. Aim

In the amygdala, inhibitory circuits control the acquisition, expression and extinction of conditioned fear (LeDoux, 2000; Ehrlich et al., 2009; Pape and Pare, 2010). GABAergic cells are found in all the nuclei of rodent amygdala including the BLA and ITC clusters (McDonald et al., 1993; Pare and Smith, 1993a). Given their physiological importance, variety of functions and lack of sufficient understanding of GABAergic component of amygdala microcircuit, I aimed to study two distinct cell types found in the amygdala.

A significant progress in understanding the cellular organization of amygdala would be to clarify the physiological role of Im that is unknown despite the high number of cells it contains. Recent data show that the activation of Im neurons is stronger after extinction and extinction retrieval (Busti et al., 2011), suggesting for the first time a specific role of this nucleus in emotional processing. In the first part of this study I therefore aimed to investigate (1) electrophysiological responses, (2) axo-dendritic patterns and (3) synaptic inputs/outputs of Im neurons.

The BLA is the most important site of integration of synaptic inputs during fear conditioning (Herry et al., 2008). Interactions between PNs and GABAergic interneurons determine the computation of emotional memories (Ehrlich et al., 2009). The role of the GABAergic cells of the BLA is particularly important, yet we are still in the process of discovering specific cell-types and their connectivity. In the second part of my studies I tested if a specific type of interneuron, called NGFC is present in the BLA (1). Furthermore, I aimed to investigate the kinetics of IPSCs evoked by NGFCs in PNs of the BLA (2) and answer to what mechanisms govern that process (3). My work led to the discovery of a novel type of NPY-containing GABAergic cell in the BLA. I suggest that this cell type is an essential component of amygdala network actively contributing to the maintenance of its high inhibitory tone.

3. Part I: Functional connectivity of the main intercalated nucleus of the mouse amygdala

3.1. Introduction

In the amygdala, inhibitory circuits control the acquisition, expression and extinction of conditioned fear (LeDoux, 2000; Ehrlich et al., 2009; Pape and Pare, 2010). GABAergic cells are found in all the nuclei of rodent amygdala including the BLA and ITC clusters (McDonald and Augustine, 1993; Pare and Smith, 1993b; Ehrlich et al., 2009). The latter contain the highest density of GABAergic cells in the amygdala, and are organised in clusters surrounding the BLA that are well preserved along mammals (Millhouse, 1986; Nitecka and Ben-Ari, 1987; Pare and Smith, 1993b, a; Alheid, 1994; Pitkanen and Amaral, 1994; Royer et al., 1999; Marowsky et al., 2005). The ITC clusters can be further divided in mITC, lITC, and Im, based on the different location of the nuclei. The Im nucleus, the biggest cluster among ITC, is located ventro-medially to the BLA (Millhouse, 1986; Pare and Smith, 1994).

Recently, the ITC clusters have been shown to play a crucial role in emotional regulation and attracted strong and widespread interest. Key observations are: 1) selected lesions of mITCs result in a marked deficit in the expression of fear extinction (Likhtik *et al.*, 2008); 2) excitability of mITCs is enhanced after fear extinction (Amano *et al.*, 2010); 3) mITCs and lITCs are directly inhibited by DA (Marowsky *et al.*, 2005), providing a cellular substrate for the DA-induced disinhibition of the amygdala at systemic level (Rosenkranz and Grace, 1999); 4) the anxiolytic-like effect of NPS is mediated by enhanced synaptic excitation of mITCs but not of other cell types of amygdala (Jungling *et al.*, 2008).

Recent studies have reported some functional diversity within and between ITC clusters. Neurons of the mITC cluster are synaptically coupled with variable

synaptic strengths dependent on the frequency of presynaptic cell firing and this functional diversity is correlated to peculiar dendritic and axonal patterns of different cell pairs (Geracitano *et al.*, 2007). Recently, three types of mITCs have been recognised based on their axonal projections, the biggest population innervates the Im nucleus, forming functional GABAergic synapses (Busti *et al.*, 2011). Moreover, different activation amongst ITC clusters has been detected in rodents displaying an impairment of fear extinction (Hefner *et al.*, 2008).

A significant progress in understanding the cellular organization of amygdala would be to clarify the physiological role of Im that is unknown in spite of the high number of cells contained in this nucleus. Recent data shows that the activation of Im neurons is stronger after extinction and extinction retrieval (Busti *et al.*, 2011), suggesting for the first time a specific role of this nucleus in emotional processing. I report here electrophysiological responses, axo-dendritic patterns and synaptic inputs/outputs of Im neurons in order to start clarifying their role in the fear-learning related amygdala circuits.

3.2. Materials and Methods

3.2.1. Acute slice preparation

All procedures involving animals were performed according to methods approved by the UK Home Office and The Animals (Scientific Procedures) Act 1986. Acute coronal slices were prepared from C57BLn mice (postnatal day 16-25; Charles River, Margate, UK). Animals ($n = 98$) were decapitated under deep isoflurane anaesthesia (4% in O_2), and their brains were rapidly removed and placed in ice cold sucrose artificial cerebrospinal fluid (ACSF) cutting solution (containing in mM: 75 sucrose, 87 NaCl, 25 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4 , 0.5 $CaCl_2$, 7 $MgCl_2$, 25 glucose, saturated with 95% O_2 , 5% CO_2 , at pH 7.3-7.4). Slices (350 μm) containing amygdala (at ~ -0.9 mm Bregma level) were cut (Leica VT 1000S, Leica Microsystems GmbH, Nussloch, Germany) and transferred to a nylon mesh where they were maintained in a chamber initially containing sucrose ACSF cutting solution at 37°C for 30 min. During this period the cutting solution was substituted with normal ACSF (containing in mM: 130 NaCl, 24 $NaHCO_3$, 3.5 KCl, 1.25 NaH_2PO_4 , 2.5 $CaCl_2$, 1.5 $MgSO_4$, 10 glucose saturated with 95% O_2 , 5% CO_2 , at pH 7.3). Slices were then maintained at room temperature (20-22°C).

3.2.2. Electrophysiology and analysis

Acute slices were secured under a nylon mesh, submerged and superfused with ACSF in a 2 ml chamber mounted on the stage of an upright microscope (Axioskop, Zeiss, Jena, Germany). Slices were visualized with a 10 $\times$ /0.3 NA or 40 $\times$ /0.8 NA water-immersion objective (Zeiss, Oberkochen, Germany) coupled with infrared and differential interference contrast (DIC) optics linked to a video camera (Newvicon C2400, Hamamatsu, Hamamatsu City, Japan). Initially only cells with subsequent anatomical confirmation of their position (see below) were used for further analysis.

However, in a minority of experiments the position of Im was identified online with infrared and DIC optics without further anatomical confirmation of the position of the recorded neuron within Im boundaries. Somatic whole-cell patch-clamp recordings (33–35°C) were made from visually identified cells using borosilicate glass capillaries (GC120F, 1.2 mm o.d., Clarke Electromedical Instruments, Reading, UK), pulled on a DMZ puller (Zeitz-instrumente GmbH, Munich, Germany) and filled with a filtered intracellular solution consisting of (in mM): 126 K-gluconate, 4 KCl, 4 ATP-Mg, 0.3 GTP- Na_2 , 10 Na_2 -phosphocreatine, 10 HEPES and 0.5% w/v biocytin (all from Sigma-Aldrich Co. Ltd, Poole, UK), osmolarity 270–280 mosmol l^{-1} without biocytin, pH 7.3 with KOH. A caesium-based intracellular solution was used to isolate AMPA and NMDA receptor-mediated excitatory postsynaptic currents (AMPA-, NMDA-EPSCs) consisting of (in mM): 126 Cs-methansulfonate, 4 CsCl, 10 HEPES, 10 Na_2 -Phosphocreatine, 4 Mg-ATP, 0.3 Na-GTP and 0.5% w/v biocytin (all from Sigma-Aldrich), osmolarity 270–280 mosmol l^{-1} without biocytin, pH 7.3 with CsOH. Biocytin was added to allow *post hoc* visualization of the recorded neurons. Resistance of the patch pipettes was 5–6 M Ω , and recordings were accepted only if the initial seal resistance was greater than 1 G Ω . The series resistance (R_s) was compensated online by 60–70% in voltage-clamp mode to reduce voltage errors, and cells were only accepted for analysis if the initial R_s was less than or equal to 25 M Ω (range, 14 - 25 M Ω) and did not change by more than 20% throughout the recording period. Throughout the text and figures, membrane potentials for all whole-cell recordings have been corrected for an experimentally determined liquid junction potential of –12 mV for recordings with the potassium gluconate intracellular solution.

All electrophysiological signals were amplified (10 mV pA^{-1} , EPC9/2 amplifier HEKA Elektronik, Lambrecht, Germany, PULSE software); low pass filtered at 2.9 kHz, digitized at 5 or 10 kHz. The amplifier was controlled from a personal computer running the PULSE data acquisition and analysis programme (HEKA).

Currents/voltages were acquired online with Pulse software (HEKA) and analysed offline with Pulsefit (HEKA) and IGOR Pro5.05 (Wavemetrics Inc, Oregon, USA). The input resistance (R_{in}) was calculated from the slope of a line fitted to the subthreshold range on a plot of the injected current *versus* the steady-state membrane voltage when a family of hyperpolarising and depolarising current injections were applied (range, -30 / +120 pA). The apparent membrane time constant (τ) was calculated by fitting a single exponential to the response of the cell to a current injection of -50 pA in current clamp mode. Membrane capacitance was calculated as τ/R_{in} . To study the kinetics of action potentials, a depolarising current step (3 ms, 100-150 pA) was applied. Peak amplitude and membrane afterhyperpolarization (AHP) were measured from the initial point of the action potential raising phase by a user. Action potential half-width were measured from the initial point of the action potential raising phase by a user-defined program in IGOR. Below is the code of the procedure, accompanied by description of functions (in brackets).

Function FindHalfWidth()

Function to calculate the half width of an action potential in current clamp at 50% amplitude. The event to be tested should be in the upper graph window, with cursor A at the start of the event, cursor B at the peak.

Variable declarations (these are the names use later on when storing these variables, or using them in calculations).

Variable MediumLimit (The lower limit of the rise-time interval required e.g. 0.2 = 20%)

Variable t_start (The start time of the event)

Variable t_peak (The peak time of the event)

Variable y_start	(The starting y-value of the event)
Variable y_peak	(The peak y-value of the event)
Variable delta_y_event	(The amplitude of the event)
Variable y_medium_limit	(y-value at the lower limit of the required interval)
Variable t_medium_limit	(Time at the first level crossing at y_medium_limit)
Variable t_halfwidth	(The difference between the second level crossing and the first)
Variable t_second_limit	(The x values of the second level crossing)

(This tells the function to analyse the wave that the cursors are on)

Wave DataWave = CsrWaveRef(A)

(These variables set the medium % to calculate the halfwidth over)

MediumLimit = 0.5

(This stores the times for the start and peak of the event)

t_start = hcsr(A)

t_peak = hcsr(B)

(This stores the y-values for the start and peak of the event)

y_start = vcsr(A)

y_peak = vcsr(B)

(This calculates the medium y-values for the interval required)

delta_y_event = y_peak - y_start

y_medium_limit = y_start + (MediumLimit * delta_y_event)

(This searches the data between the start and peak of the event, to find the time at which it crosses the medium y-value of the interval required and the number of crossings to be searched)

FindLevels /Q/R = (t_start)/N = 2 DataWave, y_medium_limit

Wave W_FindLevels

t_medium_limit = W_FindLevels[0]

t_second_limit = W_FindLevels[1]

(This calculates the x difference between the two level crossings)

t_halfwidth = t_second_limit-t_medium_limit

Print t_halfwidth

End Function

The sag ratio was calculated from the membrane potential at the end of a 1 s hyperpolarising pulse divided by the largest membrane potential change observed in response to a current step (range, -80 / -120 pA). The adaptation index was calculated as the ratio between the first and last interspike intervals evoked by a 1 s depolarising current pulse. The maximal (max.) firing rate was the number of action potentials elicited by a strong depolarising current pulse (range, 250 - 500 pA). Steps of depolarising current were injected into cells (200 ms, 50-100 pA) to evaluate the action of dopamine, D1 and D2 receptors antagonists on Im neurons. The values of the resting membrane potential and number of action potentials were measured from the averaged last ten trials of control and bath application of the drugs.

Extracellular stimulation was conducted by applying rectangular pulses of current (0.1 ms, range, 5 - 50 μ A) delivered through an isolation unit (A360 Stimulus

Isolator, World Precision Instruments, Stevenage, UK) to a monopolar patch pipette filled with ACSF. The stimulating electrode was placed in different sites of the amygdala to evoke inhibitory postsynaptic currents (eIPSCs) or excitatory postsynaptic currents (eEPSCs). Extracellular stimulation experiments were conducted in the presence of kynurenic acid (3 mM) or bicuculline metachloride (5-30 μ M) during recordings of eIPSCs or eEPSCs, to block AMPA/kainate/NMDA or GABA_A receptors, respectively. To determine the lowest intensity at which eIPSCs/eEPSCs could be detected, an input-output protocol was performed (range, 5 - 150 μ A, step of 5 - 10 μ A). The PSC kinetics analysis was performed on synaptic responses evoked by lowest possible stimulus intensities resulting in a detectable response. No response was judged by absence of any detectable current in single sweeps following (2-100 ms) the stimulus artefact. The values of peak amplitude, latency, 20–80% rise time, decay time and area of events were measured from the average of three consecutive sweeps. The values of synaptic latency jitter were calculated from the standard deviation of the synaptic latency of ten consecutive eEPSCs. The event peak amplitude was visually delimited and then measured using Pulsefit. The paired-pulse ratio was calculated as the mean peak amplitude of the response to the second stimulus divided by the mean peak amplitude of the response to the first stimulus, measured from the average of ten consecutive sweeps. The latency of events was determined as the time between the onset of stimulation artefact and the onset of the postsynaptic response. The decay time (fitted with a single exponential), jitter, and area of the synaptic currents were analysed by a user in IGOR. The 20–80% rise time was analysed with a user-defined program in IGOR. The measurement of the rise time of a current starts by manually setting the event's starting point and the peak amplitude of this current. It is followed by the execution of Igor procedure, described above. If an artefact of stimulation is present in the recording, the initial point is set at a level equal to the

control current. It is important to keep in mind that such a technique may under/over-estimate measured rise time.

Below is the code of the procedure, accompanied by description of functions (in brackets).

Function FindRiseTime()

Function to calculate the rise time of an event, over a defined interval (20 - 80 %)

The event to be tested should be in the upper graph window, with cursor A at the start of the event, cursor B at the peak.

Variable declarations (these are the names use later on when storing these variables, or using them in calculations).

Variable LowerLimit (The lower limit of the rise-time interval required e.g. 0.2 = 20%)

Variable UpperLimit (The upper limit of the rise-time interval required e.g. 0.8 = 80%)

Variable t_start (The start time of the event)

Variable t_peak (The peak time of the event)

Variable y_start (The starting y-value of the event)

Variable y_peak (The peak y-value of the event)

Variable delta_y_event (The amplitude of the event)

Variable y_lower_limit (y-value at the lower limit of the required interval)

Variable y_upper_limit (y-value at the upper limit of the required interval)

Variable t_lower_limit (Time at the lower limit of the required interval)

Variable t_upper_limit (Time at the upper limit of the required interval)

Variable t_risetime (Rise time for the required interval)

(This tells the function to analyse the wave that the cursors are on)

Wave DataWave = CsrWaveRef(A)

(These variables set the lower and upper % to calculate the rise-time over)

LowerLimit = 0.2

UpperLimit = 0.8

(This stores the times for the start and peak of the event)

t_start = hcsr(A)

t_peak = hcsr(B)

(This stores the y-values for the start and peak of the event)

y_start = vcsr(A)

y_peak = vcsr(B)

(This calculates the lower and upper y-values for the interval required)

delta_y_event = y_peak-y_start

y_lower_limit = y_start+(LowerLimit*delta_y_event)

y_upper_limit = y_start+(UpperLimit*delta_y_event)

(This searches the data between the start and peak of the event, to find the time at which it crosses the lower y-value of the interval required)

FindLevel /Q/R = (t_start,t_peak) DataWave, y_lower_limit

t_lower_limit = V_LevelX

(This searches the data between the start and peak of the event, to find the time at which it crosses the upper y-value of the interval required)

FindLevel /Q/R = (t_start,t_peak) DataWave, y_upper_limit

t_upper_limit = V_LevelX

(The calculates and prints the interval rise-time)

$t_{\text{risetime}} = t_{\text{upper_limit}} - t_{\text{lower_limit}}$

Print t_{risetime}

End Function

In the occlusion test, stimulating electrodes were placed within Im and IC or EC. The actual and predicted sums of values of peak amplitude eEPSCs elicited by two stimuli after independent and simultaneous stimulation were compared. If the latency of two events were different we corrected the onset of stimulation online in the way that the two stimulus artefacts appeared simultaneously. The values of peak amplitude of events recorded during occlusion test were measured from ten trials average.

To isolate AMPA- and NMDA-EPSCs we placed the stimulating electrode in Im stimulating intensity range, (5 - 50 μA) and recorded in voltage-clamp mode at holding potentials (V_H) range, -70 / +40 mV. AMPA-EPSCs were recorded in the presence of bicuculline (30 μM) and NMDA-EPSCs in the presence of 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX; 50 μM) and bicuculline (30 μM). The peak amplitude of the eEPSCs was measured using Pulsefit from the average of three sweeps for each V_H . The reversal potential was calculated from the slope of a line fitted to a plot of values of V_H versus the peak amplitude of recorded currents.

3.2.3. Histological processing of recorded cells

Immediately after electrophysiological recordings, slices were immersed overnight at 4 °C in fixative composed of 4% paraformaldehyde, 15% (v/v) saturated picric acid and 0.1M phosphate buffer (PB; pH 7.2-7.4). Gelatine-embedded slices were re-

sectioned into 60 µm thick sections. To confirm the location of the recorded cells in Im, we processed the sections containing biocytin-loaded cells with horseradish peroxidase method and counterstained the tissue with Nissl staining or immunohistochemistry reaction anti-NeuN. Briefly, biocytin-filled cells were incubated in avidin horseradish peroxidase complex (1:100 dilution; ABC Kit; Vector Laboratories, Burlingame, CA) followed by a peroxidase reaction using diaminobenzidine (DAB, Sigma-Aldrich; 0.05%) as the chromogen and H₂O₂ (1%) as the substrate. Nissl staining was performed by dehydrating/rehydrating the sections and immersing them into a cresyl violet acetate solution (1 g/400 ml H₂O; pH 3.6), dehydrating sections were then immersed in xylene-based mounting medium (Entellan; Merck, Darmstadt, Germany) and coverslipped. Alternatively to Nissl staining, anti-NeuN immunoperoxidase was performed to visualise the amygdala nuclei boundaries. In this case, only neuronal cells nuclei and somata were stained, resulting in a more accurate determination of CE nucleus subdivision boundaries (McDonald, 1982a). Non-specific antibody binding was blocked by incubation in 20 % normal goat serum (NGS; Vector Laboratories Ltd., UK) diluted in Tris-buffered saline (TBS; 0.9% NaCl, 0.05M Tris, pH 7.4 containing 0.2 % Triton X-100 for 45 min). Mouse monoclonal antibody Anti-NeuN (1:3000, Chemicon, Millipore; MAB377) was diluted in TBS, containing 1% NGS, 0.2 % Triton X-100, and applied overnight (at 4°C). The sections were subsequently washed in TBS and incubated overnight (at 4°C) in biotinylated, secondary goat anti-mouse IgG (diluted 1:200, Chemicon, Millipore, MA), diluted in TBS, containing 1% NGS and 0.2 % Triton X-100. The sections were washed in PB and processed for peroxidase-based visualisation of the amygdala nuclei boundaries and mounted on slides for permanent storage (see above). Neurolucida system (MicroBrightField, Inc., Magdeburg, Germany) was used to reconstruct the neurons (100x or 63x oil immersion objective).

3.2.4. Electron microscopy

Some recorded slices were processed for electron microscopy by Dr Daniela Busti and Prof Francesco Ferraguti, (Dept. Pharmacology, Innsbruck Medical University). After extensive washes with TBS, biocytin was revealed by ABC-HRP reaction. Contrast was enhanced using 2% osmium tetroxide v/v (Agar Scientific, Stansted, UK) in PB 0.1M for 40 min at RT, and 1% uranylacetate w/v (Agar Scientific, Stansted, UK) in 50% ethanol for 30 min at RT. The sections were dehydrated in increasing gradients of ethanol, immersed in propylene oxide and embedded in epoxy resin (Durcupan ACM; Sigma-Aldrich) on greased glass slides. Regions of interest were dissected under a stereo-microscope and re-embedded in epoxy resin (Durcupan ACM). Serial ultrathin sections (70 nm) were cut with an ultramicrotome (Ultracut S; Leica, Vienna, Austria) and collected on formvar-coated copper slot grids. The ultrastructural analysis of the specimens was performed using a Philips CM 120 electron microscope equipped with a Morada CCD TEM camera (Soft Imaging Systems, Münster, Germany).

3.2.5. Statistical tests

Statistical analyses were carried out using SPSS 17.0 (SPSS, Chicago, IL). All values are expressed as mean $\pm$ SEM. Statistical comparisons were made using a paired Student's t-test. For all analyses, statistical significance was at $p < 0.05$. Unsupervised, hierarchical cluster analysis using Ward's method (Ward, 1963) and Euclidian distance was performed to classify subtypes among Im cells. Ward's method minimizes the error sum of squares of any pair of clusters formed at a given step; this maximizes between-group differences and minimizes within-group differences. The formula for the error sum of square (ESS) is:

$$ESS = x_i^2 - 1 / n (\sum x_i)^2$$

Where x_i is the score of the i^{th} case and 'n' is a data point.

In the first step of the clustering process each case is in its own cluster (ESS = 0). The method works by joining those groups that results in the minimum increase in the ESS. Here an individual data point is compared for each variable against the cluster means for that variable. Before clustering, each electrophysiological property was converted into standardized z-scores. This achieves a normal distribution and prevents variables with larger ranges from having a greater influence on the cluster solution than variables with small ranges. Statistical differences between three groups were estimated based on one-way ANOVA with post hoc Bonferroni test, $p < 0.05$. Cluster analysis methods are designed to organize data points into relatively homogeneous groups. In order to do so they estimate similarities between data points. I validated the classification results by using ANOVA and post hoc tests.

3.2.6 Chemicals and drugs

All drugs were applied to the slice via the bath solution. Salts used for the patch pipette and ACSF were obtained either from VWR International (Lutterworth, UK) or Sigma-Aldrich (Dorset, UK). Drugs obtained from Tocris (Cookson Ins., Avonmouth, UK) were added at the following concentrations: (-) bicuculline metachloride, 5 or 30 μM ; NBQX, 50 μM ; D-AP5, 100 μM , SR95531, 5 μM ; SCH 23390 hydrochloride, 5 μM ; (-)-Quinpirole hydrochloride, 1 μM . Drugs obtained from Sigma-Aldrich were added at the following concentrations: kynurenic acid, 3mM; DA, 30 μM .

3.3. Results

3.3.1 Basic electrophysiological responses of Im neurons

The position of the Im was identified online with infrared and DIC optics, and could be recognized as a cluster of densely packed cells located ventrally to the BLA nucleus (Fig. 3.1 A). All slices used were prepared from bregma level ~ 0.9 mm (Paxinos and Franklin, 2007) (Fig. 3.1 A, B, C). Post-hoc anatomical confirmation of the location of the somata of the recorded neurons within the Im was performed in the great majority of the experiments (see materials and methods). ITC clusters consist of two cell types, so-called medium and large ITCs (Millhouse, 1986). The majority of ITCs consist of medium ITC (approx. 95%) that have round to elongate somata, round to bipolar dendritic tree bearing dendritic spines (Millhouse, 1986; Royer et al., 1999, 2000b; Marowsky et al., 2005; Geracitano et al., 2007). All cells were recorded using K-gluconate based internal solution at $V_H = -65$ mV. In the first part of the experiments I measured basic electrophysiological responses of Im cells ($n = 45$), (Fig. 3.1, Table 3.1), and found that their passive membrane properties were typical of small neurons (e.g. $R_{in} = 641 \pm 20$ M Ω ; $C_m = 30 \pm 2$ pF). The half-width AP duration and amplitude was $\sim 0.8 \pm 0.05$ ms and 75.7 ± 1 mV, respectively; the firing discharge accommodated to variable extent when evoked with a 1 s depolarising current pulse. Due to the small size of Im neurons and their short survival time not in every experiment I recorded a complete set of passive and active membrane responses. Therefore data point numbers differ between parameters, indicated in Table 3.1. These results are consistent with previously published data on mITCs (Royer et al., 1999; Marowsky et al., 2005; Geracitano et al., 2007).

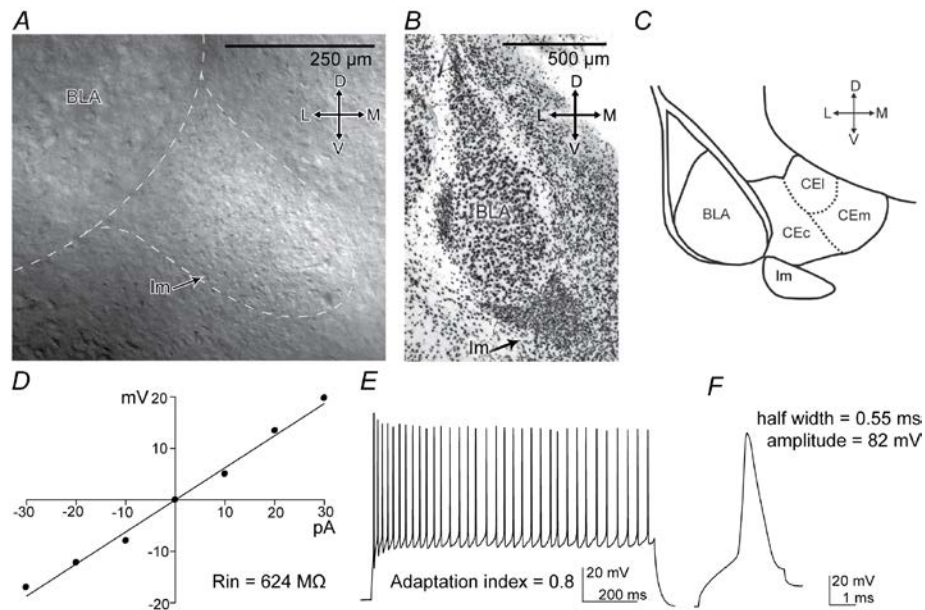


Figure 3.1. Location and electrophysiology of mouse Im neurons.

A-C, the Im nucleus can be identified as a cluster of densely packed cells, located ventro-medially to the BLA. A, identification of Im nucleus on mouse coronal slice with IR-DIC microscopy (10×/0.3 NA water-immersion objective). B, anti-NeuN staining revealing the Im. C, scheme of the amygdala depicting the position and the size of the Im at -0.9 bregma level. D-F, passive and active electrophysiological responses of a representative Im cell. D, the I/V plot used to measure the cell R_{in} . E, sustained firing pattern in response to a depolarising current stimulus (150 pA, 1 s). F, action potential evoked by a short depolarising current pulse (100 pA, 3 ms). Abbreviations: D, dorsal; L, lateral; M, medial; V, ventral; BLA, Basolateral complex; CEc, Central nucleus, capsular; CEI, Central nucleus, lateral; CEm, Central nucleus, medial; Im, main intercalated nucleus; R_{in} , input resistance.

Parameter	Average value $\pm$ S.E.M.	(n)
RMP (mV)	-71.2 ± 1.1	37
R_{in} (M Ω)	641 ± 20	43
Membrane τ (ms)	20.3 ± 0.8	42
C_m (nF)	0.03 ± 0.002	42
AHP (mV)	-12.8 ± 0.6	45
AP amplitude (mV)	75.7 ± 1	45
AP width (ms)	0.8 ± 0.05	44
Sag ratio	0.86 ± 0.03	42
Adaptation index	0.6 ± 0.04	38
Max. firing (Hz)	37.3 ± 2	38

Table 3.1. Table summarizing passive and active membrane responses of *in vitro* recorded Im neurons. Abbreviations: AHP, afterhyperpolarization; AP, action potential; C_m , membrane capacitance; Max. firing, Maximal firing; RMP, resting membrane potential; R_{in} , input resistance.

3.3.2. Im neuron diversity based on cluster analysis

To investigate the existence of possible Im neurons subtypes, electrophysiological parameters were used in an unsupervised cluster analysis. The following parameters were analysed: RMP; R_{in} ; τ ; AHP; AP amplitude; AP width; sag ratio; adaption index and maximal firing rate. A total of 27 neurons were included in this analysis after rejecting cells lacking some of the variables (Fig. 3.2, Table 3.2). The resulting dendrogram indicated the presence of three groups of Im neurons. Comparison amongst groups shows significant differences between seven out of nine measured parameters (Table 3.2). Group III consisted of cells with the resting V_m much more hyperpolarized than cells of group I and II. Moreover, their R_{in} as well as adaptation index is higher than cells of group I and II. Cells in group II had broader AP, slower membrane time constant and smaller value of adaptation index than cells of group I and III. Group I consisted of neurons with smallest AP amplitude among the three groups. The values of AHP and sag ratio remained constant among the three groups. Although our analysis could not indicate the number of neuronal types among Im neurons, it definitely suggests potential functional diversity among Im neurons. Unfortunately, it was not possible to fully reveal axo-dendritic arborisation of cells used in this study that prevented correlation of detected physiological variations with morphological characteristics of three groups.

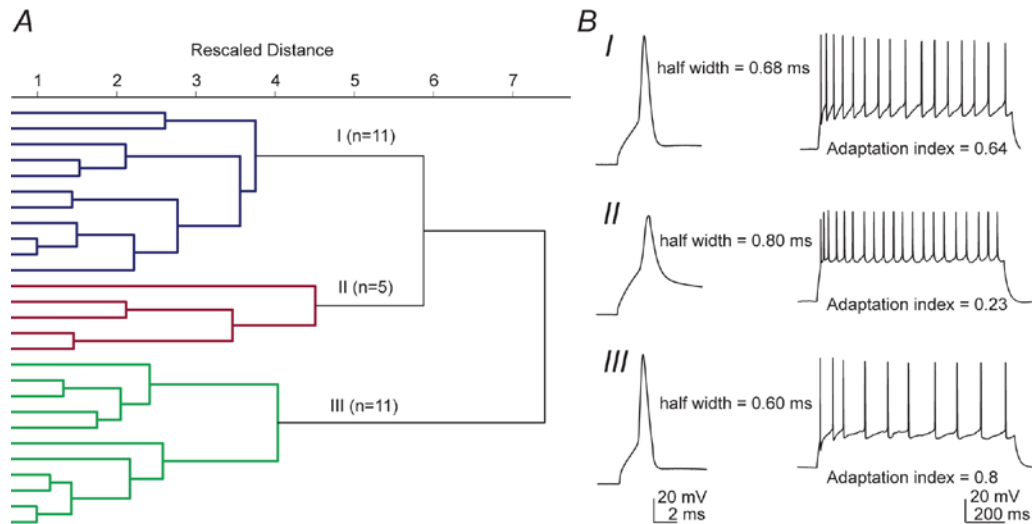


Figure 3.2. Im neuron diversity based on cluster analysis.

A, dendrogram illustrating the results from a hierarchical cluster analysis. Intersection of the dendrogram branches with the y-axis represents individual neurons. The x-axis is the rescaled distance (squared Euclidian) between group centroids at each branch point. Longer horizontal lines indicate greater distance. Note the existence of three groups of Im neurons: I ($n = 11$; blue); II ($n = 5$; red); III ($n = 11$; green). B, representative Im cells belonging to group I, II or III. Left panel: AP evoked by a short depolarizing current pulse (100 pA, 3 ms). Right panel: sustained firing pattern in response to a depolarizing current stimulus (150 pA, 1 s).

Parameter	Group I (n=11)	Group II (n=5)	Group III (n=11)
RMP (mV)	-68.2 ± 1.5	-70.8 ± 2.5	-77.8 ± 1.4
	III	III	I, II
R _{in} (MΩ)	562 ± 31	593 ± 50	777 ± 28
	III	III	I, II
Membrane τ (ms)	19.4 ± 0.9	25.0 ± 1.4	17.8 ± 1.1
	II	I, III	II
AHP (mV)	-13.5 ± 1.0	-12.7 ± 1.9	-12.2 ± 0.7
AP amplitude (mV)	71.9 ± 1.7	82.1 ± 2.8	80.1 ± 1.3
	II, III	II	I
AP width (ms)	0.67 ± 0.04	1.0 ± 0.2	0.61 ± 0.03
	II	I, III	II
Sag ratio	0.86 ± 0.02	0.92 ± 0.01	0.89 ± 0.01
Adaptation index	0.59 ± 0.05	0.24 ± 0.08	0.78 ± 0.04
	II, III	I, III	I, II
Max. firing (Hz)	32.7 ± 1.9	34.2 ± 5	43.3 ± 2.7
	III		I

Table 3.2. Table summarizing intrinsic membrane responses of *in vitro* recorded Im neurons belonging to group I, II and III based on cluster analysis. All values are shown as mean ± SEM. Statistical differences between the three groups, estimated based on ANOVA with *post hoc* Bonferroni test, are indicated under the corresponding parameter, $p < 0.05$. Abbreviations: AHP, afterhyperpolarization; AP, action potential; Cm, membrane capacitance; Max. firing, Maximal firing; RMP, resting membrane potential; R_{in}, input resistance.

3.3.3. Effect of dopamine on Im neurons

All ITC clusters receive the densest afferents from the mesencephalic dopaminergic system in the amygdala (Asan, 1998; Fuxe et al., 2003; Marowsky et al., 2005; Jacobsen et al., 2006). The neuromodulator DA elicits a characteristic inhibition of the excitability of mITCs and IITCs (Marowsky et al., 2005), in contrast to the other GABAergic cells of the amygdala tested so far that are depolarized (Rosenkranz and Grace, 1999; Bissiere et al., 2003). The action of DA on mITC and IITC clusters has been proposed to be a key cellular event underlying the DA-mediated disinhibition of the BLA and CE nuclei leading to fear-related behaviour (Marowsky et al., 2005). To test whether Im neurons could participate to this behavioural effect I superfused DA (30 μ M, $n = 21$) while injecting depolarising current pulses into recorded Im cells (50-100 pA for 200 ms). All cells were recorded using K-gluconate based internal solution at $V_H = -65$ mV. I observed a reversible membrane hyperpolarization ($p < 0.05$, Fig. 3.3 A, B) accompanied by a significant decrease in the number of APs ($p < 0.05$, Fig. 3.3 A, B) and value of R_{in} ($p < 0.05$, Fig. 3.3 A, B). Due to the specific experimental conditions data point numbers differ between parameters (Fig. 3.3 B). To assess DA receptor subtype mediating this effect I superfused Im neurons with DA type 1 receptor (DA D1; $n = 4$) or DA type 2 receptor (DA D2; $n = 4$) antagonist in the presence of DA. DA D1 receptor antagonist combined with DA blocked action of DA (Fig. 3.3 C). Consistently, DA D2 receptor antagonist combined with DA mimicked action of DA (Fig. 3.3 C). This result together with anatomical data (see below) suggests that Im, IITC and mITC clusters share common characteristics, and that Im neurons could participate in behavioural effects of DA.

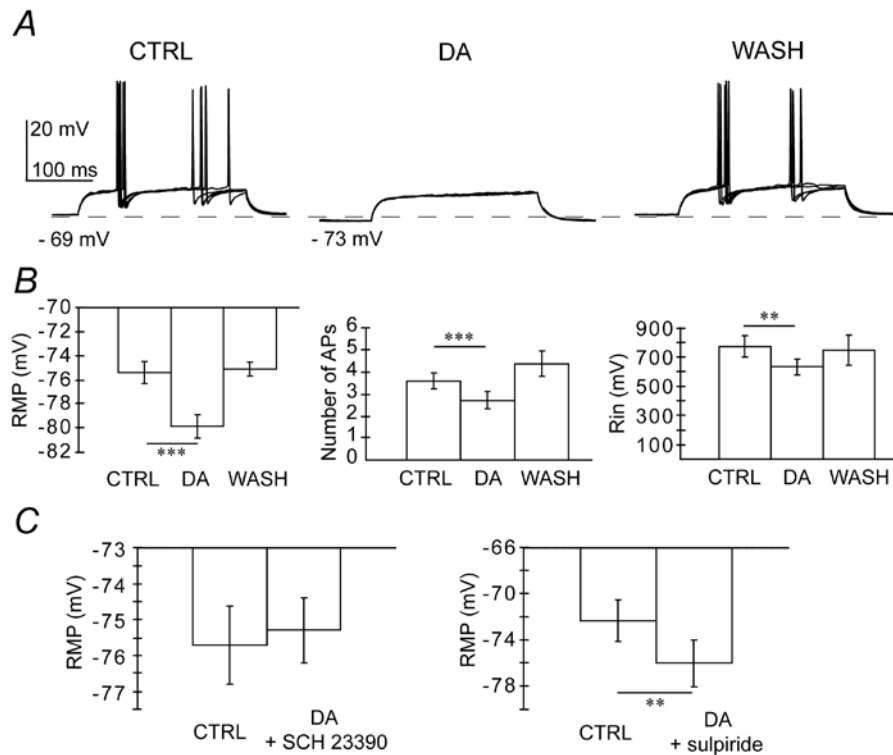


Figure 3.3. Hyperpolarizing effect of dopamine on the Im cells.

A, response of Im cell to a depolarizing current pulses (60 pA, 200 ms) during control (CTRL), dopamine bath application (DA, 30 μ M) and washout. B, average ($\pm$ SEM) effect of DA on the resting membrane potential (RMP, $n = 21$), number of action potentials (APs; $n = 21$) and input resistance (R_{in} ; $n = 7$). Comparison between control and DA bath application (* $p < 0.05$, ** $p < 0.01$; Paired Sample t-test). C, summary of the effects of applied drugs on RMP and number of APs. Note that DA and DA D2 antagonist in the presence of DA evoked hyperpolarizing response. Comparison between drugs bath application (* $p < 0.05$, ** $p < 0.01$; Paired Sample t-test).

3.3.4. Synaptic output of Im cells

It is crucial to our understanding of the physiological role of Im neurons to identify their target neurons. In order to describe efferent projection of mouse Im neurons, their axonal arbours were studied. Neurons were digitally reconstructed with the Neurolucida system ($n = 9$), and four representative cells with the longest recovered axonal processes are shown in Fig. 3.4. Neurons with the soma in the Im shared common characteristics that could be described as poorly branching dendritic tree (round to bipolar), that mostly remained confined within the boundaries of the Im, spiny dendrites and a projection axon. I invariably detected cut axons at the bottom or at the upper surface of the slice preparation, clearly suggesting that Im cells were projection neurons. In line with this, little axon was observed in the Im itself. Importantly, I detected the presence of axon branches in several amygdala nuclei, with striking distribution diversity between cells. Axon could be detected in all three subdivisions of CE nucleus as well as in the BLA, medial and extended amygdala (Fig. 3.4 A-C), as well as toward IITC (Fig. 3.4B) and mITC clusters (Fig. 3.4D). No correlation was observed between positions of cell soma in the latero-medial plane with site of its projection. Preparation of slices from constant bregma level (~ 0.9 mm) prevented from possibility of relating position of the cell in rostro-caudal level with their projection sites.

To functionally confirm synaptic output of Im cells, I placed an extracellular electrode in the Im and recorded from CE and BLA cells while stimulating with increasing strength (Fig. 3.5). All kinetics data refer to responses evoked by lowest stimulus intensities eliciting a detectable response. All cells were recorded using K-gluconate based internal solution at $V_H = -65$ mV. The eIPSCs were recorded in the presence of kynurenic acid (3 mM) and were abolished by bath application of the GABA_A receptor antagonist SR95531 (5 μ M). Monosynaptic connectivity was detected when recording from the CE (2/8 cases) and BLA (2/5 cases). Representative examples of extracellular stimulation of Im as well as recording sites

are shown in Fig. 3.6. Responses detected in the CEC had peak amplitude of 21.1 and 10.8 pA; rise time of 2.6 and 2.0 ms; decay time of 25.8 and 20.8 ms respectively. Responses detected in the BLA nucleus had peak amplitude of 129.2 and 27.8 pA; rise time of 3.1 and 4.3 ms; decay time of 32.1 and 23.3 ms respectively. These data confirm that Im neurons have significant outputs in the central nucleus and BLA, in line with my anatomical observations.

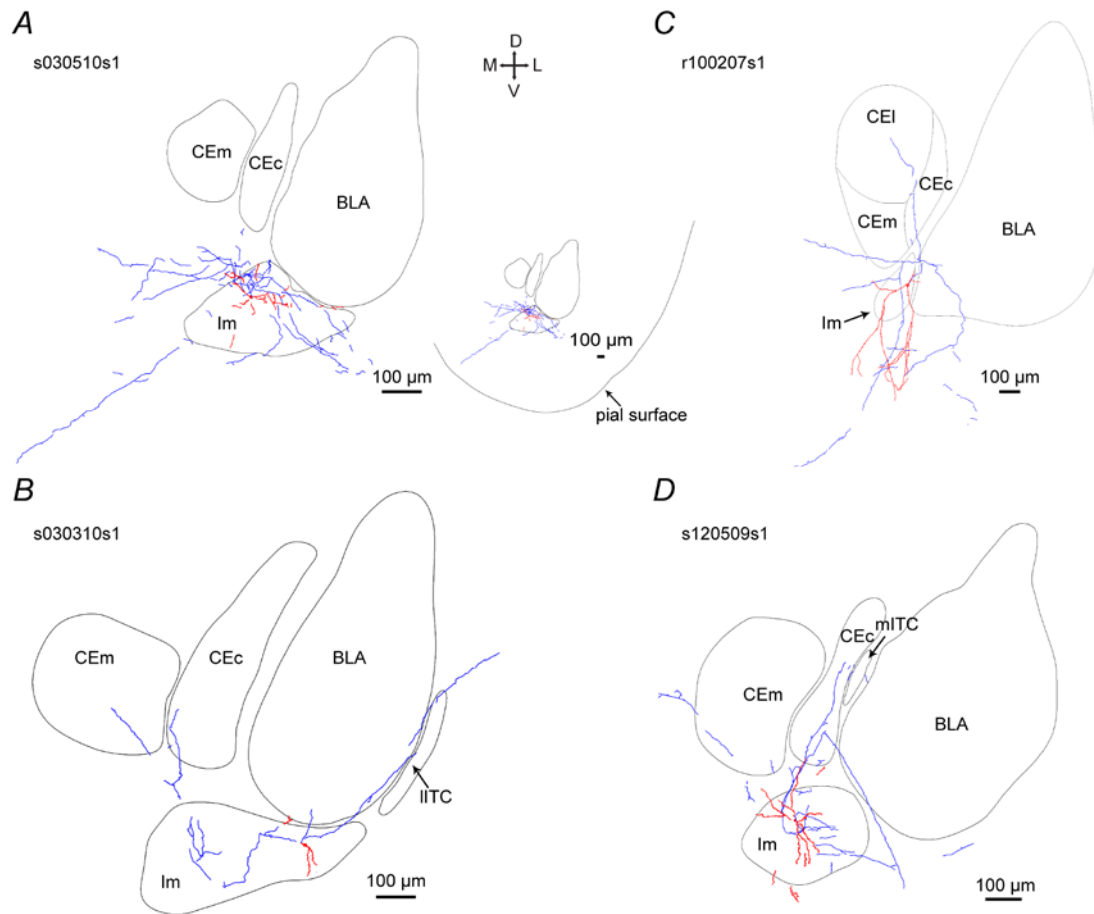


Figure 3.4. Reconstruction of Im neurons.

Neurolucida system was used to reconstruct the neurons (100x oil immersion objective for cells code s030510s1 (A); s030310s1 (B); s120509s1 (D) and 63x oil immersion objective for cell code r100207 (C). Red- soma and dendrites; blue – axon; black – nuclei boundary. The inset of panel A, illustrates the reconstruction of cell s030510s1 at lower magnification to document the presence of its long axon projecting outside the amygdala. Abbreviations: D, dorsal; L, lateral; M, medial; V, ventral; BLA, Basolateral complex; CEc, Central nucleus, capsular; CEI, Central nucleus, lateral; CEm, Central nucleus, medial; Im, main intercalated nucleus, mITC, medial intercalated cell cluster; IITC, lateral intercalated cell cluster.

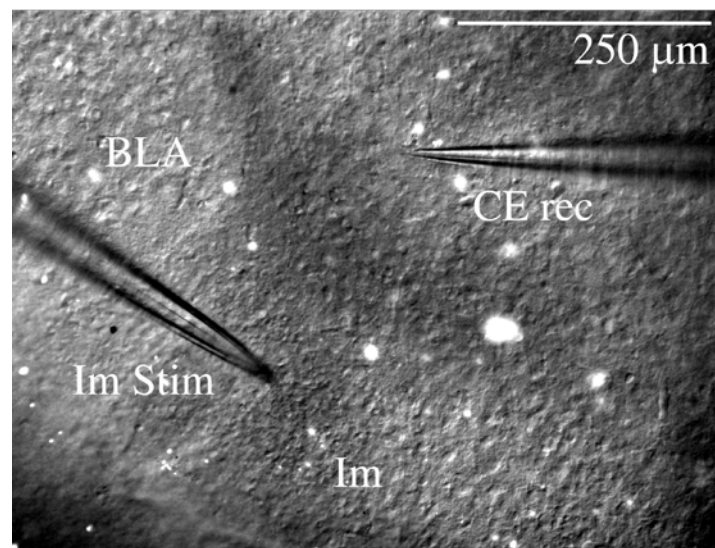


Figure 3.5. Location of stimulating and recording electrode.

Low magnification picture under a 10×/0.3 NA water-immersion objective. Abbreviations: BLA, Basolateral complex; CE, Central nucleus; Im, main intercalated nucleus; Rec., recording electrode, Stim., stimulating electrode.

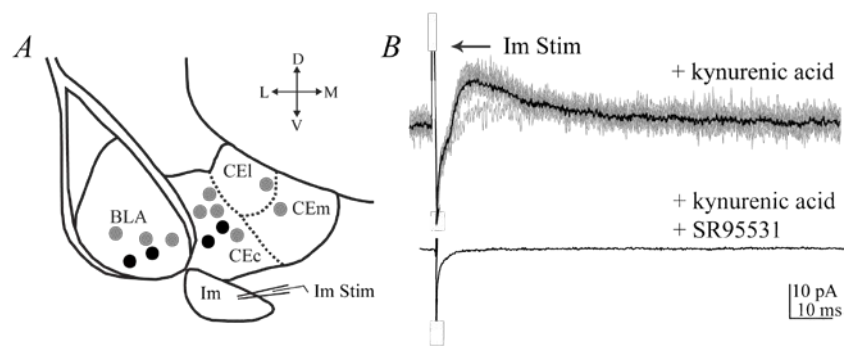


Figure 3.6. Synaptic output of the Im.

A, scheme of the amygdala depicting the location of the stimulating electrode and recording sites. Black dots represent detected eIPSCs, grey dots represent recording locations where eIPSCs could not be observed. B, representative voltage clamp recording showing eIPSCs in CE after Im stimulation (upper trace; 100 μ A intensity of stimulation; in the presence of 3 mM kynurenic acid) and subsequent blockade by application of SR95531 (5 μ M). Individual eIPSCs are shown in grey; the average of 10 traces is shown in black. Abbreviations: D, dorsal; L, lateral; M, medial; V, ventral; BLA, Basolateral complex; CEc, Central nucleus, capsular; CEI, Central nucleus, lateral; CEm, Central nucleus, medial; Im, main intercalated nucleus; Stim., stimulating electrode.

3.3.5. Inhibitory inputs to the Im

Recent data suggests that mITCs could be divided in three groups based on the presence of axon in different nuclei of amygdala (Busti et al.). Interestingly, the largest of these groups is represented by neurons whose axons innervate the Im (17 out of 51 neurons). I aimed to test if this connectivity is functional and to determine other possible sources of synaptic inhibition to the Im. I placed an extracellular electrode in the IC (where mITC are located), Im, BLA and EC (where IITCs are located). Next, I recorded from Im cells while stimulating with increasing strength (Fig. 3.7C2; 3.8B, C). All cells were recorded using K-gluconate based internal solution at $V_H = -65$ mV. All kinetics data refer to responses evoked by lowest possible stimulus intensities eliciting a detectable response. The IPSCs were evoked in the presence of kynurenic acid (3 mM) and were abolished by bath application of bicuculline (5 μ M; Fig. 3.7 C1), indicating they were mediated by GABA_A receptors. Summary of quantitative analysis of some aspects of the eIPSC is reported in Table 3.3. I detected monosynaptic eIPSCs when placing the stimulating electrode within the IC (2/6 cases; Fig. 3.7). In keeping with the evoked IPSCs in Im neurons after stimulation of the mITC, our collaborators (Dr Daniela Busti and Prof. Francesco Ferraguti) demonstrated that the axon terminals of biocytin-filled MCp-mITC neurons formed symmetrical synapses with dendritic profiles of Im neurons (Fig. 3.7 A,B), which further confirmed anatomically a direct inhibitory connectivity between mITC and Im.

I always detected monosynaptic eIPSCs when placing the stimulating electrode within the Im (6/6 cases; Fig. 3.8). When two stimuli were applied in short succession (50 ms) an average paired pulse ratio of 1.2 ± 0.12 was observed. Detected eIPSCs may have mixed origin coming from local Im or remote GABAergic neurons. To distinguish between these two possibilities, I performed paired recordings of Im cells. However I never detected synaptically-connected cell pairs ($n = 10$, data not shown), showing the eIPSCs were evoked from neurons located

outside the Im. This is consistent with the poor axonal and dendritic arborisation of these neurons within the Im. Finally, I also observed eIPSCs when stimulating the EC, where IITCs are located (2/6 cases) or the BLA (1/6 cases, Table 3.3), suggesting additional sources of synaptic inhibition.

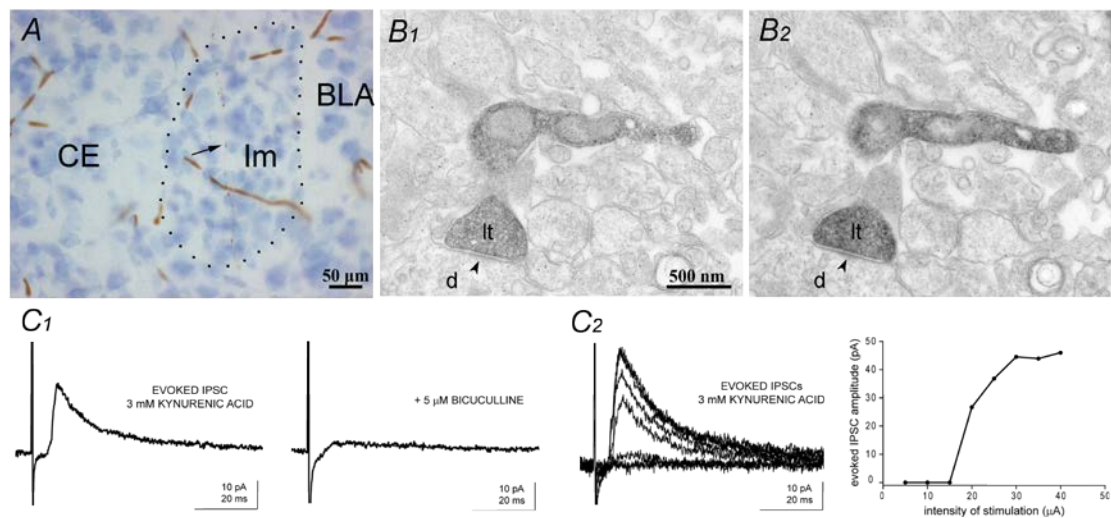


Figure 3.7. Direct inhibitory connectivity between mITC and Im.

A, light micrograph of Im containing a portion of a biocytin filled axon (arrow) originated from a MCp-mITC neuron. B1,2 electron micrographs of serial sections of a biocytin labelled axon terminal making a symmetrical synapse (arrowhead) with a small dendritic shaft in the Im. d, dendrite; It, labelled terminal. C1, representative IPSCs elicited in the Im upon extracellular stimulation in the mITC. The traces represent the average of 10 sweeps. The response was mediated by GABA_A receptors, since it was abolished by 5 mM bicuculline. C2, IPSCs evoked in the same cell at increasing stimulation intensities, within the range of 5-40 mA. The input-output curve shows the relation between the intensity of stimulation used and the peak amplitude of the IPSCs. Each point of the graph is the average value of three sweeps. Note the presence of failures elicited by lower stimulation intensities and the presence of a plateau for higher stimulation intensities. Abbreviations: BLA, Basolateral complex; CE, Central nucleus; Im, main intercalated nucleus. Panel A and B prepared by Dr Daniela Busti.

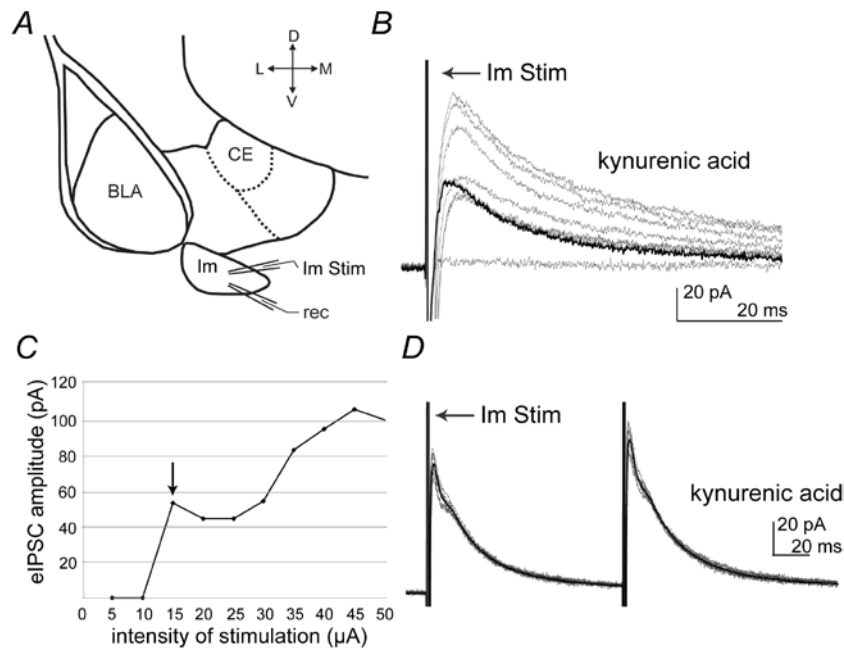


Figure 3.8. eIPSCs recorded in the Im.

A, scheme of the amygdala depicting the location of the stimulating and recording electrodes. B, voltage clamp recording showing IPSCs evoked by Im stimulation at increasing intensities. The average of 3 traces for each intensity (range, 5 - 50 μA , step of 5 μA) is shown in grey. An eIPSC after 15 μA stimulation is illustrated in black. C, the input-output curve shows the relation between the intensity of stimulation used and the peak amplitude of the eIPSCs. Each point of the graph represents the average value of three sweeps. Note the presence of failures elicited by lower stimulation intensities. The arrow indicates response evoked by "minimal" stimulation intensity. D, voltage clamp recording showing paired pulse facilitation of IPSC evoked by Im stimulation. Individual eIPSCs are shown in grey; the average of 10 traces is shown in black. All eIPSCs were recorded in the presence of kynurenic acid (3 mM), and were mediated by GABA_A receptors, since they were abolished by bicuculline (5 μM ; not shown). Abbreviations: D, dorsal; L, lateral; M, medial; V, ventral; BLA, Basolateral complex; CE, Central nucleus; Im, main intercalated nucleus; Rec., recording electrode, Stim., stimulating electrode.

Stimulation site	Success rate	Minimal peak amplitude (pA)	Stimulation intensity (μ A)	Area (fC)	Risetime (ms)	Decay (ms)	Latency (ms)
Im	6/6	94.9 $\pm$ 31.3	15	3.3 $\pm$ 0.6	0.8 $\pm$ 0.2	15.8 $\pm$ 1.5	0.8 $\pm$ 0.1
BLA	1/6	162.5	15	2.24	0.23	11.3	0.95
EC	2/6	48.9 $\pm$ 26.7	10 $\pm$ 5	3.3 $\pm$ 1.5	1.3 $\pm$ 0.8	12.5 $\pm$ 0.1	0.7 $\pm$ 0.1
IC	2/6	17.9 $\pm$ 8.2	17.5 $\pm$ 2.5	2.3 $\pm$ 1.0	1.7 $\pm$ 0.5	12.9 $\pm$ 2.7	3.3 $\pm$ 2.7

Table 3.3. Table summarizing quantitative analysis of eIPSC elicited by the lowest intensity of stimulation able to evoke a detectable current. All values are shown as mean $\pm$ SEM. Abbreviations: BLA, Basolateral complex; EC, external capsula; IC, intermediate capsula; Im, main intercalated nucleus.

3.3.6. Excitatory inputs to the Im

Next, excitatory inputs to the Im were studied by evoking EPSCs in response to extracellular minimal electrical stimulation. All cells were recorded using K-gluconate based internal solution at $V_H = -65$ mV. The eEPSCs were monitored in the presence of the GABA_A receptor antagonist bicuculline (5 μ M), and were abolished by kynurenic acid (3 mM, data not shown), indicating that they were mediated by ionotropic glutamate receptors. The eEPSCs were always detected when stimulating within the Im (6/6 cases). When two stimuli were applied in short succession (50 ms) in the Im an average paired pulse ratio of 1.5 ± 0.27 was observed. Furthermore, stimulation of EC or IC resulted in eEPSCs in 3/7 cases and 2/6 cases, respectively (Table 3.4). These results suggest that Im neurons can be controlled by cortical afferents, that make up the majority of fibres in EC and IC (LeDoux, 2000), as it is for mITC and lITC clusters (Quirk et al., 2003; Berretta et al., 2005; Marowsky et al., 2005). Some of the principal neurons of BLA have been observed, by using Golgi staining, to send axon collaterals to the Im (Millhouse, 1986). To test if these connections are functional, I placed a stimulating electrode in the BLA nucleus. I detected a functional monosynaptic connectivity when stimulating BLA in 2 out of 7 cases. However, the amplitude of such eEPSCs was small (11.5 and 16.4 pA) and they occurred with a marked delay time from the stimulation (6.7 and 5.9 ms). Synaptic jitter of eEPSCs latency was 0.3 ± 0.12 ms, 0.27 ± 0.15 ms, and 0.19 ± 0.06 ms when stimulating the Im ($n = 5$), IC ($n = 4$) or EC ($n = 3$), respectively, suggesting the monosynaptic nature of the responses (Doyle and Andresen, 2001). Likewise, the synaptic jitter of eEPSCs latency was 0.74 ms and 0.26 ms when the BLA was stimulated, consistent with monosynaptic excitation.

To determine whether responses after Im and EC or IC stimulation were mediated by distinct set of excitatory inputs I performed an occlusion test (Popescu et al., 2007). Two stimulating electrodes were placed in IC or EC and Im (Fig. 3.8A). The actual and predicted sums of eEPSCs amplitude evoked by two stimuli of

independent fibres were compared (Fig. 3.8B). If the response elicited by paired stimuli is smaller or bigger than the algebraic sum of individual responses, it provides evidence that the activated fibres are partly common. The response evoked by the paired stimuli of Im and IC was 115 % of the summed eEPSCs ($n = 5$). The response evoked by the paired stimuli of Im and EC was 102 % of the summed eEPSCs ($n = 5$). These data suggests that electrical stimulation of Im and IC/EC recruited distinct axonal fibres (Fig. 3.8B).

As a final point, I aimed to define the postsynaptic receptor components mediating eEPSCs observed in Im neurons. To examine the voltage dependence of eEPSCs, recordings were performed in the presence of bicuculline ($30\ \mu\text{M}$) to block GABA_A receptor activity and by using caesium-based intracellular patch solution to reduce outward potassium current and block GABA_B receptors (Gahwiler and Brown, 1985). In all cells tested ($n = 6$) the stimulation of the Im resulted in eEPSCs mediated by both AMPA- and NMDA-receptors components (Fig. 3.9A1). The peak amplitude of the eEPSCs mediated by AMPA-receptors components was measured 5 ms after stimulation artefact, at the peak of the response. The AMPA-receptor mediated component at these synapses had a linear $I-V$ relationship and reversal potential of $3.4\ \text{mV}$ (Fig. 3.9A2). However, due to the isolation of the current based solely on the time of its occurrence, I could notice a small NMDA-receptor mediated component, seen as altered of $I-V$ plot. NMDA-EPSCs were isolated by blocking AMPA receptors with NBQX ($50\ \mu\text{M}$) in the presence of bicuculline ($30\ \mu\text{M}$; Fig. 3.9B1). The peak amplitude of the NMDA-EPSCs was measured 10 ms after stimulation artefact, at the peak of the response. Application of D-AP5 ($100\ \mu\text{M}$, a selective NMDA receptor antagonist) in the presence of bicuculline and NBQX abolished the response, indicating that it was entirely mediated by NMDA receptors (data not shown). The NMDA-receptor mediated component at these synapses had a nonlinear $I-V$ relationship, with a region of negative slope between -50 and $+10\ \text{mV}$ and reversal potential of $8.1\ \text{mV}$ (Fig. 3.9B2).

Stimulation site	Success rate	Minimal peak amplitude (pA)	Stimulation intensity (μA)	Area (fC)	Risetime (ms)	Decay (ms)	Latency (ms)
Im	6/6	42.6 ± 14.7	19.2 ± 2.4	4.7 ± 1.3	2.0 ± 0.7	7.2 ± 1.2	1.36 ± 0.7
BLA	2/7	13.9 ± 2.5	42.5 ± 0.8	0.8	0.8 ± 0.1	10.1 ± 4.4	7.8 ± 1.1
EC	3/7	47.7 ± 27.8	22.5 ± 6.6	1.5 ± 0.3	1.3 ± 0.3	8.4 ± 1.4	2.9 ± 1.4
IC	2/6	32.2 ± 19.9	27.5 ± 7.5	27.5 ± 7.6	0.9 ± 0.7	3.6 ± 1.9	3.6 ± 3.2

Table 3.4. Table summarizing quantitative analysis of eEPSC elicited by the lowest intensity of stimulation able to evoke a detectable current. All values are shown as mean ± SEM. Abbreviations: BLA, Basolateral complex; EC, external capsula; IC, intermediate capsula; Im, main intercalated nucleus.

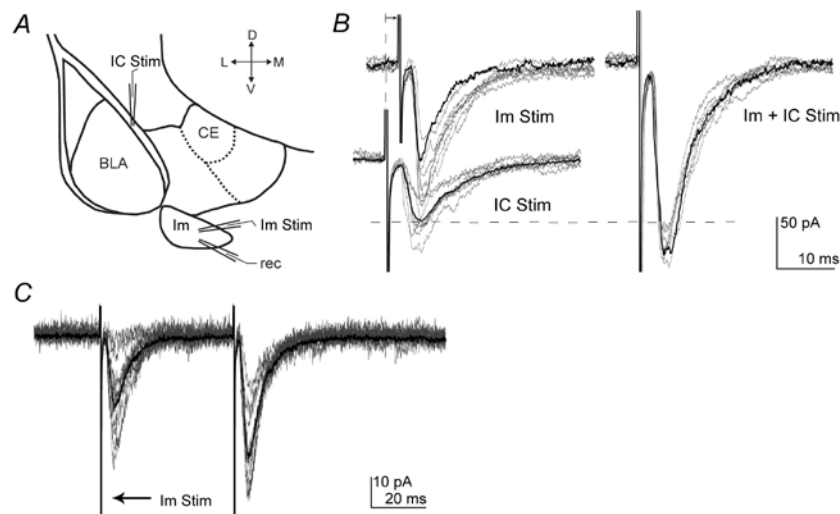


Figure 3.9. eEPSCs recorded in the Im - occlusion test.

A, scheme of the amygdala depicting the location of the stimulating and recording electrodes. B, eEPSCs recorded in the presence of bicuculline (5 μ M), individual eEPSCs are shown in grey; the average of 10 traces is shown in black. Left panel: representative EPSCs evoked by the independent stimulation of Im (peak amplitude = 100.9 pA) and IC (peak amplitude = 54.7 pA). The arrow next to the top traces indicates the time shift applied online to achieve simultaneous stimulation of IC and Im. Right panel: simultaneous activation of Im and IC (peak amplitude = 172.9 pA). In this representative example, the response evoked by the paired stimuli of Im and IC was 111% of the summed individual eEPSCs. C, voltage clamp recording showing paired pulse facilitation of EPSC evoked by Im stimulation. Individual eEPSCs are shown in grey; the average of 10 traces is shown in black. Abbreviations: D, dorsal; L, lateral; M, medial; V, ventral; BLA, Basolateral complex; CE, Central nucleus; IC, intermediate capsula; Im, main intercalated nucleus; Rec., recording electrode, Stim., stimulating electrode.

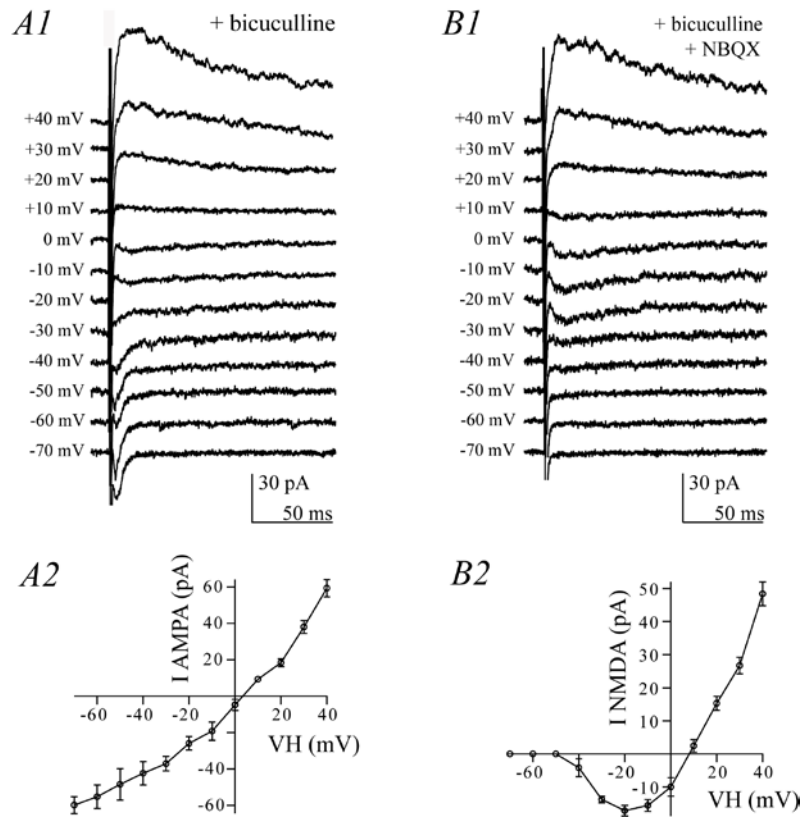


Figure 4.0. AMPA/NMDA receptors mediated eEPSCs recorded in the Im.

Voltage clamp recording after Im stimulation at V_H ranging from -70 mV to +40 mV. A1, AMPA receptor-mediated synaptic currents in the presence of bicuculline (30 μ M). A2, plot of the peak amplitude of AMPA receptor-mediated eEPSCs against V_H with the standard error indication. In this representative example, the EPSCs reversal potential was 3.4 mV. B1, NMDA receptor-mediated eEPSCs in the presence of bicuculline (30 μ M) and NBQX (50 μ M). B2, plot of the peak amplitude of NMDA receptor-mediated eEPSCs against V_H with the standard error indication. In this representative example, the EPSCs reversal potential was 8.1 mV.

3.4. Discussion

In this study, I provide new information about the Im nucleus of mouse amygdala.

3.4.1. Intrinsic membrane features of Im cells

I found that the passive membrane responses of Im cells, characterised by high R_{in} and low membrane capacitance, were typical of those measured in small neurons, and similar to those previously reported in other ITCs (Royer et al., 1999; Marowsky et al., 2005; Geracitano et al., 2007). One exception was RMP that was more depolarised in Im cells than in other ITCs. Furthermore, active membrane responses recorded from Im cells were also comparable to those recorded in other ITCs *in vitro*; for example, the presence of APs of rather low peak amplitude (~70 mV) (Royer et al., 1999). Cluster analysis of Im electrophysiological properties indicated the presence of three groups of Im neurons that differed in seven out of nine parameters. We previously noticed heterogeneity amongst mITCs in terms of short-term plasticity (Geracitano et al., 2007) as well as axonal patterns.

I also found that DA, which heavily innervates ITCs (Asan, 1998; Fuxe et al., 2003), reversibly hyperpolarised Im cells and depressed their AP frequency *via* DA D1, as reported in mITCs and lITCs (Marowsky et al., 2005), but not in other GABAergic cells of the BLA (Rosenkranz and Grace, 1999; Bissiere et al., 2003). Furthermore, ITCs displays a similar immunoreactivity for the μ OR (Likhtik et al., 2008), the alpha 3 GABA_A receptor subunit (Marowsky et al., 2004), the D1 receptor (Fuxe et al., 2003; Jacobsen et al., 2006), and several transcription factors (Kaoru et al., 2010). Taken together, this data suggests that ITC clusters share common characteristics and perhaps form a distinct system from other GABAergic cells of the amygdala.

3.4.2 Synaptic output of Im cells

Anatomical characterization of the recorded cells indicates that Im cluster consists of small to medium size, spiny neurons with bipolar dendritic organization and projecting axon. Since axons of Im cells were cut in the acute slice preparation they are likely to project outside the mouse amygdala, as it occurs in the cat (Pare and Smith, 1994). Axonal branches were detected in several amygdala nuclei within the slices. Axons showed variable lengths and trajectories in all subdivisions of the CE nucleus and in the BLA, and in the IC and EC where mITC and lITC clusters are located, respectively. Since electrophysiological data suggests that functional synapses originate from these axonal branches, this indicates that Im cells inhibit the BLA and CE nuclei, and possibly mITCs and lITCs. These findings add complexity to the view that the flow of information in amygdala is organized in dorso-ventral direction and that the ITC clusters serve mainly as a feedforward inhibitory gate (Pare et al., 2004; Amano et al., 2010).

3.4.3 Synaptic inputs of Im cells

In order to study this topic I placed an extracellular stimulating electrode in different areas of the amygdala and examined EPSCs or IPSCs evoked by minimal stimulation intensity. With this method, local neurons nearby the stimulating electrode are primarily stimulated, but it cannot exclude that other excitable processes such as dendrites or axons belonging to distant neurons could also have been activated. Local stimulation always elicited robust eIPSCs in Im cells, although local connectivity may be low since I failed to detect unitary IPSCs when pairs of Im neurons were tested. This is also consistent with an observation of relatively poor dendritic and local axonal branching of Im cells within the Im. Since I report here monosynaptic eIPSCs in some cases also after the stimulation of the IC and EC (where the mITC and lITC cluster are located) and BLA, it is likely that these events were evoked by GABA released from neurons with the soma outside the Im. The

relative fast kinetics of the eIPSCs recorded in the Im cells after “minimal stimulation” experiments were comparable to those we previously reported for most unitary IPSCs in paired recordings of mITCs (Geracitano et al., 2007). Presented results support the idea that ITC clusters are functionally connected in a predominantly dorso-ventral direction (Royer et al., 1999, 2000b). Surprisingly, an eIPSC was also detected when stimulating the BLA, suggesting that interneurons of the BLA may also provide extrinsic, in addition to local, inhibition (Bienvenu et al., 2012)

When synaptic inhibition was pharmacologically blocked, eEPSCs were always recorded after the local stimulation of the Im. Since there is no evidence that this nucleus contains glutamatergic cells, the eEPSCs are likely due to the stimulation of extrinsic glutamatergic fibres. The eEPSCs were probably monosynaptic owing to low synaptic jitter detected (Doyle and Andresen, 2001). Interestingly, the synaptic latency of the eEPSCs after the stimulation of the BLA was much longer than after the stimulation of the other nuclei of amygdala. This could be due to slow axonal conduction velocity and/or high axonal length as well as to the passive propagation of BLA principal cell's stimulation. Where do excitatory inputs come from? Potential excitatory sources include the IL, the thalamus and the BLA, the latter has been shown to send axon collaterals to the Im (Millhouse, 1986). It is important to keep in mind that the Im can also be contacted by axon collaterals emitted by passing fibres en route to other structures (especially to the central and medial nuclei of amygdala) (Millhouse, 1986).

Since mITCs undergoes NMDA-dependent long term depression and potentiation (Royer and Pare, 2002), I studied the type of excitatory receptor(s) expressed by Im cells. I found that the eEPSCs resulting from the Im stimulation had both a fast AMPA- and a slow NMDA-receptor component. Future studies should address the interesting possibility that Im cells display NMDA-dependent synaptic plasticity.

Furthermore, to understand how Im gates the flow of information, I investigated whether excitatory inputs onto the Im interact with each other using an occlusion test (Popescu et al., 2007). Two stimulating electrodes were placed within Im and IC or EC. The actual and predicted sums of values of peak amplitude eEPSCs elicited by two stimuli after independent and simultaneous stimulation were compared. If the latency of two events were different I corrected the onset of stimulation online in the way that the two stimulus artefacts appeared simultaneously. Alternatively, one can measure cross facilitation to test whether responses after Im and EC or IC stimulation were mediated by distinct set of excitatory inputs. Paired pulse facilitation (PPF or depression) is a measure of a short-term synaptic plasticity. In this paradigm, two presynaptic spikes are evoked in close succession and the responses of the postsynaptic cell are measured. The PPF index is the ratio of the postsynaptic response to the second presynaptic spike over the response to the first one. It reflects the release probability of the presynaptic cell. If the release probability is high, then the first pulse will deplete the available transmitter, and the second pulse will cause fewer transmitters to be release, leading to a low PPF. If the release probability is low, then the first spike will cause a small postsynaptic response, but the build-up of calcium in the presynaptic terminal will lead to an increased release probability on the second spike. In this test, one can measure if two sets of inputs are overlapping by stimulating them separately, in sequence with a short time interval. If the two set of inputs are overlapping then one could observe an interaction expressed as PPF index. The main limitation of both methods is the use of the extracellular stimulating electrode. Even with the use of minimal stimulation intensity one cannot exclude that adjacent regions are not also activated. Care has been taken to place the electrodes in similar position across all experiments. The results of the occlusion test were consistent with the stimulation of independent set of inputs. Within the limitation of the used approach, data suggests that heterogeneous excitatory inputs impinging

on Im cells may be spatially segregated along their somato-dendritic domain. Future studies by stimulating presynaptic fibres expressing channelrhodopsin or anatomical methods will be needed to further test this important point.

3.4.4. Physiological implications

The data suggest a role for the Im in relation to other nuclei of amygdala and therefore acquisition, expression and extinction of conditioned fear (LeDoux, 2000; Ehrlich et al., 2009; Pape and Pare, 2010). The Im projects to the basal forebrain (Pare and Smith, 1994), which in turns projects to the entire cortical mantle. In this way the Im could indirectly affect the activity of vast cortical areas, such as switching the fear response from freezing to risk assessment and exploration (Gozzi et al., 2010) or influence activity of basal forebrain afferents required for fear learning (Letzkus et al., 2011). How the Im could in principle affect fear memories? One possibility is that Im neurons serve as additional GABA release site, during emotional states that require increased level of GABA in the amygdala microcircuit. In this respect, *in vivo* studies have shown that increased level of GABA in the amygdala is a crucial feature of fear extinction (Chhatwal et al., 2005; Heldt and Ressler, 2007), and that BLA plays a critical role in fear extinction learning (Herry and Mons, 2004; Herry et al., 2008). Principal neurons of the BLA consist of discrete cell populations, one of them is active during fear extinction (Herry et al., 2008). Principal cells of the BLA send excitatory inputs onto mITCs that in turn provide feedforward inhibition to the CE, and this path is strengthened during fear extinction (Royer et al., 1999; Amano et al., 2010). The present findings that the Im also receives an excitatory input from the BLA and projects axonal branches to the CE indicate that the Im could be another source of feedforward inhibition to the CE. Consistent with this view, it was observed that extinction training and consolidation lead to the induction of an immediate early gene in the Im that persists during extinction retrieval (Busti et al., 2011). Moreover, fear extinction is dependent on

NMDA receptor activity, as *in vivo* injection of a NMDA agonist facilitates the acquisition of fear extinction (Mao et al., 2006). I show here that Im cells displayed NMDA receptor-mediated eEPSCs. This leads to the possibility that excitatory synapses onto Im neurons could express NMDA receptor-dependent LTP and that this plasticity would contribute to fear-related memories.

3.4.5. Conclusion

In summary, Im cells of the mouse amygdala have specific anatomical and functional features enabling them to actively participate in emotional processing. I suggest that the Im nucleus represents an additional site of integration of the distributed network underlying fear memories comprising the IL, amygdala and hippocampus (Myers and Davis, 2007; Quirk and Mueller, 2008).

3.5. Future work

The results obtained in this project represent the first electrophysiological characterisation of Im cells reported so far. However, several intriguing questions about the Im function remain to be elucidated. Future experiments ideally should combine the analysis of anatomy and physiology of these neurons with the investigation of their role in emotionally-relevant behaviours.

Analysis of the anatomy of the recorded cells showed the presence of axon branches in several amygdala nuclei, including other ITC clusters. Further studies should aim to fully reveal the anatomy of single cells within the Im. It would be extremely interesting to perform juxtacellular recording and labelling of single Im cells *in vivo* (Pinault, 1996) in rodent followed by reconstruction of the axonal arborisation and the identification of the postsynaptic targets. Furthermore, a combination of retrograde and anterograde dyes injection in different nuclei of the amygdala could provide further insight into the connectivity of the Im. A current approach would be to use optogenetic tools to characterise the physiological role of anatomically identified long-range projections to/from Im (Zhang et al., 2007). Fully elucidating the connectivity of the Im nucleus is essential to further hypothesise on its specific role in the brain during fear learning as well as in anxiety disorders.

My result indicate that Im cells displayed NMDA receptor-mediated eEPSCs. As fear extinction is dependent on NMDA receptor activity (Mao et al., 2006), my finding raises the question of whether excitatory synapses onto Im neurons express NMDA receptor-dependent LTP or LTD during fear extinction. In addition to the modulatory effect induced by D1-R agonist, it would be also interesting to study the modulation

of Im elicited by other receptors (such as μ OR) that are selectively expressed by neurons of the Im, especially during behaviourally relevant tests.

Moreover, the present data show that the Im receives an excitatory input from the BLA and projects to the CE. Recently, the formation of discrete neuronal populations, within main input and output nuclei of amygdala, was shown to contribute to distinct aspects of fear conditioning (Herry et al., 2008; Ciocchi et al., 2010). Can it be that Im neurons are targeted by specific subpopulation of BLA PNs? Although this is a very relevant question, currently we do not know any markers allowing distinguishing PN subpopulation in BLA, which would be unable to test this hypothesis.

Moreover there are two populations of CEm neurons that can be divided based on their distinct projection to the periaqueductal grey area (which is implicated in the freezing response) and to the dorsal vagal complex (which modulates cardiovascular responses (Viviani et al., 2011)). Similar specialisation of neurons based on their projections can be found in layer 5 of prefrontal cortex (Morishima and Kawaguchi, 2006) or visual cortex (Brown and Hestrin, 2009). Interestingly, in entorhinal cortex subpopulations of principal cells have different local inhibitory input depending on their long-range connectivity (Varga et al., 2010). This leads to the question of whether Im neurons selectively innervate distinct subpopulations of CE neurons, therefore influencing specific behaviours. It is possible to distinguish neurons within CEI by their electrophysiological characteristics or response to the oxytocin (Viviani et al., 2011), and this property may be used in the future to answer to this question.

My data suggest that the Im can be an additional source of GABA, required during various emotional states. In addition, our collaborators observed that extinction

training and consolidation lead to the induction of an immediate early gene in the Im that persists during extinction retrieval (Busti et al., 2011). It would be of interest to directly test the role of Im neurons during fear conditioning and extinction protocols. Since similar to the mITC neurons, Im neurons also express postsynaptic μ ORs, it would be possible to perform selective chemical lesion using peptide-toxin conjugate that target only Im (Likhtik et al., 2008). If activation of Im neurons and resulting release of GABA is crucial for behaviour, then the selective lesion of the Im region should impair the expression of fear extinction.

4. Part II: Neurogliaform cells of amygdala: a source of slow phasic inhibition within basolateral complex

4.1. Introduction

The BLA is the most important site of integration during fear conditioning (Herry *et al.*, 2008). This nucleus is a cortical-like structure consisting of glutamatergic PNs and local GABAergic inhibitory neurons (Pape and Pare, 2010). Interactions between these two cell classes determine the computation of emotional memories in the BLA. The role of GABAergic cells is particularly important, since their inhibitory input provides constant silencing of PNs of the BLA (Gaudreau and Pare, 1996). Suppression of inhibition can increase the firing rate and synchronisation of PNs during fear (Pare and Collins, 2000). Conversely, pharmacological impairment of GABAergic transmission induces anxiogenic-like effects (Makkar *et al.*, 2010).

In cortical structures diverse interneuron populations regulate the activity of PNs, therefore governing the state of neuronal assemblies (Klausberger and Somogyi, 2008). Specialised types of interneurons target specific sub-cellular domains of postsynaptic cells with high temporal precision (Klausberger and Somogyi, 2008). The IPSCs evoked by different interneuron types and recorded in postsynaptic cells show marked kinetic diversity (Buhl *et al.*, 1994; Nusser *et al.*, 2001a). Some phasic IPSCs are very fast (Bartos *et al.*, 2002), but others are 5-10 times slower (rise time > 3 ms, and decay time > 30 ms) (Tamas *et al.*, 2003). The slower events have been referred to as GABA_{A,slow} (Capogna and Pearce, 2011). This signal has been reported in many brain areas including hippocampus (Pearce, 1993; Banks *et al.*, 1998; Price *et al.*, 2005; Prenosil *et al.*, 2006; Price *et al.*, 2008; Karayannis *et al.*, 2010; Armstrong *et al.*, 2011; Markwardt *et al.*, 2011), neocortex (Tamas *et al.*, 2003; Olah *et al.*, 2007; Szabadics *et al.*, 2007; Olah *et al.*, 2009;

Wozny and Williams, 2011; Suzuki and Bekkers, 2012); striatum (Ibanez-Sandoval et al., 2011; English et al., 2012); thalamus (Huntsman et al., 1999; Schofield and Huguenard, 2007); cerebellum (Crowley et al., 2009); and brainstem (Kuo et al., 2009). The role of GABA_{A, slow} is likely to differ from that of the classical fast inhibition (Capogna and Pearce, 2011). This includes: modulation of NMDA-dependent synaptic plasticity (Kapur et al., 1997); control of glutamate release in neocortex (Olah et al., 2009); participation in hippocampal theta oscillations (Fuatealba et al., 2010); depolarisation of new born granule cells in the dentate gyrus (Markwardt et al., 2011); gating nicotinic inputs onto spiny projection neurons of striatum (English et al., 2012). A specific type of interneuron, termed NGFC is a source of GABA_{A,slow} in neocortex, hippocampus and striatum (Tamas et al., 2003; Price et al., 2005; Ibanez-Sandoval et al., 2011), in addition to the related Ivy cell type in the hippocampus (Fuatealba et al., 2008).

I report here that a novel type of the NPY-containing GABAergic cell, with dendritic and axonal patterns typical of the NGFC is the source of GABA_{A,slow} in the BLA. I have also investigated the mechanisms underlying slow IPSCs. The data suggest that low concentration and spillover of GABA to extrasynaptic sites are likely to underlie the slow kinetics of the IPSCs.

4.2. Materials and Methods

4.2.1. Acute slice preparation

All procedures involving animals were performed according to methods approved by the UK Home Office and The Animals (Scientific Procedures) Act 1986. Acute coronal slices were prepared from bacterial artificial chromosome (BAC) transgenic mice that express the humanized *Renilla* green fluorescent protein (hrGFP) under the control of the mouse NPY (stock 006417; The Jackson Laboratory) (Fig. 4.1.). Animals (postnatal day 16-25) were decapitated under deep isoflurane anaesthesia (4% in O₂), and their brains were rapidly removed and placed in ice-cold sucrose-containing ACSF cutting solution (containing in mM: 75 sucrose, 87 NaCl, 25 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 0.5 CaCl₂, 7 MgCl₂, 25 glucose, saturated with 95% O₂, 5% CO₂, at pH 7.3-7.4). Slices (325 µm thickness) containing amygdala were cut (Microm HM 650 V, Thermo Fisher Scientific Inc., Germany) and transferred on a nylon mesh where they were maintained in a chamber initially containing sucrose ACSF cutting solution at 37°C for 30 min. During this period the cutting solution was substituted with normal ACSF (containing in mM: 130 NaCl, 24 NaHCO₃, 3.5 KCl, 1.25 NaH₂PO₄, 2.5 CaCl₂, 1.5 MgSO₄, 10 glucose saturated with 95% O₂, 5% CO₂, at pH 7.3). Slices were then resting at room temperature (18-22°C).

4.2.2. Electrophysiology and analysis

Acute slices were secured under a nylon mesh, submerged and superfused with ACSF in a chamber mounted on the stage of an upright microscope (Axioskop, Zeiss, Jena, Germany). Slices were visualized with a 4×/0.1 NA or 60×/0.9 NA water-immersion objective (LUMPlanFI; Olympus, Tokyo, Japan) coupled with infrared and differential interference contrast (DIC) optics linked to a video camera

(CCD Camera C7500, Hamamatsu, Hamamatsu City, Japan). A mercury vapour short-arc lamp (100W, N HBC 103, Zeiss) was connected to the epifluorescence system to visualise the GFP-expressing neurons. Somatic whole-cell patch-clamp recordings (at $\sim 33^{\circ}\text{C}$) were made from visually identified cells using borosilicate glass capillaries (GC120F, 1.2 mm o.d., Clarke Electromedical Instruments, Reading, UK), pulled on a DMZ puller (Zeitz-instrumente GmbH, Munich, Germany) and filled with a filtered intracellular solution consisting of (in mM): 42 K-gluconate, 88 KCl, 4 ATP-Mg, 0.3 GTP- Na_2 , 10 Na_2 -phosphocreatine, 10 HEPES and 0.5% w/v biocytin (all from Sigma-Aldrich Co. Ltd, Poole, UK), osmolarity 270–280 mosmol without biocytin, pH 7.3 adjusted with KOH. Reversal potential (E_{Cl}) for Cl^- ions was -11 mV; the IPSC polarity was inward at the holding potential (V_h) of -65 mV. In a subset of experiments EGTA (10 mM; Sigma-Aldrich) was added to the internal solution of the presynaptic cell. A caesium-based intracellular solution was used to block K^+ channels consisting of (in mM): 42 Cs-methansulfonate, 88 CsCl, 10 HEPES, 10 Na_2 -Phosphocreatine, 4 Mg-ATP, 0.3 Na-GTP and 0.5% w/v biocytin (all from Sigma-Aldrich), osmolarity 270–280 mosmol without biocytin, pH 7.3 adjusted with CsOH. Biocytin was added to allow *post hoc* visualization of the recorded neurons. Resistance of the patch pipettes was 4-5 M Ω . Recordings were accepted only if the initial seal resistance was greater than 1 G Ω and series resistance did not change by more than 20% throughout the recording period. No correction was made for the junction potential between the pipette and the ACSF. All electrophysiological signals were amplified (10 mV pA^{-1} , EPC9/2 amplifier HEKA Elektronik, Lambrecht, Germany, PULSE software), low pass filtered at 2.9 kHz, digitized at 5 or 10 kHz. The amplifier was controlled from a personal computer running the PULSE data acquisition and analysis programme (HEKA). Currents/voltages were acquired online with Pulse software (HEKA) and analysed offline with Pulsefit (HEKA) and IGOR Pro5.05 (Wavemetrics Inc, Oregon, USA). The input resistance (R_{in}) was calculated from the slope of a line fitted to the subthreshold range on a plot of the

injected current *versus* the steady-state membrane voltage when a family of hyperpolarising and depolarising current injections were applied (range, -30/+120 pA). The apparent membrane time constant (τ) was calculated by fitting a single exponential to the response of the cell to a current injection of -50 pA in current clamp mode. Membrane capacitance was calculated as τ/R_{in} . To study the kinetics of action potentials, a depolarising current step (3 ms, 100-150 pA) was applied. Peak amplitude and membrane afterhyperpolarization (AHP) were measured from the initial point of the action potential raising phase by a user. Action potential half-width were measured from the initial point of the action potential raising phase by a user-defined program in IGOR. The code of the procedure, accompanied by description of functions is written in chapter 3.2.2. The adaptation index was calculated as the ratio between the first and last interspike intervals evoked by a 1 s depolarising current pulse. Presynaptic and postsynaptic neurons were voltage clamped at $V_h = -65$ mV. After obtaining whole-cell configuration, I waited 5 min before starting a protocol to ensure diffusion of internal solution containing Cl^- into the recorded neurons (Fig. 4.2.). Action currents were elicited in a presynaptic cell (3 ms, 100 pA) and the corresponding unitary IPSC was recorded in synaptically coupled postsynaptic neuron or in the presynaptic neuron as an autaptic IPSC. The inter-stimuli interval was usually 120s to ensure stable and non-depressing IPSCs during repeated stimulation, consistent with other NGFC-mediated IPSCs (Fig. 4.3.) (Tamas et al., 2003; Price et al., 2005). A longer time of 180s was used as the interval between 5Hz or *in vivo* firing stimulation (see below). Kinetic analysis and exponential fitting of synaptic responses were performed in Igor Pro. The measurement of the rise time of a current starts by manually setting the event's starting point and the peak amplitude of this current. If an artefact of stimulation is present in the recording, the initial point is set at a level equal to the control current. It is important to keep in mind that such a technique may under/over-estimate

measured rise time. In a subset of recordings, evoked action current partially masked the presence of the rising phase of the autaptic IPSC. In this case, the rise time was estimated from a line fitted to the remaining trace, only if it constituted more than 20% of the total peak amplitude. Spontaneous IPSC (sIPSC) were recorded at $V_h = -65$ mV for 60s and analysed using Minianalysis software (Synptosoftware, Decatur, GA, USA). The peak amplitude, the 20-80% rise time, decay time was calculated by fitting a single exponential in each trace. The decay of the averaged postsynaptic and autaptic uIPSCs could be also fit with a double exponential, and the weighted decay time constant was calculated using the following formula: $\tau_w = \tau_1 A_1 + \tau_2 (1 - A_1)$, where τ_w is the weighted decay time constant, τ_1 and τ_2 are the time constants of the first and second exponential functions, respectively, and A_1 is the proportion of the peak amplitude of the averaged uIPSC that is contributed by τ_1 .

In vivo firing patterns of NPY+ cells were recorded in anaesthetised rats with the juxtacellular/extracellular method (Bienvenu et al., 2012). Eleven action potentials recorded in NPY+ during theta oscillations were injected into whole-cell patch-clamped cells as voltage pulses (1 ms, 100 pA) using Matlab (The MathWorks) and Pulse (HEKA) software. The stimuli evoked a train of action potentials *in vitro* that exactly matched the sequence of action potentials detected *in vivo*.

Statistical tests and analysis were performed using SPSS 17.0 (SPSS, Chicago, IL). Data are presented as mean $\pm$ S.E.M.; non-parametrical two-tailed tests (Mann-Whitney, Wilcoxon single ranks, Kruskal-Wallis) were used to assess the significance of differences between groups of data.

4.2.3. Histological processing of recorded cells

After recordings, the slices were immersed for 14-18h in a fixative solution containing: 4% paraformaldehyde, 15% (v/v) saturated picric acid and 0.1 M phosphate buffer (PB; pH 7.2-7.4; at 4°). Gelatine-embedded slices were re-sectioned into 60 µm thick sections. Sections with biocytin-filled cells were incubated in avidin-biotinylated horseradish peroxidase complex (1:100 dilution; ABC Kit; Vector Laboratories, Burlingame, CA) followed by a peroxidase reaction using DAB (Sigma-Aldrich; 0.05%) as the chromogen and 1% H₂O₂ as the substrate. Sections were then mounted on gelatine-covered slides, air-dried, immersed in xylene-based mounting medium (Entellan; Merck, Darmstadt, Germany) and cover-slipped. Neurons were drawn (63x magnification) using a drawing tube attached to a light microscope.

4.2.4. Electron microscopy

After recordings, slices were immersed for 14-18h in a fixative solution containing: 4% paraformaldehyde, 0.05 glutaraldehyde, 15 % (v/v) saturated picric acid and 0.1 M PB (pH 7.2-7.4; at 4°). Slices were incubated in 1% H₂O₂ to block endogenous peroxidase activity, embedded in gelatine, and re-sectioned at 60 µm thickness. After cryoprotection in 0.1M PB with 10% and 20% sucrose (at 4° for 1h) the sections were freeze-thawed in liquid nitrogen. Sections with biocytin-filled cells were incubated in avidin-biotinylated horseradish peroxidase complex (1:100 dilution) followed by a peroxidase reaction using DAB (Sigma-Aldrich; 0.05%) as the chromogen and 1% H₂O₂ as the substrate. All sections were post-fixed in 1% osmium tetroxide (Oxkem, UK) in 0.1 PB for ~45 min. The sections were dehydrated through a graded series of ethanol dilutions (50%, 70%, 90%, 95%, 100%; 2x10 min for all dilutions). To increase contrast in the EM 1% uranyl acetate was included in the 70% ethanol solution (~45 min). Next, sections were washed (2x20 min) in propylene oxide and infiltrated with resin for ~8 h (Durcupan; Fluka Chemical).

Sections were mounted in resin on glass microscope slides and polymerized at 60° for 24 h. The soma, axon and dendrites of labelled neurons were examined at high magnification, under a light microscope. The areas of interest were cut from the embedded sections and re-embedded in Durcupan blocks for sectioning. Ultrathin (70 nm) serial sections were collected on Pioloform-coated single slot copper grids. The ultra-thin sections were then contrasted with lead citrate for 1-2 min and examined using a Philips CM-100 electron microscope.

4.2.5. Chemicals and drugs

All drugs were applied to the slice *via* bath solution. Salts used for the patch pipette and ACSF were obtained either from VWR International (Lutterworth, UK) or Sigma-Aldrich (Dorset, UK). Kynurenic acid (3 mM, Sigma-Aldrich) was added routinely to ACSF. Drugs obtained from Tocris (Cookson Ins., Avonmouth, UK) were added at the following concentrations: SR95531 (also termed gabazine, 5 μ M), TPMPA (75 μ M), dextran (1.25 mM), CGP35348 (50 μ M), CI218872 (1 μ M), L655708 (25 nM). RO4938581 (500 mM) was donated by Hoffmann-LaRoche (Basel, Switzerland).

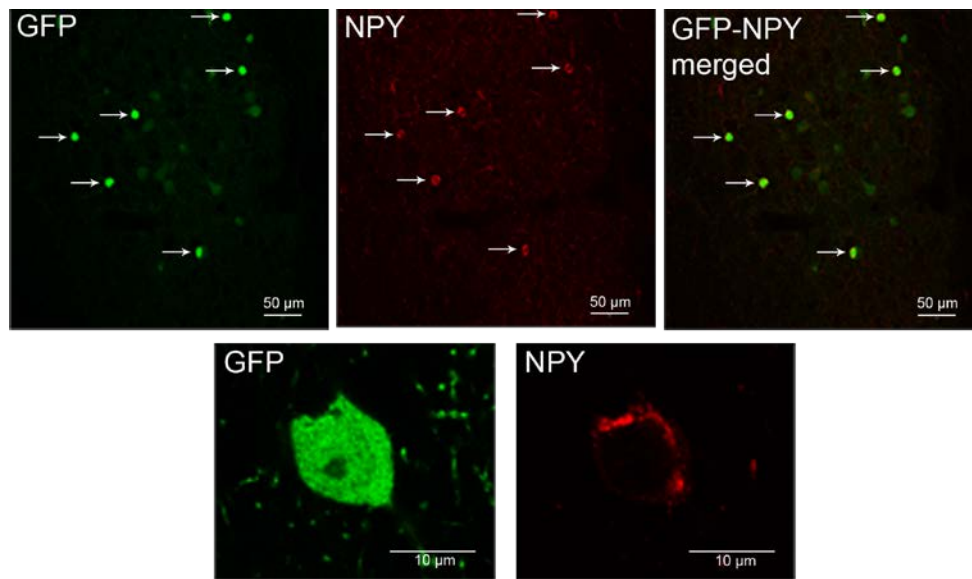


Figure 4.1. NPY-hrGFP BAC transgenic mice. Neuropeptide Y immunoreaction on control tissue. Confocal images, 20x, from BLA region (upper panels) and 63x (lower panels).

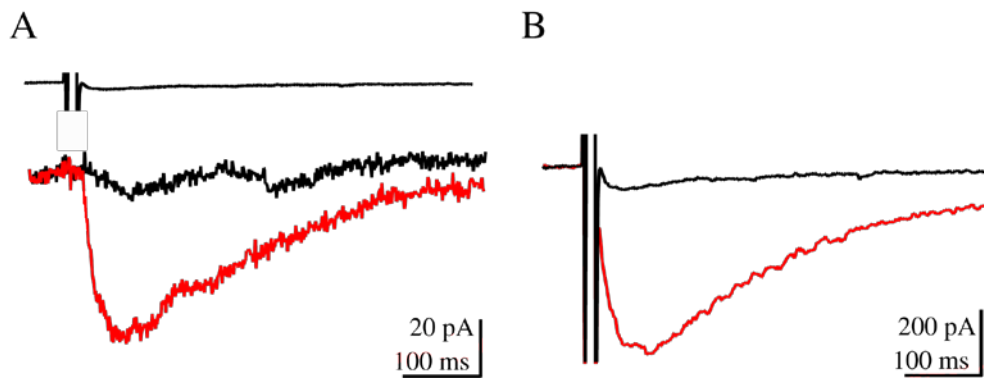


Figure 4.2. Diffusion of intracellular Cl^- contained in the pipette solution.

Change in the amplitude of uIPSCs (A, postsynaptic current; B, autaptic current; top trace in A shows a truncated presynaptic action current) from the onset of whole-cell patch clamp recording (black trace) and after 5 min (red trace). Note the increase in the size of the uIPSCs.

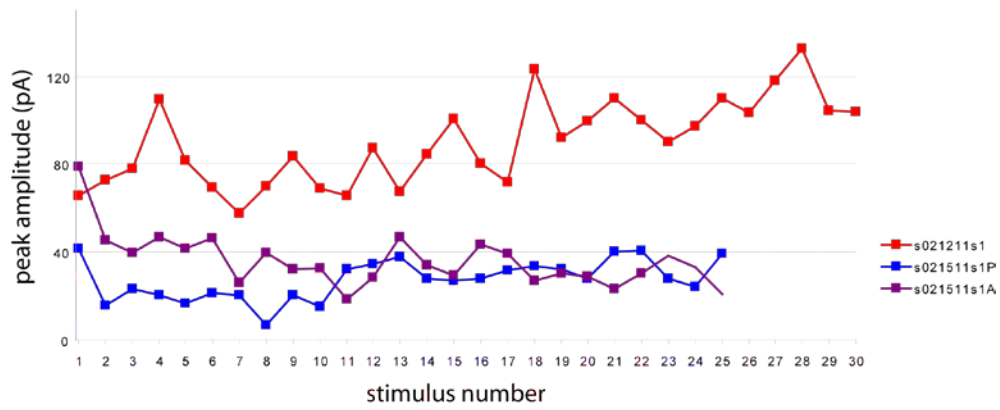


Figure 4.3. Stability of the uIPSCs.

Three uIPSC during ~ 1h of repeated stimulation (25 or 30 stimuli). The inter-stimuli interval is 120s to ensure stable and non-depressing IPSCs.

4.3. Results

4.3.1. Neurogliaform cells are the source of slow phasic inhibition in the BLA

All recorded NPY-GFP+ cells were filled with biocytin to analyse their dendritic and axonal aspects (Fig. 4.4., Fig. 4.5.). I observed that these neurons had a round cell body and short, sparsely spiny dendrites arranged in a stellate fashion around the soma. The axon branched profusely mostly close to the soma, displaying frequent, small *en passant* boutons. It also produced a dense local plexus that is the landmark of these cells (Fig. 4.4., Fig. 4.5). The axon and dendrites were segregated within the BLA suggesting an interneuron-like, local function. The soma appearance as well as the dendritic and axonal patterns closely resembled NGFCs reported by anatomical studies of amygdala in other species (Tombol and Szafranska-Kosmal, 1972; McDonald and Culberson, 1981; McDonald, 1982b), and I will refer to them as such. All cells were recorded using Cl⁻ based internal solution at $V_H = -65$ mV, unless stated otherwise. The injection of hyperpolarising-depolarising current pulses from the resting membrane potential revealed some intrinsic functional aspects of these neurons ($n = 13$, Fig. 4.6., *Table 4.1.*). In particular, NGFCs exhibited a relatively broad action potential (Fig. 4.6. *B*), a relatively low input resistance (Fig. 4.6 *E,F*), pronounced spike AHP and a delayed firing in response to a depolarising current pulse of the rheobase intensity (Fig. 4.6. *A,C*). Larger depolarising current steps elicited accommodating spike trains (Fig. 4.6. *D*). Next, I performed whole-cell patch-clamp recordings of pairs between presynaptic NPY-GFP+ cells and postsynaptic PNs of the BLA. These experiments were usually performed in the presence of kynurenic acid, an antagonist for the ionotropic glutamate receptor (3 mM) to prevent excitatory network activity, but identical results were obtained in experiments performed in control ACSF. An action current in the presynaptic cell elicited a unitary IPSC in the postsynaptic cell (postsynaptic uIPSC, 98/128 cases, Fig. 4.7.A). This protocol also elicited an inward current in the presynaptic cell

occurring shortly after the action current (therefore referred as an autaptic uIPSC, 86/98 cases, Fig. 4.7.B). The reconstruction of a representative connected pair is shown in figure 4.8. On average, the postsynaptic and autaptic uIPSCs peak amplitude was 143.1 ± 17.6 pA ($n = 58$) and 135.7 ± 14.0 pA ($n = 51$); the 20-80% rise time was 3.00 ± 0.15 ms ($n = 58$) and 4.02 ± 0.55 ms ($n = 37$); the fitted decay time constant was 41.60 ± 2.02 ms ($n = 58$) and 74.80 ± 6.00 ms ($n = 45$), respectively (Fig. 4.7.C). When the decay of the IPSCs was fitted with τ_w the values were similar (41.51 ± 2.83 ms and 71.66 ± 5.73 ms, for postsynaptic and autaptic uIPSCs, respectively). The fitted decay of autaptic IPSC was significantly slower than that of postsynaptic IPSC ($p < 0.001$), but the rise time and the peak amplitude did not significantly differ ($p > 0.05$). Rise time and decay time constant of the IPSCs were positively correlated (Pearson correlation, $R = 0.68$ for postsynaptic and $R = 0.63$ for autaptic uIPSCs, $p < 0.001$, Fig. 4.7.D). Since the kinetics of postsynaptic and autaptic IPSCs matched closely that described in the literature as GABA_{A,slow} (Capogna and Pearce, 2011) I refer to them as such or as 'slow uIPSCs'. Notably, presynaptic action currents evoked slow uIPSCs always without failures (Fig. 4.7.A, B). Both currents were abolished by the GABA_A receptor antagonist SR95531 (5 μ M) and no other synaptic component was detected ($n = 17$, Fig. 4.7.A, B). The remaining outward current seen in the presynaptic cell (Fig. 4.7.B) was mediated by Ca²⁺-dependent voltage gated K⁺ channels, as this current reversed at V_h of -90 mV ($n = 8$, Fig. 4.9.). I tested the potential influence of the AHP outward current on the autaptic uIPSC kinetics. In the following subset of experiments, cells were recorded using Cl⁻ and EGTA based internal solution at $V_h = -65$ mV. The inclusion of the Ca²⁺ chelator EGTA (10 mM) into the pipette internal solution blocked the AHP outward current, as expected (Faber and Sah, 2002). In the presence of EGTA the rise time and decay of the autaptic uIPSC was not significantly different from that recorded with control patch solution ($n = 5$, $p > 0.05$ for all tested parameters, Fig. 4.9.). Moreover, no electrical synapses were detected in synaptically-coupled cells

(n = 6, data not shown), as a hyperpolarising current pulse injected in a NPY+ cell did not evoke a detectable voltage deflection in a PN.

As previously mentioned, some presynaptic NPY+ cells did not evoke uIPSCs (30/128), namely they were not synaptically-coupled to PNs. We noticed that in a few cases (n = 8, Fig. 4.10.), some passive and active membrane responses in these NPY+ cells were different than those observed in NGFCs. Specifically, the R_{in} was $514 \pm 37 \text{ M}\Omega$ ($p < 0.001$ compared to NGFC data shown in Table 4.1), the membrane τ was $38.7 \pm 7.9 \text{ ms}$ ($p < 0.001$), and the adaptation index was 0.54 ± 0.08 ($p < 0.01$). This suggests that these NPY+ cells were not NGFCs consistent with the heterogeneity of NPY+ cells observed in other brain areas (Karagiannis et al., 2009; Ibanez-Sandoval et al., 2011). However, since the aim of my study was to identify the cell type and the mechanisms involved in $\text{GABA}_{A,slow}$ in the amygdala, I have not further examined this issue.

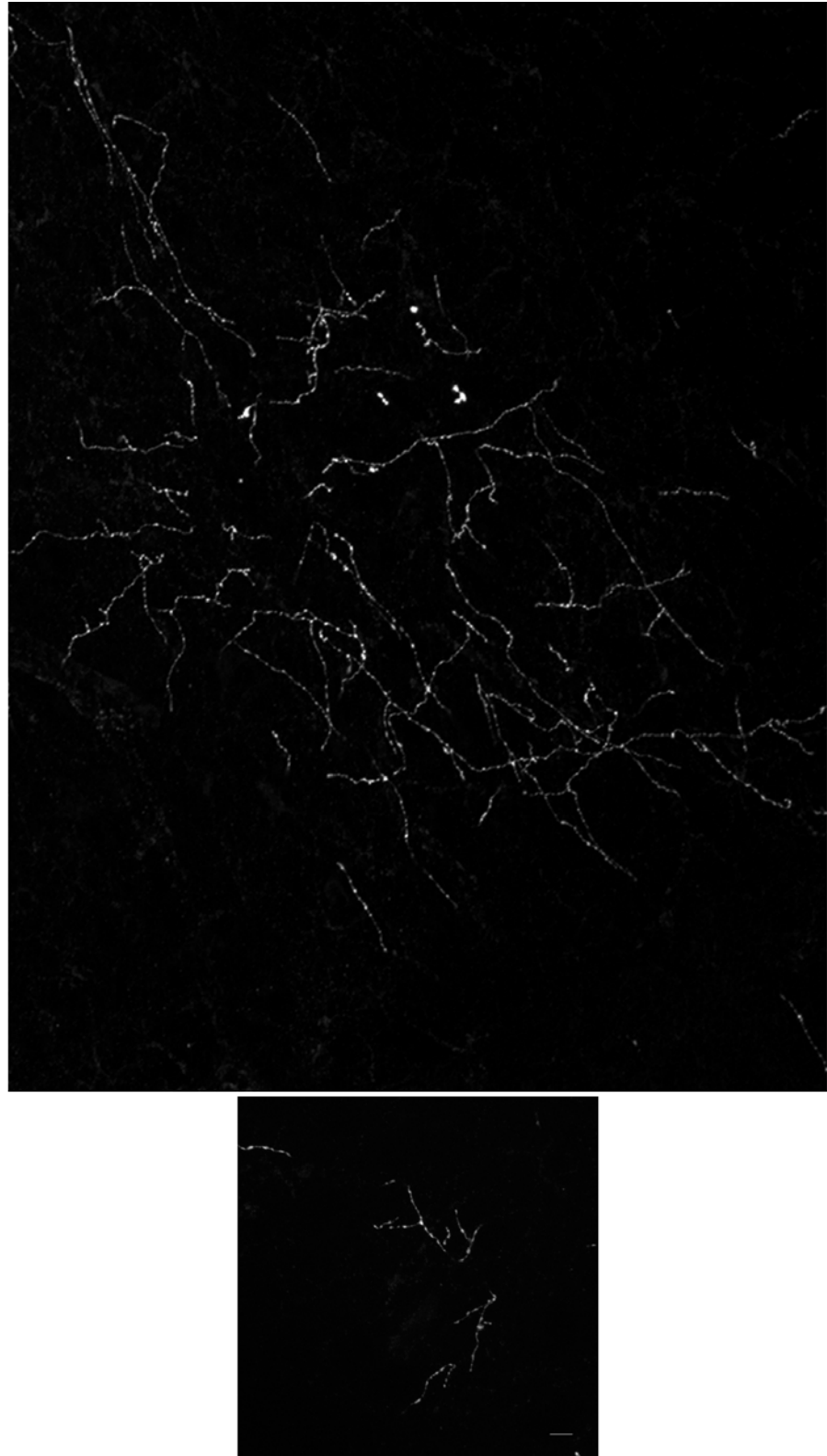


Figure 4.4. Axonal arborisation of an *in vitro* recorded NPY-GFP positive cell. Single section apotome picture, 40x, of the axon, third section after the soma (60 μm thickness). Confocal image of axon, 63x, fourth section from the soma.

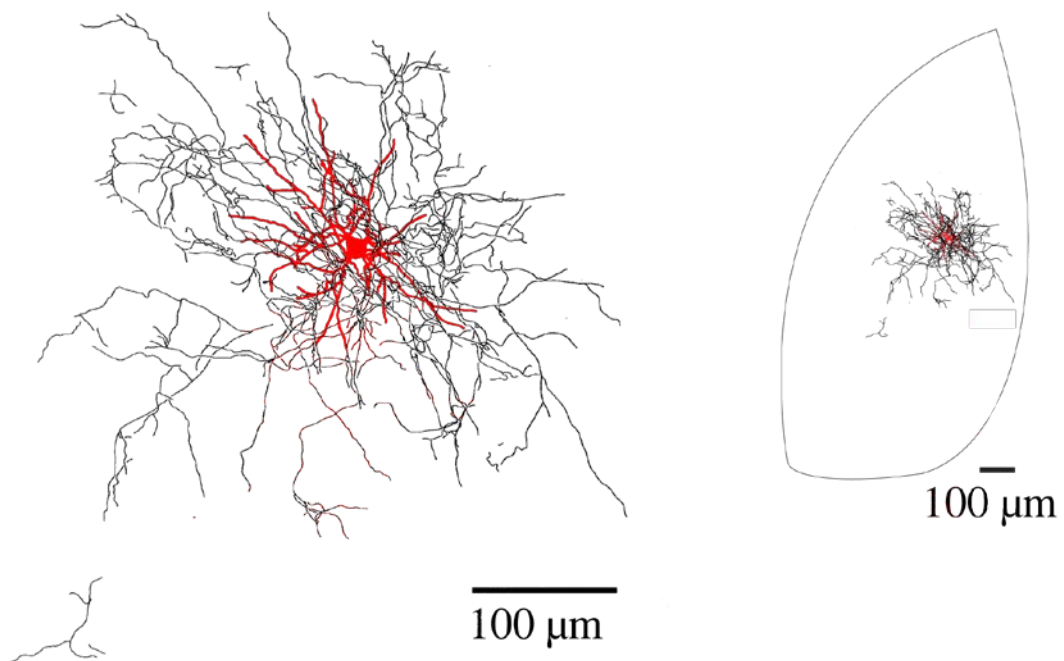


Figure 4.5. Dendritic and axonal patterns of NGFC in the BLA.

Light microscopic reconstruction (63x) of a biocytin-labelled NPY+ cell (red, soma and dendrites; black, axon). The location of the reconstructed cell within the BLA boundaries are also illustrated.

parameter	AVG $\pm$ SEM (n= 13)
R _{in} (M Ω)	287.9 $\pm$ 23.3
AP width (ms)	0.92 $\pm$ 0.06
AP amplitude (mV)	64.9 $\pm$ 4.1
AHP amplitude (mV)	33.9 $\pm$ 1.8
Adaptation index	0.27 $\pm$ 0.04
Membrane τ (ms)	13.6 $\pm$ 1.4
C _m (nF)	0.05 $\pm$ 0.01

Table 4.1. Summary of passive and active membrane responses of NPY-GFP+ neurons. Data shown as mean $\pm$ SEM. R_{in}, input resistance; C_m, membrane capacitance; AHP, afterhyperpolarization; AP, action potential.

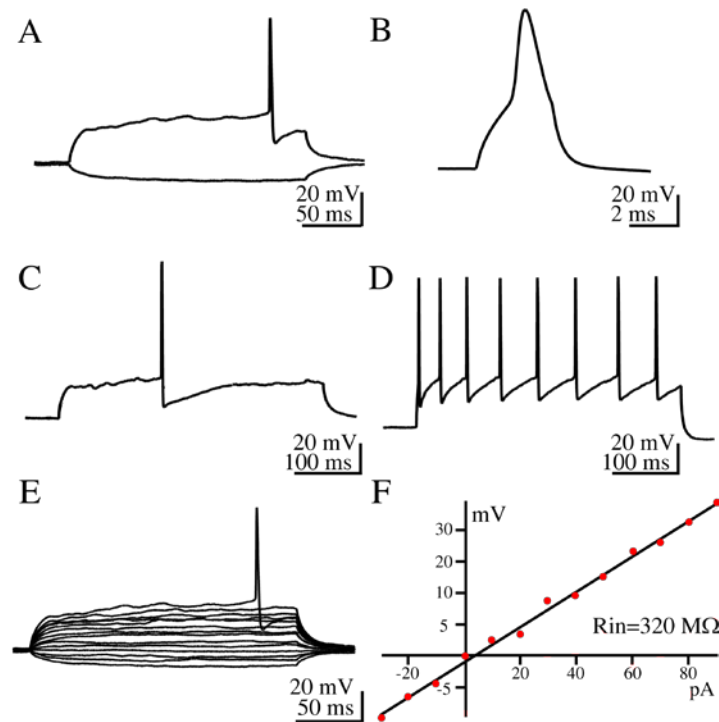


Figure 4.6. Firing patterns of NGFCs in the BLA.

A-E, passive and active electrophysiological responses of a representative NPY+ cell. A, responses of a NPY+ cell to injected hyper- and depolarising current pulses (- 30 pA and 70 pA, 200 ms), note late firing. B, action potential evoked by a short depolarising current pulse (100 pA, 3 ms). C, D, responses of a NPY+ cell to injected depolarising current pulses, showing late firing (120 pA, 400 ms, C) and frequency adaptation (280 pA, 400 ms, D). E, F, voltage traces in response to current pulses (- 30 to + 90 pA, 400 ms, E) and the I/V plot used to measure the cell R_{in} (F).

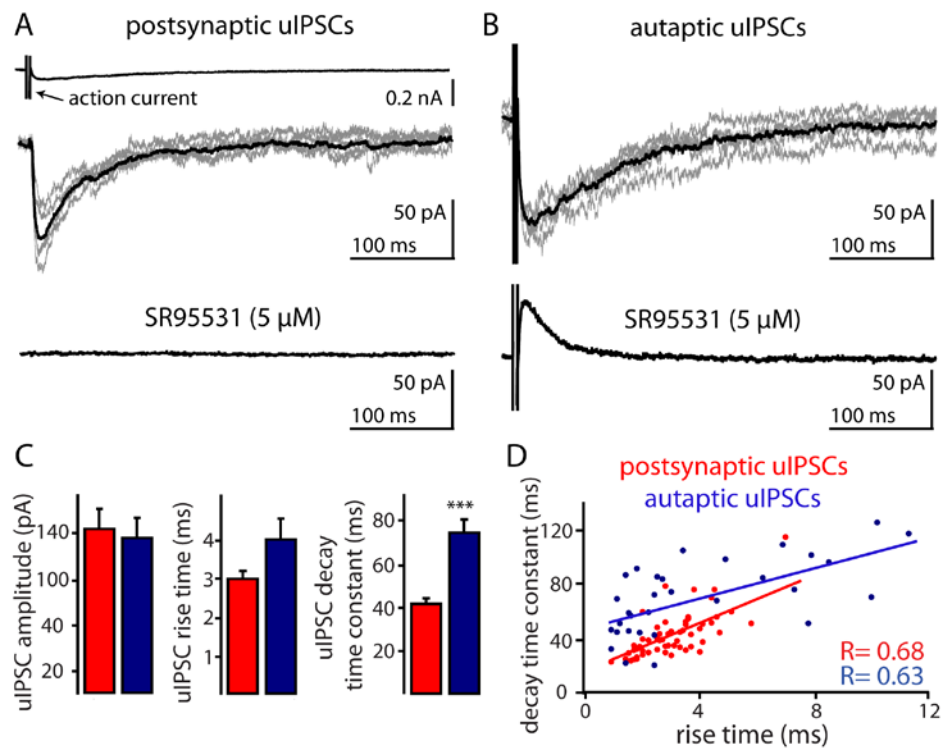


Figure 4.7. NGFCs evoke postsynaptic and autaptic slow uIPSCs.

A, short square depolarising voltage pulse applied to a presynaptic NPY-GFP+ cell elicited an action current (top trace), which evoked a slow uIPSC in a postsynaptic PN of the BLA (middle trace) and an autaptic uIPSCs in the NPY-GFP+ cell, B. Individual uIPSCs are shown in grey; average of 5 traces is shown in black. Bath application of the GABA_A receptor antagonist SR95531 (5 μ M) abolished postsynaptic (A, lower trace) and autaptic uIPSCs (B, lower trace). The outward current (B) is mediated by voltage gated K⁺ channels. C, quantification of 20-80% rise time, decay time and peak amplitude of postsynaptic and autaptic uIPSCs. Statistical significance is noted (Mann-Whitney test; *** $p < 0.005$). Data shown as mean $\pm$ SEM. D, scatter plot of decay time versus rise time of autaptic and postsynaptic uIPSCs (Pearson correlation, $R = 0.68$ for postsynaptic and $R = 0.63$ for autaptic uIPSCs, $p < 0.001$).

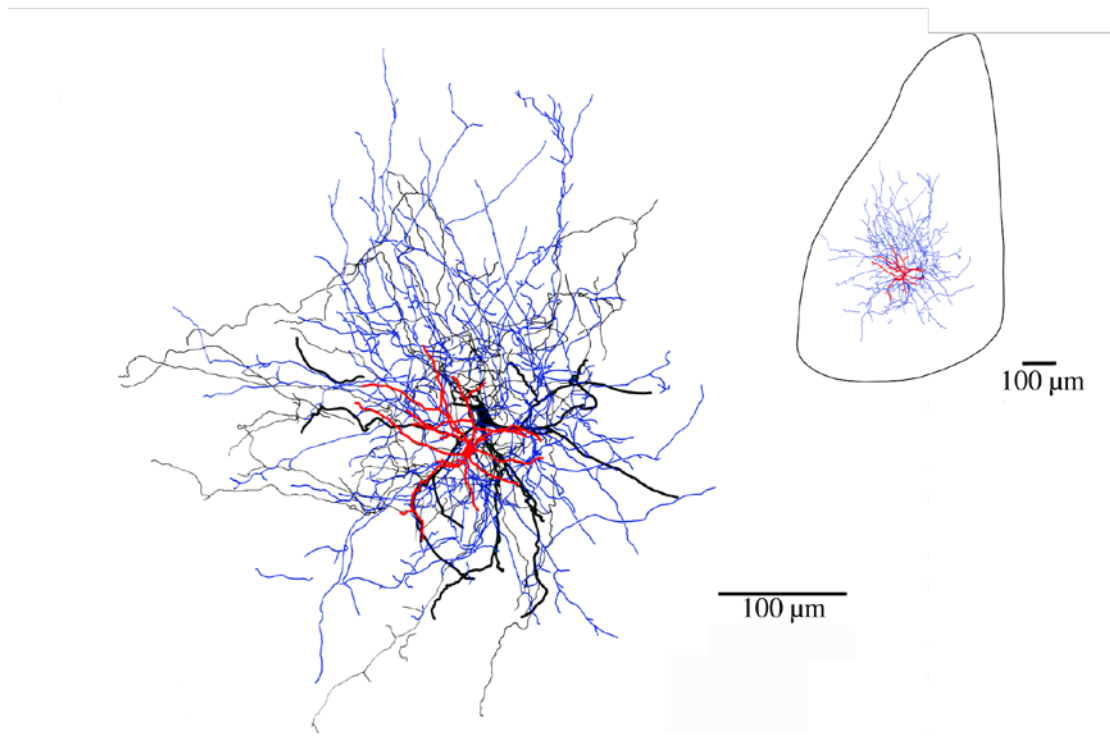


Figure 4.8 Light microscopic reconstruction (63x magnification) of a biocytin-labelled presynaptic NPY+ and postsynaptic PN cells.

Soma and dendrites (thick), of the presynaptic cell are shown in red, the axon (thin) is shown in blue; soma and dendrites (thick), axon (thin) of the postsynaptic cell are shown in black. The insert shows the location of the reconstructed cell within the BLA boundaries.

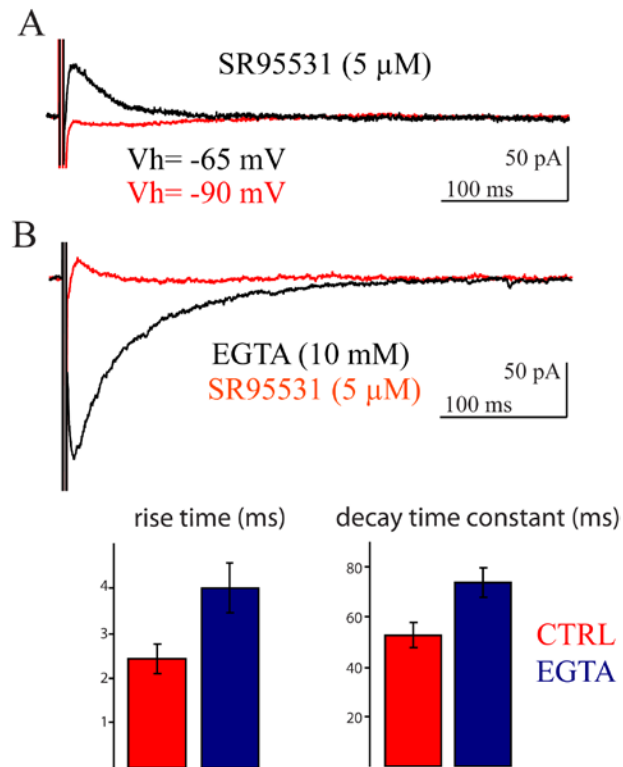


Figure 4.9. Voltage gated K^+ channels do not influence autaptic uIPSC kinetics.

A, bath application of GABA_A receptor antagonist (5 μ M SR95531) abolished autaptic uIPSCs (control current not shown). The remaining outward current is mediated by voltage gated K^+ channels as it is abolished by change of the holding potential (V_h) from -65mV to -90 mV, a value close to the E_K (red trace, $n = 9$). B, example of an autaptic uIPSC recorded at V_h of -65 mV with the addition of EGTA (10 mM) into the patch pipette solution. The Ca^{2+} chelator EGTA strongly reduces the outward current but does not make the autaptic uIPSCs faster ($n = 5$, $p > 0.05$, unpaired t-test). Bath application of 5 μ M SR95531 abolished autaptic uIPSCs unmasking a small residual outward current (red trace, $n = 5$). Histograms show the effect of the addition of EGTA into the patch pipette solution on kinetics of slow sIPSCs (in blue) compared to control patch pipette solution (in red).

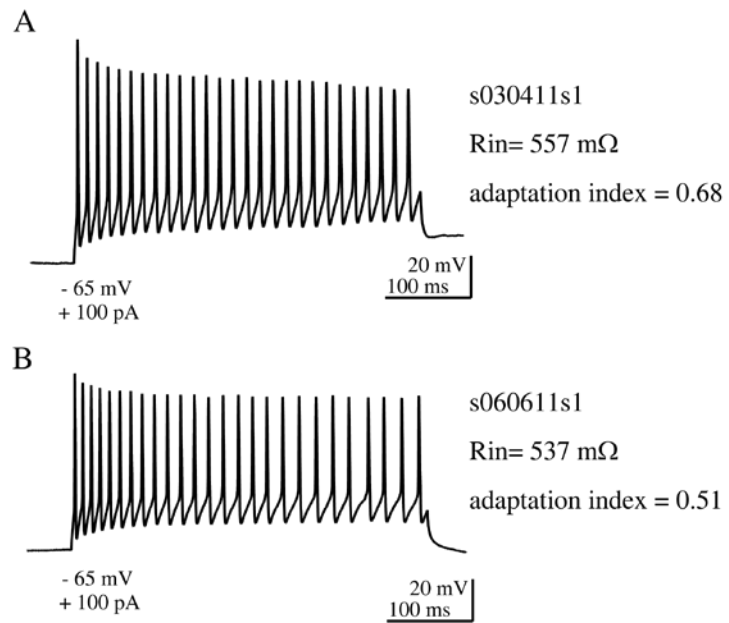


Figure 4.10. Firing patterns of NPY+ non-NGFCs in the BLA.

A, B, responses of two NPY+ cells to injected depolarising current pulse.

4.3.2. Low concentration of extracellular GABA causes slow NGFC-IPSC

The kinetics of slow uIPSC after stimulation of NGFCs of the BLA was much slower than uIPSCs evoked by any type of interneuron of the BLA tested so far (Woodruff and Sah, 2007a; Spampinato et al., 2011). What causes such prolonged synaptic inhibition? I hypothesised that the specific spatiotemporal concentration profile of GABA at the synapse determines the IPSC kinetics. To test this idea and assess the transmitter concentration at the postsynaptic receptors of PNs, first, I compared the effect of a low affinity, fast unbinding (~ 0.6 ms) (Jones et al., 2001) competitive GABA_A receptor antagonist TPMPA (75 μ M) on the slow uIPSC and on fast spontaneous IPSCs (fast sIPSCs) recorded from PNs. This antagonist should be more effective on IPSCs evoked by lower extracellular GABA concentration (Barberis et al., 2011). Bath application of TPMPA reduced the amplitude of slow uIPSC ($n = 16$, decrease to 41.9 % of initial value, $p < 0.05$, Fig. 4.11.A, C), but produced a non-significant decrease on the amplitude of fast sIPSC ($n = 5$, to 81.6 % of initial value, $p > 0.05$, Fig. 4.11.B, C). Moreover, TPMPA had no significant effect on the rise time and decay time of slow uIPSC or fast sIPSC (all p values > 0.05 , Fig. 4.11.C), although it tended to decrease the decay of slow uIPSC, a result consistent with transmitter spillover (Overstreet and Westbrook, 2003). Summary of data obtained with TPMPA is presented in Table 4.2.

The GABA_A receptor $\alpha 1$ subunit agonists potentiate the IPSC amplitude to various extents depending on the degree of receptor occupancy (Hajos et al., 2000). If NGFC elicits a lower concentration of transmitter compared to other interneurons, then slow IPSCs should be more sensitive to $\alpha 1$ agonists than fast IPSCs. To test this idea I used the selective $\alpha 1$ agonist CI218872, since it displays a greater selectivity for GABA_A receptors containing the $\alpha 1$ subunit than the more widely used zolpidem (Korpi et al., 2002). The application of CI218872 (1 μ M) significantly increased the amplitude of slow uIPSCs without significantly affecting their kinetics ($n = 14$, $p < 0.05$, Fig. 4.12.A, B). In contrast, this drug had no effect on the amplitude

and kinetics of fast sIPSCs recorded from PNs ($n = 5$, $p > 0.05$ Fig. 4.12.C, D). Summary of data obtained with CI218872 is presented in Table 4.2. These results indicate that GABA_A receptors were not saturated by GABA released from NGFCs, and also suggest that the $\alpha 1$ subunit is present at these synapses.

Taken together, these results suggest that compared to other interneurons of the BLA, transmitter release from NGFCs results in lower GABA concentration at the postsynaptic receptors.

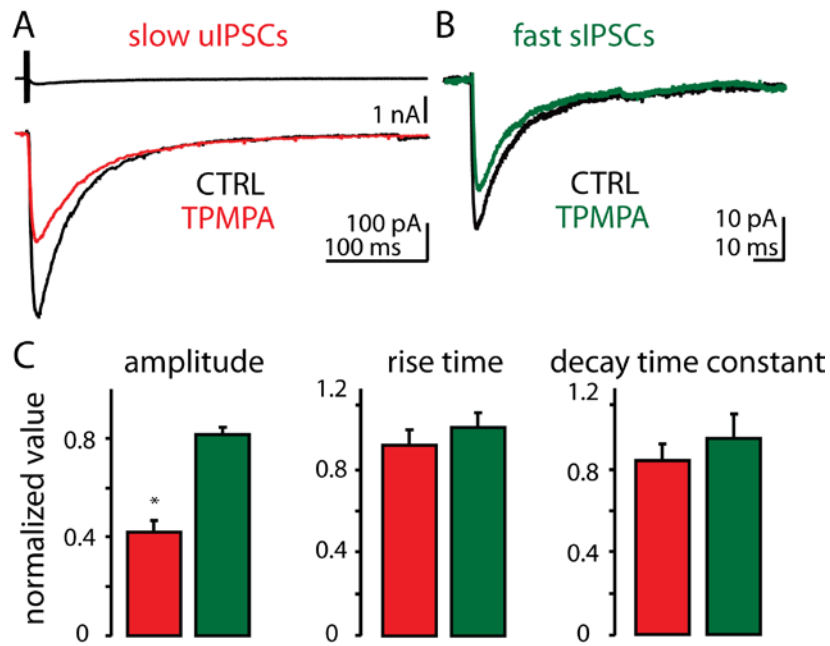


Figure 4.11. NGFCs evoke low non-saturating concentration of GABA.

A, action current in a presynaptic NPY+ cell (upper trace) evoked a uIPSC in a postsynaptic PN (black trace); the same current upon application of the fast unbinding GABA_A receptor competitive antagonist TPMPA (75 μ M, red trace). B, effect of TPMPA (green trace) on fast sIPSCs. Each trace is the average of 5 sweeps in A, and of 48 events in B.

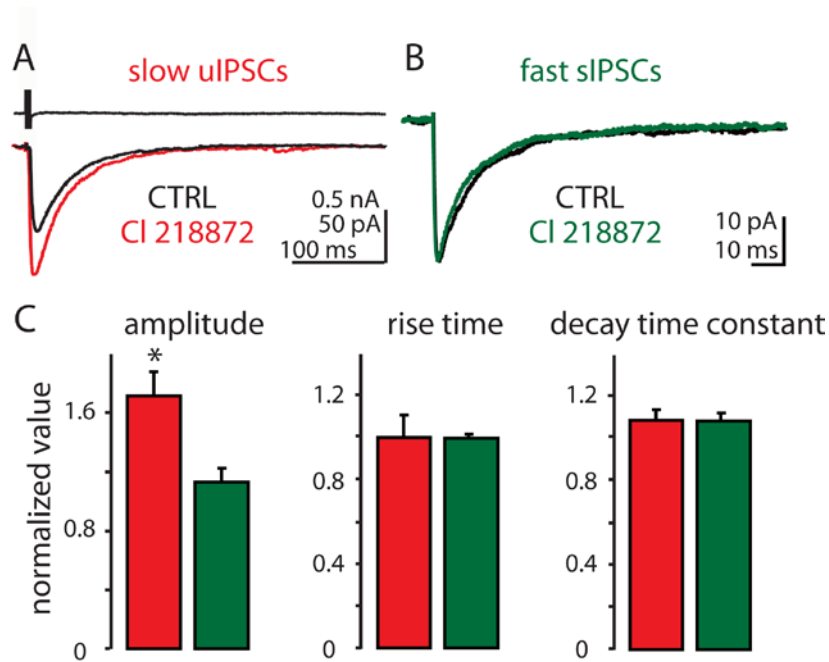


Figure 4.12. NGFCs evoke low non-saturating concentration of GABA.

A, quantification of the effects of Cl 218872 on amplitude, rise and decay time of slow uIPSCs ($n = 16$) and fast sIPSCs ($n = 5$). Mann-Whitney test, * $p < 0.05$. B, an action current in a presynaptic NPY+ cell (upper trace) evoked a uIPSC in a PN in control (black trace) and during the application of the GABA_A receptor $\alpha 1$ subunit agonist Cl 218872 (1 μ M, red trace). C, effect of Cl 218872 (green trace) on fast sIPSCs. Each trace is the average of 5 sweeps in A, and of 61 events in B. C, quantification of drug effect on amplitude, rise and decay time of slow uIPSC ($n = 14$) and fast sIPSCs ($n = 5$). Wilcoxon test, * $p < 0.05$.

		slow uIPSCs			fast sIPSCs		
		amplitude (pA)	rise time (ms)	decay time (ms)	amplitude (pA)	rise time (ms)	decay time (ms)
pre- TPMPA		220.7 ± 38.8	2.76 ± 0.43	44.35 ± 5.65	35.5 ± 4.9	1.25 ± 0.19	12.17 ± 1.95
TPMPA	* 108.5 ± 30.1 n= 16		2.35 ± 0.30	38.68 ± 7.17	28.8 ± 3.9 n= 5	1.22 ± 0.13	10.92 ± 1.62
pre- Cl218872		173.6 ± 49.2	2.93 ± 0.36	34.93 ± 3.65	38.2 ± 7.6	1.40 ± 0.15	14.00 ± 1.46
Cl218872	* 229.1 ± 48.6 n= 14		2.62 ± 0.41	39.14 ± 4.15	36.6 ± 1.5 n= 5	1.37 ± 0.16	14.75 ± 1.57

Table 4.2. Quantification of drugs effect on amplitude, rise and decay time of slow uIPSC and fast sIPSCs. Data shown as mean ± SEM. Wilcoxon test, *p< 0.05.

4.3.3. GABA spillover occurs at NGFC synapses

Next, I tested whether spillover of GABA is present at NGFC synapses of amygdala. Firstly, I used the high molecular weight molecule dextran to increase the viscosity and impair the diffusion of the neurotransmitter across the synaptic cleft (Min et al., 1998). I expected that dextran should increase intra-cleft GABA concentration and decrease spillover and activation of extrasynaptic receptors, and this should affect the amplitude of slow uIPSC rather than fast sIPSC. As predicted, the addition of dextran (1.25 mM, MW: 40000 kDa) to the ACSF significantly increased the amplitude ($n = 15$, increase to 183.1% of initial value, $p < 0.01$, Fig. 4.13.A, C) and tended to accelerate the decay time constant of slow uIPSCs (although this effect did not reach significance, $p > 0.05$). In contrast, dextran had no significant effect on fast sIPSC amplitude and kinetics ($n = 6$, 98.0% of initial value, $p > 0.05$, Fig. 4.13B, C). These results indicate that GABA released from NGFC, but not from other interneurons of the BLA, diffuses away from the releasing sites acting in paracrine manner. Summary of data obtained with dextran is shown in Table 4.3.

There is evidence for the presence of high affinity extrasynaptic GABA_A receptors on the target cells for neocortical and hippocampal NGFCs (Karayannis et al., 2010). Therefore, I tested the presence of the high-affinity $\alpha 5$ subunit. I used two different ligands: L655708, an inverse agonist selective for the $\alpha 5$ subunit up to 25 nM (Olah et al., 2009; Vargas-Caballero et al., 2010), and RO4938581, a highly selective $\alpha 5$ subunit inverse agonist (Ballard et al., 2009). None of these ligands significantly changed the amplitude or kinetics of slow uIPSCs ($n = 11$, 25 nM, L655708, Fig. 4.14.A, C, or 500 nM, RO4938581, $n = 7$, Fig. 4.14.B, C, all p values > 0.05). Summary of data obtained with these drugs is shown in Table 4.3. Therefore, GABA released from NGFCs does not appear to activate extrasynaptic $\alpha 5$ subunits, perhaps because their expression in the BLA is too low to mediate

detectable changes in cell excitability upon their inhibition (Fritschy and Mohler, 1995).

Moreover, presynaptic GABA_B receptors are located at extrasynaptic sites of neocortical (Tamas et al., 2003) and hippocampal NGFCs (Price et al., 2005). If GABA escapes the synaptic cleft it should activate these receptors. To test this hypothesis, I stimulated presynaptic cells at theta frequency (5 Hz). This protocol elicited a transient short-term depression ($n = 10$, Fig. 4.15.A, C). Application of the GABA_B receptor antagonist CGP35348 (5 μ M) partially but significantly rescued the synaptic depression ($p < 0.05$ for 2nd, 3rd and 4th stimulation, $n = 10$, Fig. 4.15.A, C). The same protocol was repeated by recording the postsynaptic cell with caesium containing patch solution, to block postsynaptic GABA_B receptors (Gahwiler and Brown, 1985). Under these conditions CGP35348 produced a similar increase of uIPSCs as in control conditions ($p > 0.05$ for 2nd, 3rd and 4th stimulation, $n = 6$, Fig. 5.15.), suggesting the presynaptic location of GABA_B receptors. I also injected into NPY+NGFC the firing from a NPY and vesicular GABA transporter immunopositive cell recorded *in vivo* during hippocampal theta activity. The application of 5 s *in vivo* firing elicited a robust synaptic depression that fully reversed after 2 min ($n = 8$, Fig. 4.16.A, B). Consistent with the theta frequency stimulation experiments described above, the application of CGP 35348 partially rescued the synaptic depression ($n = 8$; $p < 0.05$ for 2nd, 3rd, 4th and 10th slow uIPSC, Fig. 4.16.A, B). These data thus confirm that GABA released by NGFCs spills over and activates extrasynaptic receptors.

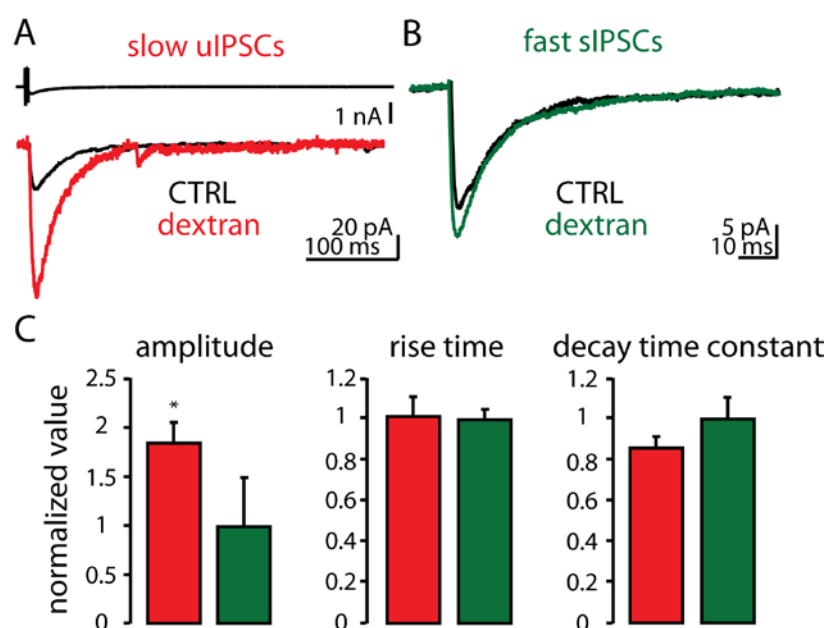


Figure 4.13. NGFCs elicit GABA spillover: actions of dextran.

A, action current in presynaptic NPY+ cell (upper trace) evoked a uIPSC in a postsynaptic PN (black trace); the same current upon application of dextran (1.25 mM, red trace). B, effect of dextran (green trace) on fast sIPSCs. Each trace is the average of 5 sweeps in A, and of 75 events in B. C, quantification of the effect of dextran on the amplitude, rise and decay time of slow uIPSCs ($n = 15$) and fast IPSCs ($n = 6$). Mann-Whitney test, * $p < 0.05$.

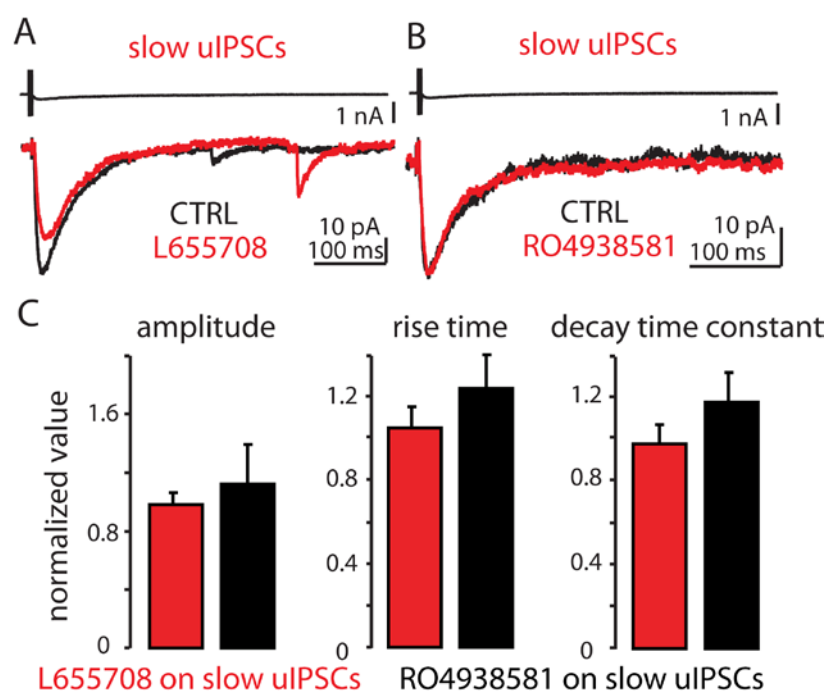


Figure 4.14. NGFCs elicit GABA spillover: actions of $\alpha 5$ GABA_A receptor ligands.

A, B, action of GABA_A receptor $\alpha 5$ subunit inverse antagonists L655708 (A, 25 nM, red trace) or RO4938581 (B, 500 nM, red trace) on slow uIPSCs. Each trace is the average of 5 sweeps. C, quantification of drug effect on amplitude, rise and decay time of slow uIPSC and fast sIPSCs (n =11, 7). Wilcoxon test, *p< 0.05.

	amplitude (pA)	slow uIPSCs rise time (ms)	decay time (ms)	fast sIPSCs amplitude (pA)	rise time (ms)	decay time (ms)
pre- dextran	183.9 ± 40.8	2.55 ± 0.39	45.95 ± 12.9	35.1 ± 4.2	1.32 ± 0.11	14.45 ± 1.79
dextran	* 292.7 ± 68.8 n= 15	2.53 ± 0.40	41.79 ± 5.38	33.1 ± 2.2 n= 6	1.32 ± 0.13	13.66 ± 1.35
pre- L655708	109.7 ± 25.9	4.47 ± 0.69	61.31 ± 7.84	-	-	-
L655708	106.6 ± 22.4 n= 11	4.42 ± 0.66	58.30 ± 8.26	-	-	-
pre- RO4938581	85.8 ± 19.9	2.01 ± 0.49	50.55 ± 6.54	-	-	-
RO4938581	78.2 ± 22.8 n= 7	2.43 ± 0.61	61.50 ± 12.85	-	-	-

Table 4.3. Quantification of drugs effect on amplitude, rise and decay time of slow uIPSC and fast sIPSCs. Data shown as mean ± SEM. Wilcoxon test, *p< 0.05.

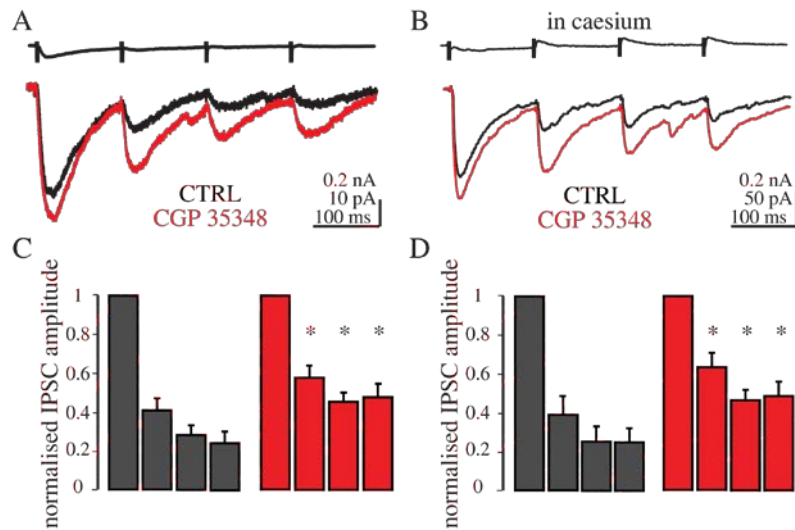


Figure 4.15. NGFCs elicit GABA spillover: presynaptic GABA_B receptor attenuates synaptic depression

A, action current in a presynaptic NPY+ cell (5 Hz stimulation, upper trace) evoked uIPSCs in a PN (lower trace, in black). Application of the GABA_B receptor antagonist CGP 35348 (50 μ M) increased the amplitude of the uIPSCs and attenuated synaptic depression (lower trace, in red). Each trace is the average of 5 sweeps. B, as in A, but the PN cell was recorded with a caesium methanesulfonate containing solution to block postsynaptic GABA_B receptors. Each trace is the average of 5 sweeps. C, histograms show the amplitude of each IPSC recorded during 5 Hz stimulation in control (black) and in the presence of CGP 35348 (red) normalised to the amplitude of the first uIPSC (n = 10). D, as in C, in presence of caesium (n = 6).

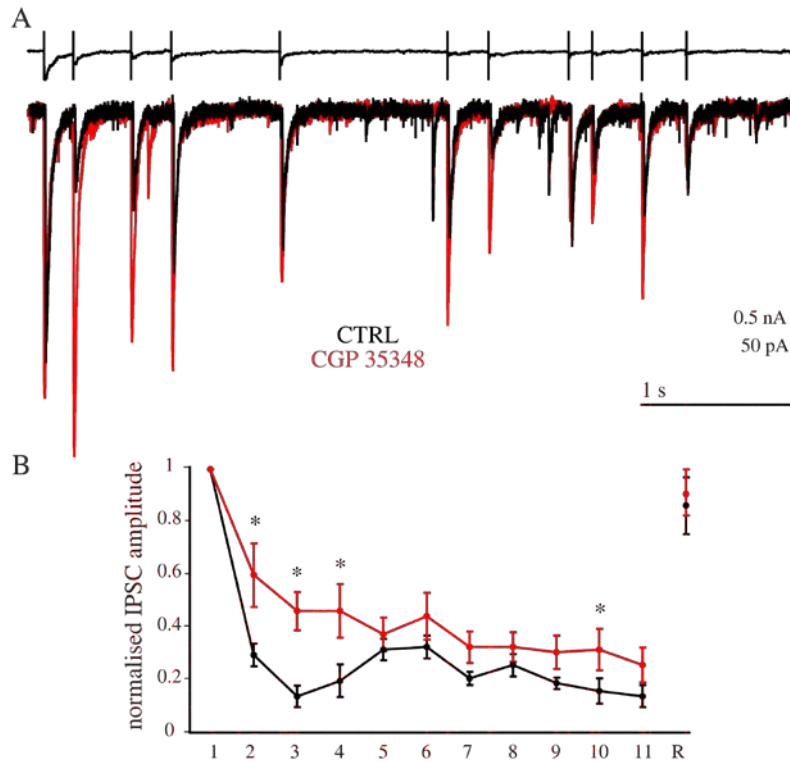


Figure 4.16. NGFCs elicit GABA spillover: presynaptic GABA_B receptor attenuates synaptic depression evoked by *in vivo* train of action potentials.

A, action current in presynaptic NPY+ cell (*in vivo* train of action potentials, upper trace) evoked uIPSCs in PN (lower trace, in black). Application of CGP 35348 (50 μ M) increased the amplitude of the uIPSCs and attenuated synaptic depression (lower trace, in red). B, graph shows the amplitude of each IPSC evoked by *in vivo* firing in control and in the presence of CGP5348 normalised to the amplitude of the first uIPSC (n = 8; the numbers on the x axis refer to the action potential occurrence in the train). The IPSC amplitude recorded 2 min after the end of the train stimulation is also shown (R, recovery). Asterisks mark significant differences with respect to the pre- drug condition; Mann-Whitney test, *p < 0.05.

4.3.4. Non-synaptic appositions are formed by NGFC terminals

The data illustrated so far suggest that the extracellular spatiotemporal concentration profile of GABA released from axon terminals is the major factor that determines the kinetics of slow uIPSC evoked by NPY-GFP+ cells of BLA. Does this have a structural correlate? We tested this issue by using electron microscopy to analyse slices in which slow uIPSCs were recorded after the stimulation of NPY-GFP+ cells filled with biocytin. Strikingly, the majority of visualised 40 terminals of NPY-GFP+ cells formed non-synaptic appositions ($n = 31$, 77.5%; Fig. 4.18): on soma ($n = 5$, Fig. 4.17.A), on dendrites ($n = 14$, Fig. 4.17.B, C, D), on another terminal ($n = 2$); further 10 appositions were formed on unidentified structures (Fig. 4.17.E). We noticed encapsulation of post-synaptic targets by pre-synaptic terminals (Fig. 4.17.B), consistent with NGFC terminals of the neocortex (Olah et al., 2009). Furthermore, we observed clear non-synaptic appositions between a terminal of NPY-GFP+ and its dendrite (Fig. 4.17.F), representing the structural substrate of autaptic uIPSC. We also identified 9 synapses formed on: dendrites ($n = 6$), soma ($n = 1$), another terminal ($n = 1$) and on a glial cell ($n = 1$). It is likely that the ratio between synaptic and non-synaptic appositions formed on the postsynaptic cells influence the kinetics of the uIPSCs. These results are consistent with the idea that GABA can diffuse from the synaptic cleft and reach receptors sensitive to low concentration of the neurotransmitter on various compartments of nearby cells.

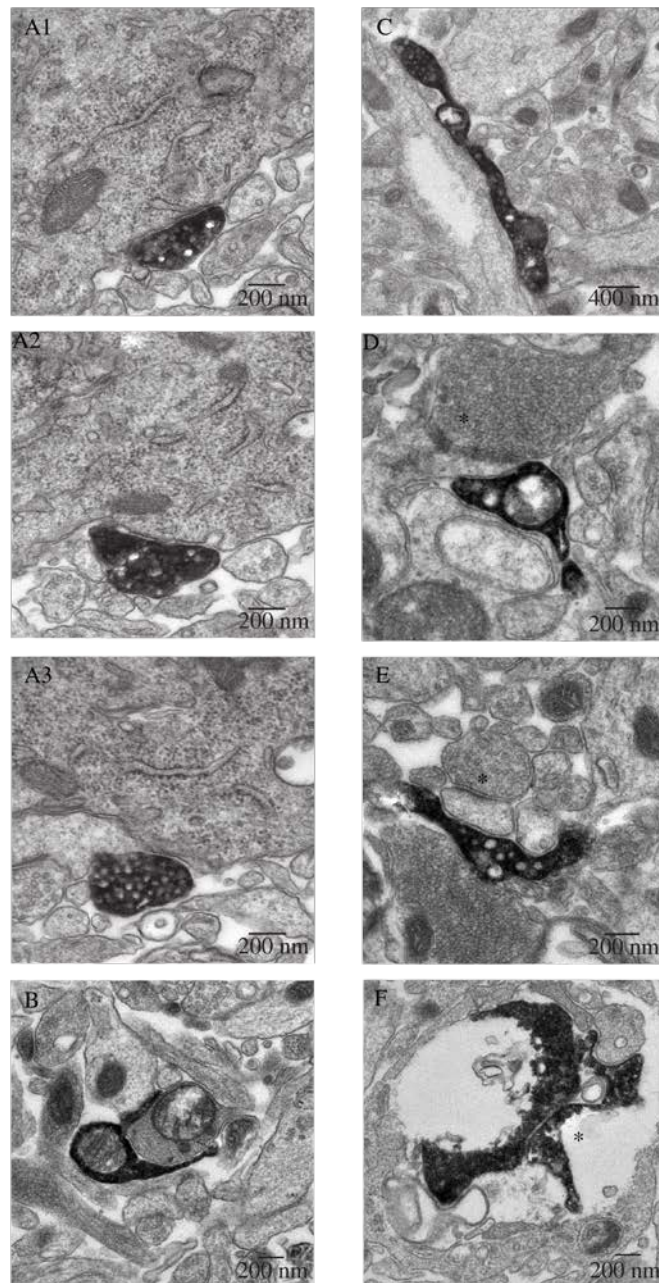


Figure 4.17. Non-synaptic appositions are formed by NGFC terminals.

A1,2,3, labelled terminal making close apposition with the soma or the initial part of a dendrite (sample of serial sections). *B*, labelled terminal making close apposition with a dendritic profile. *C*, labelled terminal making close apposition with a medium size dendrite. *D*, labelled terminal making close apposition with a spine that receives a type I synapse (asterisk). *E*, another labelled terminal making close apposition with postsynaptic structure that receives type I synapse (asterisk). *F*, labelled terminal (asterisk) making close apposition with a dendrite of the presynaptic cell (autapse). Note that all appositions are not classical synaptic structures.

4.4. Discussion

This work reports a novel type of NPY+, local inhibitory cell and demonstrates that this cell type is a source of slow phasic inhibition onto PNs of the BLA.

4.4.1. Neurogliaform cells of amygdala are the cellular source of slow phasic inhibition in the BLA

The anatomical aspect of these NPY+ cells closely resembles NGFC-like class III neurons observed in Golgi preparation in rat and opossum (McDonald and Culberson, 1981; McDonald, 1982b) and Golgi IIc cells described in the BLA of the cat (Tombol and Szafranska-Kosmal, 1972). Therefore, I have termed them as NGFC of the amygdala. It has been shown that in the amygdala NPY co-localise with SOM (McDonald, 1989), and these neurons resemble cells that produce NO (McDonald et al., 1993). Future studies should characterise the immunohistochemical markers of NPY+ cells and also test for the presence of different sub-populations in the BLA. In the hippocampus, some but not all bistratified cells, NGFC and Ivy cells express NPY (Buhl et al., 1994; Price et al., 2005; Fuentealba et al., 2008). In the striatum two types of NPY+ GABAergic cells have been recently reported: NGFC and plateau-depolarisation low-threshold spike interneuron (Ibanez-Sandoval et al., 2011).

Several types of interneurons have so far been characterised in the BLA. This classification is based mainly on the expression of calcium binding proteins and neuropeptides, single-cell RT-PCR, and electrophysiology of anatomically-identified neurons *in vitro* (Ehrlich et al., 2009; Pape and Pare, 2010; Spanpanato et al., 2011). The kinetics of IPSCs evoked by NGFCs of the BLA is much slower than any other interneuron type previously studied in the amygdala, such as PV positive cells (Woodruff and Sah, 2007b). It closely matches the kinetics of the IPSCs evoked by

NGFCs of the neocortex, hippocampus and striatum (Tamas et al., 2003; Price et al., 2008; Ibanez-Sandoval et al., 2011). The discovery of slow GABA_A receptor-mediated IPSC in the amygdala and the identification of NGFC as the cell type mediating it provides additional evidence for the wide presence and significance of this signal in the CNS (Capogna and Pearce, 2011).

4.4.2. Mechanisms underlying the unusual slow kinetics of IPSCs evoked by NGFCs

What mechanisms underlie the slow NGFC-IPSC? Because NGFC of cortex and hippocampus target the distal dendrites of pyramidal cells (Vida et al., 1998; Tamas et al., 2003; Price et al., 2008), dendritic filtering could in principle explain the slow kinetics of the synaptic currents. However, we observed with the electron microscope that the terminals of NGFC in the amygdala are opposed not only to dendritic profiles but also to peri-somatic membranes of the postsynaptic cells. This renders this possibility unlikely. The subunit composition of the GABA_A receptors can determine the kinetics of the IPSCs at CNS synapses (Farrant and Kaila, 2007). For example, slow IPSC impinging on CA1 pyramidal cells is impaired in the hippocampus of GABA_A receptor $\beta 3$ subunit null mice (Hentschke et al., 2009). It has been suggested that neocortical and hippocampal NGFC synapses express both low- and high-affinity GABA_A receptors, at both synaptic and extrasynaptic sites (Capogna and Pearce, 2011). The present results indicate the involvement of the GABA_A receptor $\alpha 1$ subunit at BLA-NGFC synapses. Since this subunit usually confers fast gating (Farrant and Kaila, 2007) it is an unlikely determinant of the IPSC slowness. A third causative factor could be the spatiotemporal concentration profile of GABA at NGFC synapses. A low-affinity, competitive GABA_AR antagonist with fast unbinding rate, TPMPA was used to test the concentration of GABA at the postsynaptic receptors. The rationale behind this experiment is that low-affinity antagonist can be competitive against low concentration of GABA to occupy the

binding site, in the presence of a high concentration of GABA its effect will be negligible. On the contrary, the application of a high-affinity, competitive GABA_AR antagonist (like gabazine) will be effective on both type of currents. In agreement with other studies, bath application of TPMPA decreased the peak amplitude of NGFCs-evoked current but had no effect on the fast current, and thus shows a clear difference in the neurotransmitter concentration at the distinct synaptic clefts (Szabadics et al., 2007; Karayannis et al., 2010). Application of three antagonists with different unbinding rates (fast unbinding TPMPA < intermediate unbinding bicuculline < slow unbinding gabazine), resulted in a decrease of the peak amplitude and increase in the rise time only in the presence of TPMPA (Karayannis et al., 2010). It shows that GABA was present long enough to activate the receptors after TPMPA dissociated hence prolonging the rise time of the current (Karayannis et al., 2010).

My results obtained with the use of TPMPA and an $\alpha 1$ subunit agonist demonstrate that the GABA released from NGFCs terminals reaches a relatively low concentration and does not saturate postsynaptic receptors (Barberis et al., 2011). This result is in agreement with the low concentration and the prolonged presence of transmitter found at neocortical and hippocampal NGFC synapses (Szabadics et al., 2007; Karayannis et al., 2010). Efforts have been made to model the temporal profile of the transmitter, or in other words the time dependent change in the concentration of GABA release from NGFCs (Karayannis et al., 2010). Model stimulations fit the experimental data with low-level of GABA transient (peak concentration $\sim 30 \mu\text{M}$) that persisted over a long time (35-73 ms), despite being limited by a GABA reuptake mechanism (Karayannis et al., 2010). Such a prolonged exposure of the receptors to the agonist can favour desensitisation. Indeed, synapses formed by NGFCs are very sensitive to the frequency of the stimulation either with a single presynaptic APs or with a train of APs that mimics their firing

pattern recorded *in vivo* (Price et al., 2005; Szabadics et al., 2007; Karayannis et al., 2010). To avoid the desensitisation of the NGFC-synapses, independent studies modulated the NGFCs with no shorter than 2 min intervals. The recovery of the slow current after stimulation with a train of 100 APs is achieved in ~20 min, therefore called mid-term depression (Karayannis et al., 2010). This mechanism, together with the presynaptic inhibition mediated by GABA_B receptors and depletion of presynaptic vesicles, are the most likely causes of the synaptic depression occurring after physiologically-relevant high-frequency stimulation of NGFCs in the BLA.

To further test the spillover of the neurotransmitter one can use dextran to slower the diffusion of the molecule away from the release site. There is experimental evidence that large molecular weighted dextran, when added to the perfusion solution, retard the movement of small inorganic ion (Prokopova et al., 1996). The group of Kullmann used dextran to slow glutamate diffusion (Rusakov and Kullmann, 1998). Their data suggested that dextran macromolecules influence the diffusion of small molecules in the extracellular space (Rusakov and Kullmann, 1998). Macromolecules act as obstacles to the movement of diffusing molecules such as glutamate, resulting in an increase in the effective viscosity of the medium and therefore in the time required for their movement away from release sites. Experimentally, they found that adding 5% dextran (40 kDa) to the perfusion solution increases the viscosity of the perfusate from 1.05 to 2.45 mPas. Moreover, dextran does not influence presynaptic transmitter release, does not act on receptors or transporters. More recently, the group of Soltesz used dextran to study the spillover of GABA at the synapses of cortical NGFCs (Szabadics et al., 2007). Their rationale was that an increased intra-cleft GABA concentration and decreased activation of extrasynaptic receptors would result from less GABA escaping from the NGFCs synapse into the extrasynaptic space in the presence of slowed diffusion. Consistent with this idea, they observed that dextran enhances the amplitude and

accelerates the decay of slow cortical NGFC-mediated IPSCs but not of fast IPSCs evoked by the activation of fast spiking basket cells.

According to the hypothesis that GABA diffuses away from the site of release, application of dextran increased the peak amplitude of the current (Szabadics et al., 2007), meaning that more transmitter molecules were available directly at the cleft and could bind to the available GABA_AR and fewer transmitters were available to the extra-synaptic receptors. In other words, slowing the diffusion of transmitter mimics the high peak concentration of the GABA at the classical synapse and thus accelerates the kinetics of the IPSCs. If GABA diffuses away from the synaptic cleft, it most likely results in a lower concentration of transmitter acting on postsynaptic receptors. The results obtained with dextran and the GABA_B receptor antagonist suggest spillover of GABA to extrasynaptic sites. In this respect, I observed that the terminals of NGFCs of the BLA form non-synaptic appositions more often than classical synaptic contacts on target neurons. Membrane-to-membrane apposition can allow diffusion of GABA from the cleft and its action as a volume transmitter onto extrasynaptic sites. Furthermore, a lack of clearly defined postsynaptic elements favours the diffusion of the transmitter and can influence the concentration of GABA reaching postsynaptic receptors. This view is consistent with ultrastructural observations made previously at neocortical (Olah et al., 2009) and hippocampal NGFCs (Karayannis et al., 2010).

4.4.3. Unique features of NGFCs of amygdala

Despite many similarities between NGFCs of different brain areas, I detected several unique features typical of NGFCs present in the BLA. Firstly, slow uIPSCs elicited by NGFCs of the amygdala, as in the striatum (Ibanez-Sandoval et al., 2011), are entirely mediated by the GABA_A receptor and lacks a GABA_B receptor component typically detected in the neocortex or the hippocampus (Tamas et al., 2003; Price et al., 2005). Secondly, NGFCs make autapses more frequently in the

BLA than in the hippocampus (~ 90% reported here *versus* ~ 50% reported in Karayannis et al., 2010), suggesting a more robust self-regulation of activity in the former. Thirdly, appositions between NGFC terminals and the soma of PNs were observed in the BLA, whereas NGFC selectively targets the dendrites of postsynaptic pyramidal cells in neocortex and CA1 hippocampus (Tamas et al., 2003; Price et al., 2008). Fourthly, NGFC-IPSC in the amygdala is not sensitive to GABA_A receptor $\alpha 5$ inverse agonists, in contrast to that evoked in neocortex and hippocampus (Szabadics et al., 2007; Karayannis et al., 2010). This difference can be explained by the lower level of expression of that subunit in BLA compared to the neocortex or hippocampus (Fritschy and Mohler, 1995). This result is consistent with the idea that the subunit composition of the GABA_A receptor is not necessarily the major determinant shaping kinetics of the slow IPSC.

4.4.4. Physiological role of slow IPSCs evoked by NGFCs in BLA

What is the physiological role of NPY+-NGFC? Recently it was observed that NPY+ cells of the amygdala fire preferentially close to the peak of the hippocampal theta cycle *in vivo* (Bienvenu and Capogna, unpublished observations). This, together with the present observation that this cell type elicits an unitary postsynaptic response lasting nearly one theta cycle, suggests a possible function in the coordination of hippocampal-amygdala theta oscillations that emerge during fear memory retrieval (Seidenbecher et al., 2003). Furthermore, it is estimated that the dense axonal arborisation of one cortical NGFC provides potential release sites equivalent to six basket cells (Olah et al., 2009). The GABA released from NGFCs inhibits the release of glutamate or GABA from axon terminals, which are located at some distance from NGFC release sites (Olah et al., 2009). Since, AP of NGFCs evoke IPSCs without failures they can have profound effect on nearby cells and be very effective in silencing spontaneous activity of PNs in the BLA (Gaudreau and Pare, 1996). Moreover, series of APs generated by NGFCs leads to the depression

of slow synaptic events and disinhibition of postsynaptic targets favouring long-term plasticity of excitatory inputs. Currently, it is postulated that GABA released from NGFC can overcome re-uptake mechanisms and act as volume transmitter (Olah et al., 2009; Karayannis et al., 2010). Therefore, the activity of NGFCs can provide an important source of accumulated extra synaptic GABA that activate presynaptic GABA_B receptor and mediate suppression of glutamatergic input to PNs of the BLA (Pan et al., 2009).

4.4.5. Are the NGFCs main source of NPY in the BLA?

I showed here that NGFCs in the amygdala express NPY, and this peptide has an anxiolytic action, as demonstrated by anxiety-related behavioural tests (Sajdyk et al., 1999). Application of NPY decreases the excitability of most PNs of the lateral nucleus via activation of Y1 receptors and subsequently G-protein coupled inwardly rectifying potassium channels (Sosulina et al., 2008). Although NPY can be found in PNs, it is clear that local GABAergic cells can be the main source of NPY in the BLA (McDonald and Mascagni, 2002). My data suggests that NGFCs are the most abundant NPY containing type of interneurons within the BLA. Therefore it is likely that they are the primary source of this peptide.

4.4.6. Conclusion

Growing evidence show that local GABAergic cells of the BLA are critically involved in the acquisition, manifestation and extinction of fear (Ehrlich et al., 2009; Pape and Pare, 2010). Identification of cell types and their connectivity within the amygdala is crucial for understanding how emotional information is processed. I propose that slow phasic inhibition evoked by NGFCs is an essential component of amygdala network actively contributing to maintain its high inhibitory tone.

4.5. Future work

To fully establish a novel neuronal cell type, the cells must ideally share the same neuroactive substance, postsynaptic target(s), input(s) and brain state-dependent temporal patterns (Gordon, 2010). According to this definition, additional work on novel NGFCs of amygdala should provide a description of their specific synaptic inputs and activity during emotionally-relevant brain states. In addition, it remains to be tested if NGFCs target other NGFCs as well as other types of BLA interneurons and if putative contacts are mediated also through gap junctions, as it occurs in the cortex (Simon et al., 2005) and in the hippocampus (Price et al., 2005; Zsiros and Maccaferri, 2005).

One progress in the definition of the NGFCs in the amygdala would be to establish their neurochemical content. Based on known markers of NGFCs (Price et al., 2005), Ivy cells (Fuatealba et al., 2008) and the expression patterns of typical proteins markers in the BLA, it would be of interest to test the co-localization of NPY with at least the following markers: SOM, CB, nNOS, α -actinin-2, COUP-TFII and reelin.

Surprisingly, my results contradict the initial expectation that NPY+ population of cells within the BLA consist of several cell types as previously observed in cortical areas or the striatum. For example in the hippocampus, NPY is expressed by different interneuron types, such as NGFCs/Ivy cells and bistratified cells (Buhl et al., 1994; Price et al., 2005; Fuatealba et al., 2008). I cannot rule out the existence of another cell type(s) within the NPY+ population, but different technical approach might be necessary to explore this possibility, such as the use of fluorescent dye in the recording pipette to visualize their axonal arborisation, to enable recording from PNs within the axonal arborisation of the presynaptic NPY interneuron (Ibanez-

Sandoval et al., 2011). However, the very large sample of collected data shows that even if other NPY+ cell types exist in the BLA their connectivity should be very low.

The data I obtained demonstrate that the frequency of detection of autaptic uIPSCs in the NGFCs of amygdala is higher than the one found in the NGFC of the hippocampus (Karayannis et al., 2010). It will be therefore worthwhile to establish the functional role of such self-regulation. There are several experiments that could be performed in order to do so, the most straightforward one would be to examine the changes of the cell firing pattern after the pharmacological blockade of the autaptic IPSC with a selective GABA_A receptor antagonist. I expect this would lead to an increase in the cell firing, especially when near-physiological recording conditions would be used (Karayannis et al., 2010).

In this thesis postsynaptic and autaptic uIPSCs have been pooled together to study the mechanisms underlying the slow kinetics of evoked currents. However, autaptic uIPSCs and postsynaptic uIPSCs significantly differed in their decay time constant, unlike it was observed in NGFCs of the hippocampus (Karayannis et al., 2010). It is very important to rigorously test the mechanism underlying such difference. Possibly the slower the current, the bigger the ratio between non-synaptic appositions *versus* synapses. I have obtained some clue that this might be the case by observing that autapses mostly mediate the slowest currents lacking clear-cut synaptic specializations. However, future experiments would be needed to obtain more accurate data distribution to test the hypothesis that a difference in ratio of non-synaptic appositions *versus* synapses positively correlates with longer IPSC decay time constant.

Recent studies showed that dendrite-targeting interneurons of hippocampus (namely bistratified and OL-M cells) strongly influence the dendritic computation through shunting inhibition and the interaction with other GABAergic cell types

(Lovett-Barron et al., 2012). This study demonstrates that future work using novel research tools (such as opto- and pharmaco-genetic) is likely to successfully address the question of the role of slow phasic inhibition in the BLA.

Cells belonging to the NGFC family express NPY and/or nNOS, however action potential-dependent release of these two important neuromodulators has not been experimentally proven. Application of NPY into the BLA has a powerful anxiolytic effect (Sajdyk et al., 1999). Although, NO is an unstable molecule and its role may be difficult to establish experimentally, it has been shown to contribute to retrograde signalling (Makara et al., 2007), to modulate LTP in the ventral tegmental area (Nugent and Kauer, 2008), and also to influence the release of GABA (Yu and Eldred, 2005). Interestingly, NPY and NO can control the neurovascular activity (Cauli et al., 2004). It would be therefore desirable in the future to study the functional role of NPY and NO released from the NGFCs of amygdala.

Finally, it was recently shown that NGFCs of the CA1 region of the hippocampus are under regulatory control of opioids (Krook-Magnuson et al., 2011). Given the possible therapeutically and addictive nature of these drugs it will be relevant to analyse the effects of opioids on NGFCs in the amygdala.

5. General discussion

5.1. Outlook

The amygdala complex is critically involved in processing emotion- and anxiety-related behaviours. Its function depends on the interplay of two major components, excitatory and inhibitory, under the control of various modulatory systems. Details of GABAergic cellular components of this circuit, their role and characteristics, are emerging but substantial progress is needed. For that reason, it is crucial to understand the GABAergic cell types and their connectivity in order to hypothesise and test the function of the amygdala during different behaviours.

In this thesis, I define several properties of two distinct and novel GABAergic cell types of the rodent amygdala: intercalated cells of the Im nucleus and NGFCs of the BLA.

In the first part of this thesis, I provide anatomical and functional evidence that Im neurons project to the CE and BLA nuclei and that these neurons are reciprocally connected with the mITC and lITC clusters. I also report that Im neurons receive excitatory inputs from the BLA and possibly the cortex. My results also indicate that the Im neurons express both AMPA and NMDA receptors, suggesting that they can undergo NMDA-dependent plasticity. I have revealed that DA hyperpolarizes Im neurons via DA1-R, therefore providing a novel cellular substrate for modulation of the amygdala at a systemic level.

In the second part of this thesis, I discover the presence of NGFCs in the BLA and demonstrate that this GABAergic cell type is the cellular source of slow phasic synaptic inhibition in the amygdala. Further, I identify a major mechanism underlying the remarkably slow kinetics of NGFC-mediated IPSCs, namely the presence of low concentration of extracellular GABA at postsynaptic receptors

accompanied by the lack of clear-cut synaptic specialisations. I propose that high and reliable functional coupling and prolonged effect on the nearby cells make NGFCs important in maintaining a high inhibitory tone within the BLA complex during network activity. Finally, I discuss how this form of inhibition may contribute to network functions.

5.2. Possible contribution of NGFCs and Im neurons during parallel information processing within the amygdala.

The aim of this thesis was to dissect specific GABAergic components of the amygdala circuitry, to study their morphology, connectivity and function. Collection, analysis and interpretation of the data regarding single components allow to further discussing putative roles of these cells during amygdala function.

How Im cells and NGFCs contribute to the amygdala function?

The body of recent studies shows that the information flow in the amygdala is far more complicated than generally considered so far. Commonly, BLA was shown to be the most important site of stimuli integration during fear conditioning. This view has to be changed in the light of the discovery that the CEI nucleus is crucial for NMDA-dependent formation of CS-US association (Ciocchi et al., 2010). It is possible since the CEI nucleus, similar to the BLA, receive direct inputs from the thalamus (Linke et al., 2000). This implies that the signal does not simply follow the dorso-ventral axis of consecutive amygdala nuclei, but it is also processed in a parallel manner. Moreover, the manifestation of fear depends on the increase in activity of CEm neurons (Ciocchi et al., 2010), controlled by the unidirectional inhibition from the CEI. The activity of the CEI nucleus is necessary to acquire fear

conditioning and the activity of the CEm nucleus is necessary to express behaviourally-relevant information. Both processes are most likely accompanied by a decrease in synaptic inhibition generated by several GABAergic cell types. In addition, inhibitory transmission is tightly regulated by CE neurons themselves. Therefore it is crucial to identify the projections of ITCs neurons to the CE nuclei subdivisions and their activity during fear conditioning and extinction (Busti et al., 2011). It is tempting to simplify the data interpretation and consider that Im is active during fear extinction and extinction retrieval and therefore decrease the output activity of the CEm and contribute to the formation of extinction memory (Ciocchi et al., 2010; Busti et al., 2011). However, it seems difficult to draw clear-cut conclusions as Im neurons also project toward the mITC, all subdivisions of the CE nucleus and the medial amygdala. It means that the activity of the Im nucleus during fear extinction and extinction retrieval inhibits not only to the CEm nucleus, but at the same time also disinhibits the CEm via the inhibition of the CEI nucleus and the mITC.

Moreover, the data contained in this thesis show that Im neurons are hyperpolarized by DA, through DA1-Rs, in addition to mITC (Marowsky et al., 2005). My result implies that during the behavioural states with elevated DA levels, cells within the cluster have differential effect depending on their target structure. In theory, a decrease in ITC activity has a dis-inhibiting effect on the CE nucleus if a cell project to the CEm division and an inhibitory effect if project to the CEI division. Often the axon of ITC neurons could be found in both CE nuclei division meaning that the effect of release GABA is a result of the integration of inhibitory and dis-inhibitory components.

It is important to keep in mind that discrete neuronal populations are active both within the CEI (Ciocchi et al., 2010) and BLA (Herry et al., 2008) during fear conditioning. Based on my data, I suggest that neurons of the Im represent novel GABAergic cell population contributing to the acquisition, expression and extinction

of conditioned fear. Their final effect would depend on the functional plastic properties of their inputs as well as from the cell populations they target. Both elements are largely unknown.

It is likely that local inhibitory cells are the most prominent source of synaptic inhibition providing constant silencing of PNs. As suggested before (Chapter 4.3.4), NGFCs appears to be an ideal source of such widespread and powerful inhibition.

As every cortical structure there are two inhibitory elements within the BLA, feedback and feedforward, thus inhibition is relative to incoming/local excitation. The ratio of feedback and feedforward conductances is highly dynamic in time and space, and therefore their coordinated action can tune cortical neurons to specific stimuli (Isaacson and Scanziani, 2011). The stimulus selectivity is well described in the neocortex where neurons are tuned to be sensitive to particular stimuli, like frequency for auditory stimuli, chemical composition for olfactory stimuli or orientation in space for visual stimuli (Isaacson and Scanziani, 2011). Can NGFCs contribute to shaping the response of PNs to distinct stimuli?

The LA has been identified as one of the most important sites of CS-US stimuli integration. The role of NGFCs in that process likely strongly depends on their input function and properties. If the inputs to NGFCs evoke a series of APs it will most likely lead to the depression of slow synaptic events and disinhibition of postsynaptic targets favouring long-term plasticity of excitatory inputs to the BLA PNs. On the other hand, if the inputs to NGFCs evoke single or sparse APs, the decay time of resulted uIPSCs will match the decay kinetic of NMDA-R, thus they could inhibit the induction of long-term potentiation by providing shunting effect on NMDA-induced current.

Another interesting aspect of NGFCs is whether they can undergo LTP. It has been shown that interneurons within the BLA can be divided into three functional groups based on the presence and subunit composition of NMDA-Rs. The group that do not

undergo LTP, expresses mostly GluN2B subunit. The remaining cells capable of LTP, mostly express GluN2A subunit or do not express NMDA-Rs. Repetitive stimulation of glutamatergic inputs to the interneurons of BLA results in the induction of postsynaptic rise of Ca^{2+} that is independent from NMDA-Rs activation. However, the interneuron cell types of the BLA that mediate these properties have not been identified so far (Spampanato et al., 2011). Therefore, it will be relevant to study the synaptic plasticity impinging on NGFCs of the BLA.

Activity of the BLA is not only essential during fear conditioning but also during fear extinction (Herry et al., 2008). This process is dependent on the function of the hippocampus and the mPFC, probably via mITCs. Interestingly, different functional populations of the BLA PNs probably exist and they are differentially connected to the ventral hippocampus and mPFC (Herry et al., 2008). Is it possible that the activity of NGFCs have heterogeneous effects depending on the targeted cells or on their selective inputs? Answer to this important question awaits further investigation.

5.3. Concluding remarks

It is likely that the amygdala processes information by means of several parallel circuits. My work has characterised two novel GABAergic cell types, namely neurons of the Im and NGFCs of the BLA. My contribution has been to elucidate basic anatomical, functional and pharmacological aspects of these neurons. Future work should focus on the role of these GABAergic cells in the neuronal network during relevant behaviours.

6. References

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