To: Network of European Neuroscience Schools (NENS)

Final report
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Period of training stay: 18 August-30 September
Introduction

Many recent studies have supported the idea that the amygdala is one of the brain structures involved in fear memory acquisition and storage (Davis 2000, LeDoux 2000, Maren 2001). The amygdala consists of several anatomically and functionally distinct nuclei including the lateral (LA) and basal (BA) nuclei, together referred as basolateral amygdala (BLA), and the central nucleus (CE), further divided in the centrolateral (CeL), centromedial (CeM) and central capsular (CeC, McDonald 1982). The intercalated cell masses (ITCs) of the amygdala are small clusters of GABA-ergic cells surrounding the BLA (Millhouse, 1986) and they have been reported to play a crucial role in gating the information flow from the BLA to the Ce (Royer, 1999) and mediating the expression of fear extinction (Likhtik, 2008). However, many functional aspects of this process remain still unclear. We believe that a better elucidation of the neuronal features of the BLA interneurons and of the ITC neurons will likely provide a better insight of the synaptic network underlying fear and extinction memories.

My PhD project, that I carry out at the Department of Pharmacology of Innsbruck, focuses on the identification and characterization of the different neuronal subtypes present in the ITCs of the amygdala. However, so far, I could characterize distinct subclasses of ITC neurons only on the basis of bidimensional reconstructions of biocytin-filled cells obtained by drawing the neuronal elements by camera lucida.

During the training stay that I carried out at the MRC Anatomical Neuropharmacology Unit in Oxford, I had the possibility to follow in vivo electrophysiological recordings of BLA interneurons in anaesthetized rats. During these experiments, single recorded neurons were also filled with neurobiotin, using the juxtacellular technique. The aim of my stay was to learn: (1) how to visualize the recorded neurons by immuno-fluorescence and (2) how to evaluate some neuronal aspects using both conventional epiflorescence and confocal microscopy. (3) This experience gave me the opportunity to improve my technical skills in tracing the cytoarchitectonic structure of ITC neurons and also to learn how to use Neurolucida. In fact, this is an advanced application that is becoming the gold standard for 3D reconstructions of neuron and quantitative characterization of neuronal morphological features.
Material and Methods

Visualization of in vivo recorded cells. Coronal sections of 60 μm thickness were cut by a vibratome from previously fixed male Sprague Dawley rat brains and the recording neurons were visualized by immunofluorescence. Briefly, free-floating sections containing processes of the recorded cell were washed 3 times with PBS (Phosphate Buffer Saline) and incubated with Cy3 (1:400, Vector Laboratories), AMCA (1:1000, Vector Laboratories) or Alexa 488-coupled streptavidin (1:1000, Molecular Probes) diluted in PBS, 0.15% Triton X 100 (TX) and 1% Normal Horse Serum (NHS, Vector Laboratories) for ~24h (4°C). Then, the sections were mounted on slides in Vectashield medium and covered with a coverslip. Micrographs of the recorded neurons were acquired on a Leica Leitz DM RB conventional epifluorescence microscope, equipped with a Hamamatsu (ORCA-ER) CCD camera using the Openlab software (Improvision, version 5.5.0).

Investigation of in vivo recorded putative basket cells. Putative basket cells were investigated for their expression of parvalbumin (PV). Specifically, after blocking for 1h at room temperature (RT) with a solution made of PBS, 0.15% TX and 20% NHS, the free-floating sections were incubated with the primary antibody PV (1:1000, goat, Swanson) diluted in PBS and 1% NHS for 48h (4°C), washed in PBS and reacted with the secondary antibody anti-goat Cy3 prepared in a solution containing PBS, 0.15% TX and 2% NHS. After abundant washing, the sections were mounted on slides and analyzed at the conventional epifluorescence microscope.

Investigation of in vivo recorded putative axo-axonic cells. Possible axo-axonic interneurons were tested for their postsynaptic immunopositivity to Ankyrin G. Briefly, free-floating sections were blocked with a solution, containing PBS, 0.15% TX and 20% NHS, for 1h at RT and reacted with the primary antibody Ankyrin G (1:4000, mouse, UC Devis) diluted in PBS and 1% NHS for 48h (4°C). After washing in PBS the sections were incubated with anti-mouse Alexa 488 prepared in a solution containing PBS, 0.15% TX and 2% NHS. Afterwards they were washed in PBS and mounted on slides and the immunofluorescence was studied using a laser-scanning confocal fluorescence microscope (LSM 510, Karl Zeiss) using a 100x immersion-oil objective.

Anatomical analysis of recorded neurons by Neurolucida. Two ITC neurons, previously recorded in vitro in acute slices, filled with biocytin and converted for light microscopy using the avidin biotinylated horseradish method, were reconstructed using the Neurolucida software.
(MicroBrightField Neuroscience 8) connected to a Nikon Eclipse 80i microscope. Specifically, all the portions of the soma, dendrites and axons present on serial sections were drawn, using a 60X oil-immersion objective, and properly overlapped. In one case some of the anatomical features were quantified by Neurolucida Explorer (MicroBrightField, version 4.70.11).

Results
To test whether the in vivo recorded and neurobiotin-filled interneurons of the BLA belong to different subtypes of cell, I investigated specific neuronal markers. To this aim I assessed, at first, the correct location of the single recorded neuron within the BLA by immunofluorescence using conventional epifluorescence microscopy (Fig.1).
In order to characterize some morphological features of the recorded interneurons, I used specific neuronal markers such as PV and Ankyrin G. In particular, I tested one recorded cell, showing basket-like axonal arbors, for PV. In fact, PV is a calcium binding protein specifically expressed in the soma and the processes of basket cells. As shown in Figure 2, a recorded interneuron of one of the rat brain that I have processed is positive for PV, corroborating its basket cell nature.
In another case, I could investigate a putative axo-axonic neuron by immunohistochemical analysis of the spectrin-binding protein Ankyrin G, a neuronal marker staining the axon initial segment. Regions of the neurobiotin filled axon of the recorded cell were found to make close apposition with Ankyrin G positive axon initial segments of other BLA neurons, as shown by laser-scanning confocal fluorescence microscopy (Fig. 3).
During my stay at the MRC, I could also fully reconstruct by Neurolucida two in vitro recorded ITC neurons (s121108 slice 1 and r100207 21-39), located in the main ITC nucleus of the amygdala. Figure 4 shows the ITC neuron s121108 slice 1, characterized by short dendrites and few axonal branches mainly restricted to the main ITC nucleus and the BA. Figure 5 presents a more complex ITC neuron (r100207 21-39) whose axonal projections extend in the main ITC nucleus, BLA, in the different subnuclei of the Ce and also in the basal medial amygdala (BMA) and in the medial nucleus (Me). Using Neurolucida Explorer, I could also quantify some anatomical features of this complex neuron, such as the axonal length in each of these regions, that are summarized in Tables 1 and 2.
Figure 1. An *in vivo* recorded interneuron located in the BLA. Immunofluorescence picture assessing the location of the *in vivo* recorded interneuron, filled with neurobiotin and revealed with AMCA-streptavidin, within the BLA complex of the amygdala. LA, lateral amygdala; BA, basal amygdala; ITC, intercalated cells of amygdala; ec, external capsule; Pir, piriform cortex; Ce, central nucleus; AStr, amygdala striatum transition region; CPu, caudate putamen.
Figure 2. PV positive basket cell interneuron of BLA. Conventional epifluorescence microscopy micrographs of one section, containing dendritic processes of the recorded neuron, reacted (A) with Alexa 488-streptavidin to visualize the neurobiotin and (B) with PV and Cy3-streptavidin. (C) Arrows in the superimposed picture indicate portions of the dendrites positive for PV.
Figure 3. Investigation of an axo-axonic interneuron of the BLA. Projection of Z-stack images obtained by confocal microscopy of (A) the axonal processes of a recorded BLA interneuron, filled in vivo with neurobiotin, revealed by Cy3-streptavidin and (B) Ankyrin G and Alexa 488-streptavidin immunoreaction. (C) White arrows indicate some neurobiotin filled axon making apposition to Ankinin G positive initial axonal segments.
Figure 4. ITC neuron s121108 slice 1 reconstructed by Neurolucida. Red, soma; green, dendrites; yellow, axon.
Figure 5. ITC neuron r100207 21-39 reconstructed by Neurolucida. Red, soma; green, dendrites; yellow, axon. *, main ITC nucleus; CeL, lateral nucleus of central amygdala; CeC, paracapsular nucleus of central amygdala; CeM, medial nucleus of the central amygdala; Me, medial amygdala; BMA, basal medial amygdala. Scale bar: 200µm.
<table>
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<th>Total Length (µm)</th>
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Table 1. Quantification of some of the main anatomical features of r100207 21-39.

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<th>Interm. capsule</th>
<th>CeC</th>
<th>CeL</th>
<th>CeM</th>
<th>Me</th>
<th>BLA</th>
<th>BMA</th>
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<td>613.2</td>
<td>625.5</td>
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Table 2. Quantification of the axonal length of r100207 21-39 in the different subnuclei of the amygdala.

Conclusions

During the time that I spent at the MRC of Oxford, thanks to the stipend received from the Network of European Neuroscience Schools (NENS), I had the possibility to improve a lot my technical skills on how to analyse in vivo recorded neurons by immunochemistry. Thus, I was trained by expert people in the laboratory on how operate confocal microscopes and also the Neurolucida.

I firmly believe that this experience was very helpful and constructive and I am sure that my future work will significantly benefit from it.
References


Acknowledgments

I would like to thank:

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– Thomas Bienvenu and Mirosława Manko for introducing me to electrophysiology, for their excellent and friendly supervision during the experiments and all the interesting discussions.

– Linda Katona, Dr. Pablo Henny, Dr. Nicolas Mallet for their excellent technical help, in particular with the Neurolucida and the confocal microscope.