Universidade do Minho
Escola de Ciências da Saúde

ICVS/3B's
Associate Laboratory
University of Minho

Final Report
Bial Scientific Research Bursaries

Translation of neuron-glia interactions in complex cognitive functions
Fellowship 61/10

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Braga, 20th of June 2015

Summary

The present document reports the progress made in the last period and sums up the findings of the research project "Translation of neuron-glia interactions in complex cognitive functions - Fellowship 61/10" carried in the Life and Health Sciences Institute of the School of Health Sciences (ICVS), University of Minho.

Looking at the progress reported here and in previous reports, we do believe that the goals established for this fellowship were fully accomplished. As initially proposed, we have used complementary state-of-the-art techniques such as in vivo electrophysiology, innovative behaviour, anatomical and molecular analysis, to seek a role of astrocytes in the computation of cognitive functions. It is noteworthy that we have made a rigorous management of the available budget, always looking for the best people to help with specific techniques, such as electrophysiology, and the more suitable/optimized protocols and processes. This effort resulted ultimately in an increased consistence of the data obtained, confirmed by the high quality publications obtained so far (other will be submitted soon), and by the positive feedback that we have received from the consultants and peers in international congresses.

Astrocytes appear as critical elements of the neuroglial network, whose function is required for the normal hippocampal function. This information accounts for new knowledge on the cognitive computation and this new player may account also for the cognitive decline observed for instance in ageing or pathological processes, arising as a possible therapeutic target for the treatment of prevention of cognitive deficits.

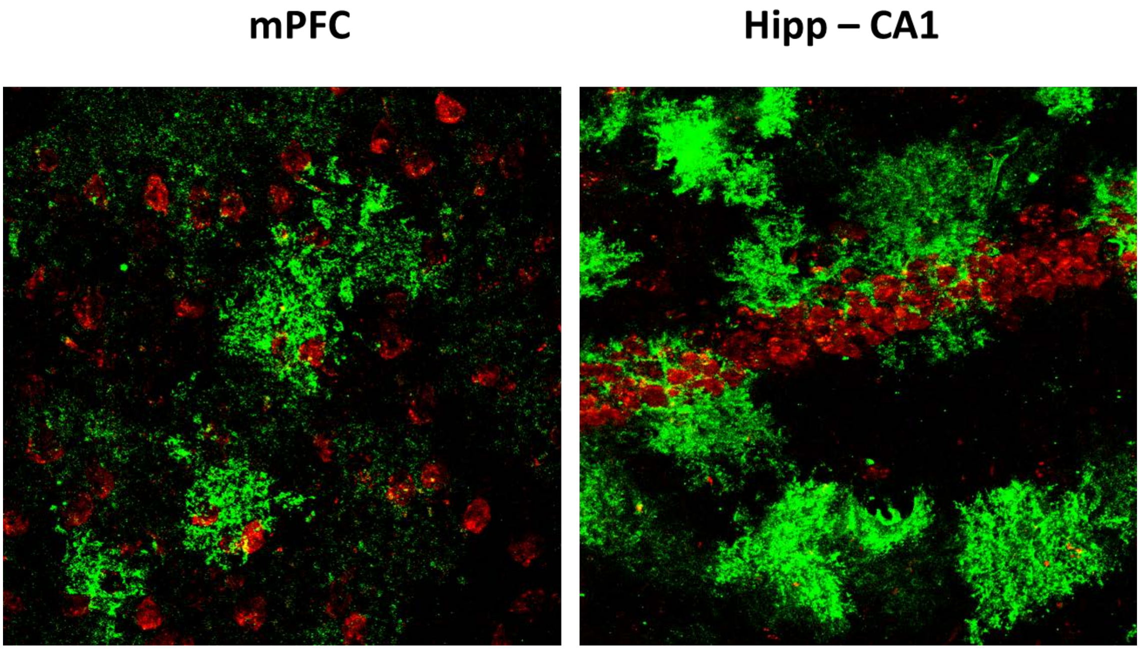
Main achievements of the last period of the project

The use of the dnSNARE mice model allows to shut down conditionally the vesicular release in astrocytes. Since the release of neurotransmitters by these cells often uses the vesicular pathway, we are able to shut down release of gliotransmiters in these animals with a minimal interference in the welfare of the animal. Therefore, this model is of major importance for the study of the role of astrocytes and their modulation of the surrounding network in the computation of complex behaviour outputs. This model was kindly supplied by Prof. Philip Haydon, Tufts University, for the purpose of this project.

The results reported previously described a putative cognitive impairment for the dnSNARE animals. The goal for this last period was to dissect that impairment and establish the electrophysiological and structural correlates that underlie this learning deficits.

<u>1. dnSNARE animals present alterations specifically in astrocytes</u>

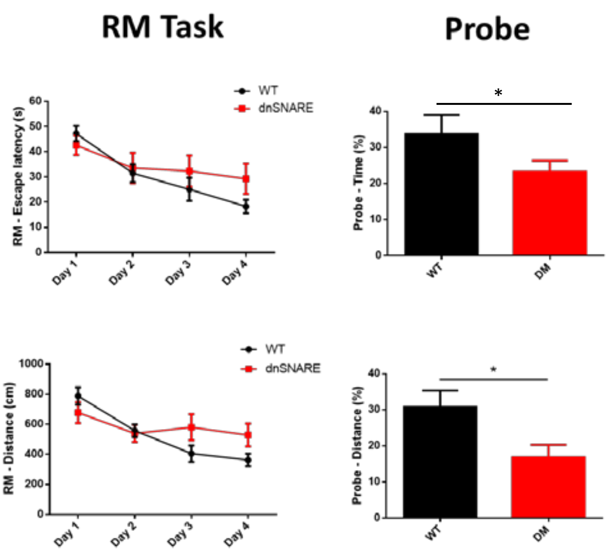
The analysis of the expression of the green fluorescent protein (GFP) that is specifically expressed in mice that lack gliotransmission allowed the confirmation that this expression is restricted to astrocytes, not affecting neurons. This was confirmed by the images bellow in which we have immunostained simultaneously neurons (red) and GFP reporter (green) and verify that they stain exclusively different structures. Additional double immunostainings showed that the GFP reporter stains GFAP+ and S100B positive astrocytes (data not presented). These results were valid for the regions that are responsible for the execution of the reference memory task.



Representative micrographs of brain tissue of dnSNARE animals. eGFP transgene expression (green) is widely observed in astrocyte-like bushy structures surrounding neuronal structures (NeuN marker; red), both in the medial prefrontal cortex (mPFC) and CA1 region of the hippocampus.

<u>2. dnSNARE animals display cognitive impairments in the Morris Water Maze</u>

In order to analyse cognitive function in the form of memory processing we used MWM both in animals with SNARE blockade (dnSNARE) and controls (WT). The MWM was applied to access spatial reference memory and reversal learning. In RMT the



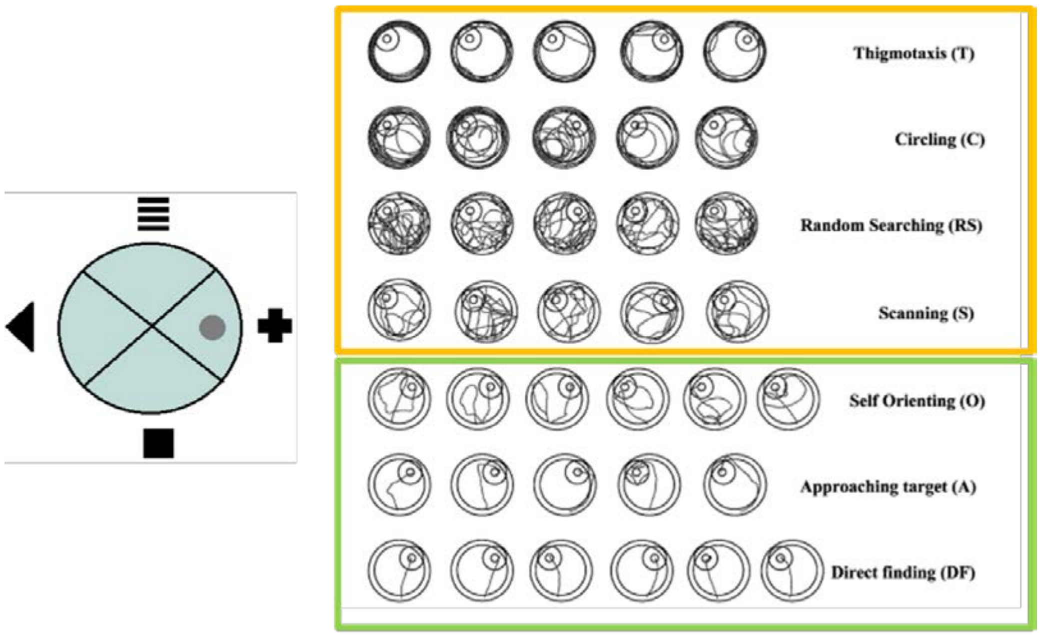
Performance in the reference memory (RM) task of the Morris Water Maze. dnSNARE animals display a tendency to longer latencies to reach the platform than wild-type controls, which lead to a decrease in time/distance swam in the quadrant where the platform used to be in the Probe trial. * $p<0.05$.

decrease in escape latency times and distance swam favors spatial learning. On the other hand RLT is considered normal when animals spend more time in the quadrant where the platform used to be after four days of RMT.

All animals show an improvement in escape latencies and distance swam throughout the four days of the RMT, as shown below. Results point out that dnSNARE mice tend to swim longer and more distance to find the submerged platform as controls, however no significant differences were found between dnSNARE mice and WT in the 4 days of test.

Results from the probe trial show that dnSNARE spent less time than WT controls in the quadrant where the platform used to be during the 4 days of the RMT. This suggests that dnSNARE animals had difficulty towards learning the task.

Additionally, we have analysed the performance of each animal in detail for each trial by analysing the number of failures of each animal and type of strategy that they use

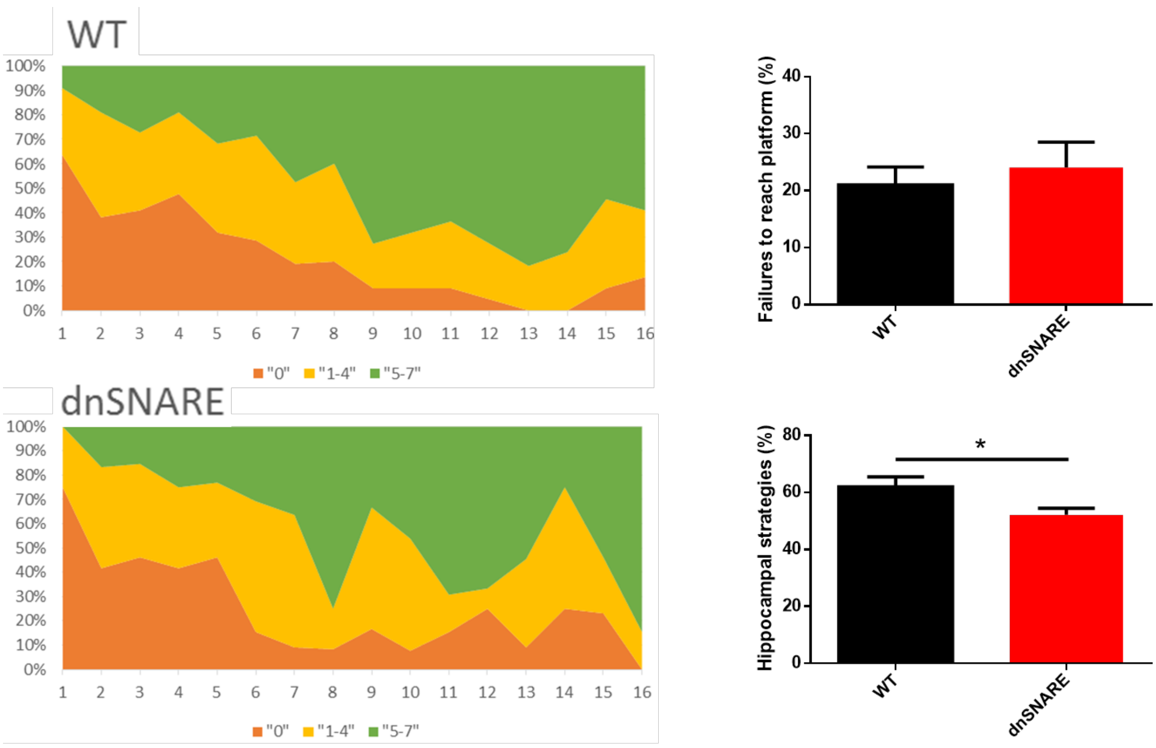


(Graziano, 2003)

Scheme used to tag strategies used by mice during the Reference Memory Task to reach the platform. Mice swim to the platform using different strategies, starting in circling-scanning approaches (orange) to more directed routes (green) as they learn how to use the spatial cues.

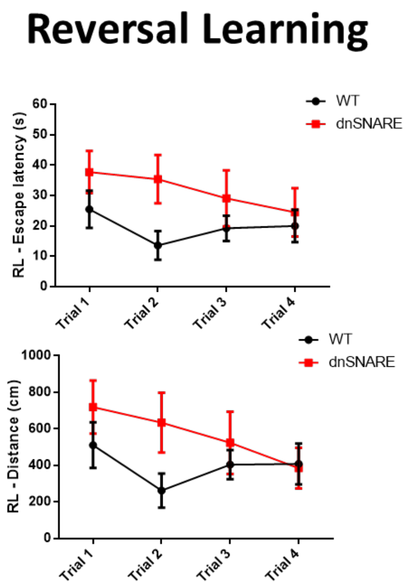
to reach the platform. The percentage of failures to reach the platform and hippocampal-dependent strategies were calculated by classifying the performance of the animals during all trials from the RM task as described avove.

The percentage of failures to reach the platform was similar between WT and dnSNARE animals as represented below, right panel. However, considering the percentage of directed strategies a significant difference was found between genotypes wherein dnSNARE use less directed strategies as compared to controls to reach the platform. This observation supports the cognitive impairment of dnSNARE mice, putatively by a decrease in hippocampal function, and recruitment of additional brain areas to promote the use of egocentric strategies.



Classification of strategies used by dnSNARE mice. Left panel, percentage of animals using directed (green) and non-directed (yellow) strategies for each of the 16 trails in the task; The percentage of animals that failed to reach the platform is depicted in brown. Right panel, group averages of the 16 trials of failures to reach the platform or directed strategies used to reach the platform by wild-type (black) or dnSNARE (red) mice. * $p<0.05$.

Regarding the reversal learning (RL), which is a task more dependent on the prefrontal cortex, it is possible to see below that there is a tendency from dnSNARE animals to spend more time and swim longer to learn the new platform position. However no statistical differences were found between genotypes. Therefore we have focused on the function of the hippocampus as coordinator of the performance of the task.



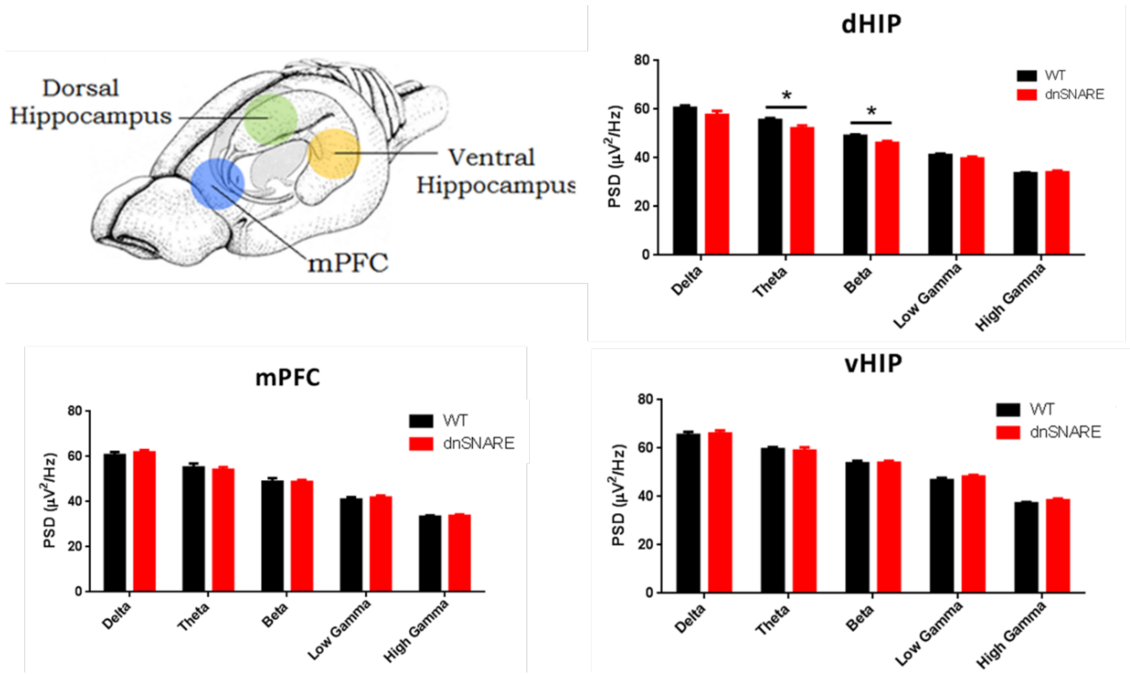
Performance in the Reversal Learning task of the Water Maze. dnSNARE animals display a tendency for longer latencies or swim more to reach the platform than wild-type controls, namely in the first trials of testing.

3. dnSNARE animals present reduced neuronal activity in the dorsal hippocampus and synchronization between this area and the medial prefrontal cortex

This task was powered by the integration of the student Vanessa Sardinha with Biomedical engineering background under the scope of this fellowship.

Power spectra translate the amplitude of the signals recorded in a brain region on the frequency domain. Power spectrum density (PSD) analysis of the recorded LFPs from the mPFC, dHIP and vHIP of all studied mice allowed the thorough characterization of power activity in a wide range of frequencies (Delta, 1 – 4 Hz; Theta, 4 – 12 Hz; Beta, 12 – 20 Hz; Low Gamma, 20 – 40 Hz; High Gamma, 40 – 90 Hz) for those regions at a basal state, as described below.

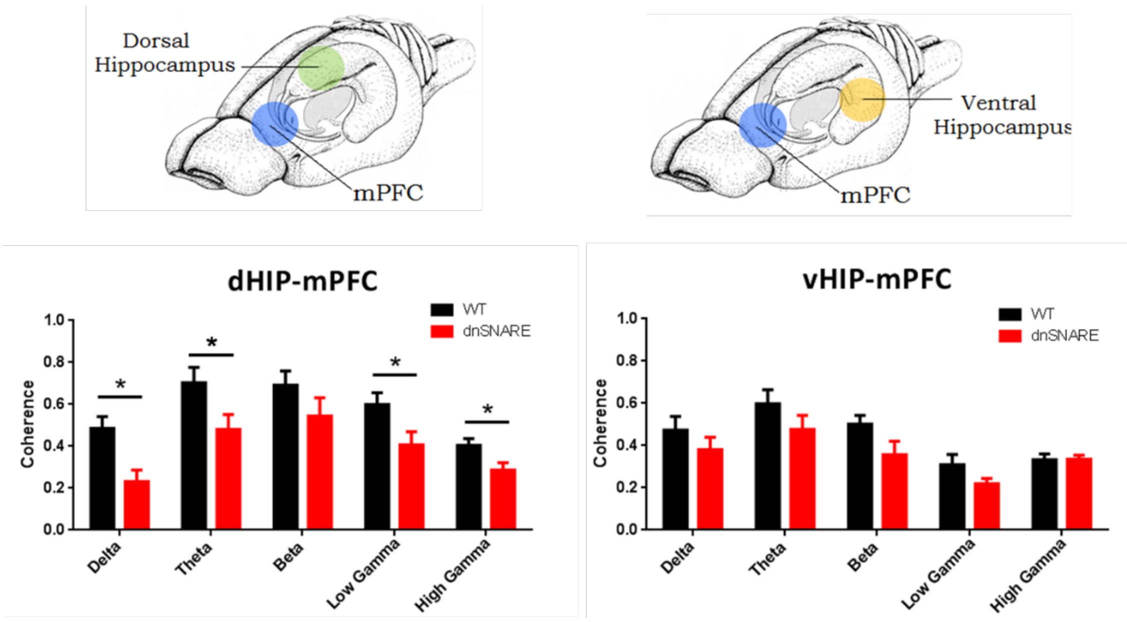
In this analysis, dnSNARE mice presented generally similar distributions of PSD in all frequency bands analyzed in the mPFC and vHIP. Of interest, the neuronal activity of these mice with gliotransmission impairment, measured by PSD, in the dHIP was reduced in the Theta and Beta bands.



Power analysis of neural activity. Power Spectral Density (PSD) values calculated from recordings obtained from the medial prefrontal cortex (mPFC), dorsal hippocampus (dHIP) and ventral hippocampus (vHIP) for control (WT) and dnSNARE mice. (Delta, 1 – 4 Hz; Theta, 4 – 12 Hz; Beta, 12 – 20 Hz; Low Gamma, 20 – 40 Hz; High Gamma, 40 – 90 Hz); * $p<0.05$.

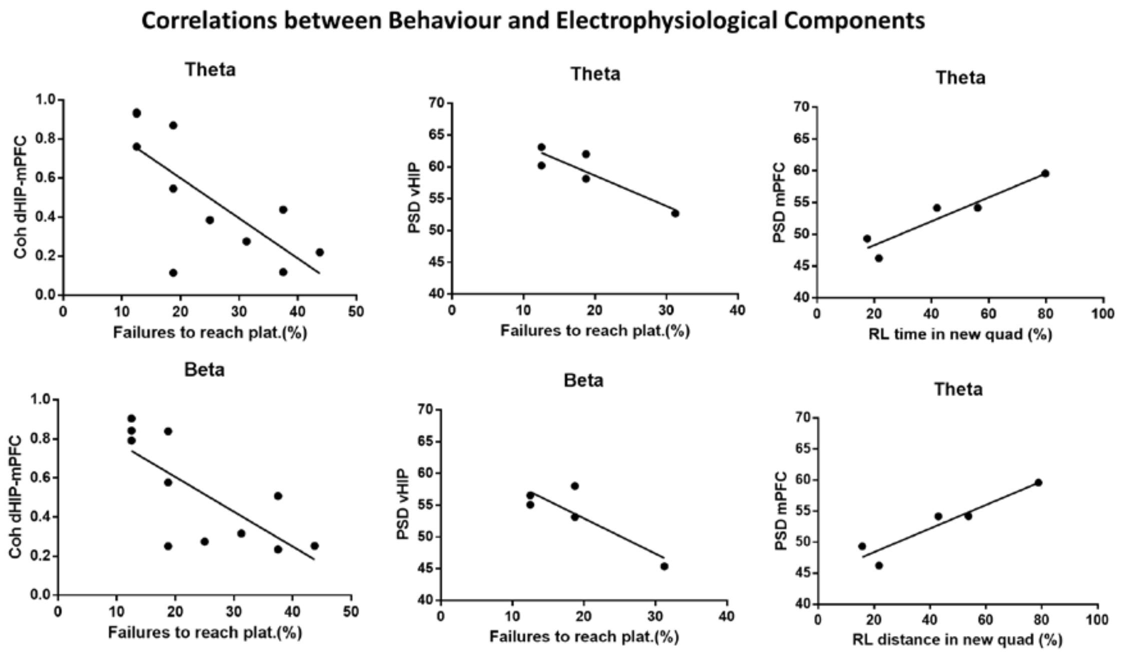
Coherence between brain regions measures the matching of temporal structure in signals recorded from those regions. The temporal structure, or phase, is a powerful measure since it does not depend on signal amplitude and two signals are said to be synchronous if their rhythms' phase match. Coherence analysis of the signals obtained simultaneously from the dHIP and mPFC, or vHIP and mPFC was used to study phase coherence between these regions of the brain (below) in WT and dnSNARE mice.

dnSNARE mice display a critical decrease of coherence generally in all frequency bands analysed when compared to the observed in WT (see below). These results suggest a critical desynchronization between these two areas that support the cognitive function analyzed. They may justify the why these animals have more difficulties to perform these tasks. Regarding the synchronization between vHIP and mPFC, dnSNARE mice displayed a tendency to decrease the entrainment of these regions, although it was not statistically significant. The vHIP is classically related with emotional functions and therefore this may also justify the absence of alterations in the mood or anxiety domains described in previous reports.



Coherence analysis of neural activity between regions. Coherence values calculated from recordings obtained simultaneously from the medial prefrontal cortex (mPFC) and dorsal hippocampus (dHIP), and mPFC and ventral hippocampus (vHIP) for control (WT) and dnSNARE mice. (Delta, 1 – 4 Hz; Theta, 4 – 12 Hz; Beta, 12 – 20 Hz; Low Gamma, 20 – 40 Hz; High Gamma, 40 – 90 Hz); * $p<0.05$.

In order to verily a link between the alterations in PSD and Coherence observed and the behaviour performance, the data resulting from these analyses was correlated. Interestingly strong positive correlations were found between coherence between mPFC-dHIP and PSD dHIP and number of platform reaches for the theta and beta bands, bands normally associated to the synchronization between cortico-limbic structures. Additionally, the neural activity in the mPFC was linked to a good performance in the reversal learning task, a task that depends clearly on the function of this area.



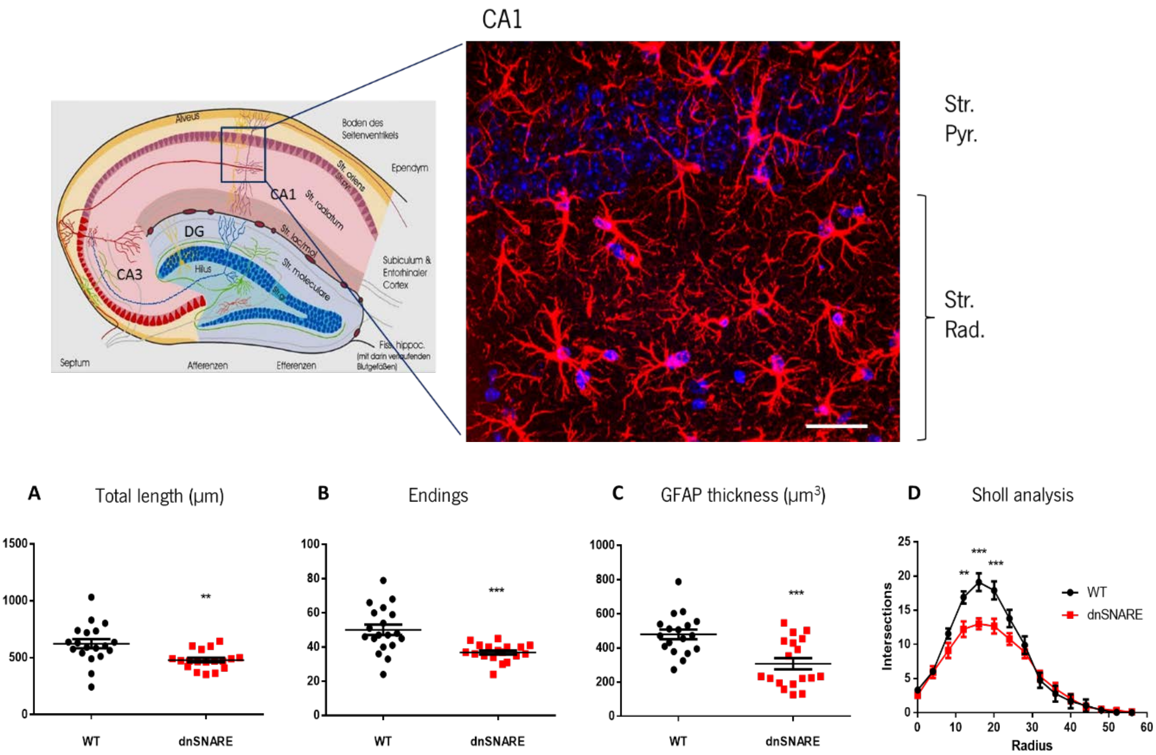
Correlations between behaviour and electrophysiological components. Significant correlations between performance in the Reference Memory task and neuronal activity in the brain regions related with this task.

Altogether, these data support that dnSNARE present hippocampal deficits probably due to impairments in the neural activity of these area and synchronization with cortical structures. Additional studies were performed to assess structural alterations in the dnSNARE model caused by the lack of gliotransmission in these animals.

4. dnSNARE animals display reduced process arborisation

This task was powered by the integration of the student Gabriela Tavares with applied biology background under the scope of this fellowship.

In order to understand in detail the impairment observed in the hippocampus, we have performed a detailed reconstruction of the 3D structure of astrocytes in this region, more specifically in the CA1 region. For that purpose, we have stained brain samples obtained from WT and dnSNARE mice after sacrifice. The staining targeted the GFAP protein, specifically expressed in the cytoskeleton of astrocytes in this area, allowing the visualization of the detailed cell structure. The Simple Neurite Tracer software is an open source tool that was adapted by the student to perform a morphometric study of astrocytes morphology in dnSNARE mice model comparing to



Astrocytes 3D reconstruction from dorsal hippocampi of WT and dnSNARE mice. dnSNARE astrocytes present a different structural morphology compared with the wild type (WT) littermates since they show shorter (**A**), less number (**B**) and thinner processes (**C**). All these features together with Sholl analysis (**D**) show that dnSNARE astrocytes present less complexity arborization compared with WT mice. n=20 astrocytes per group * p<0.05.

WT littermates. After GFAP staining the images were obtained using confocal laser scanning microscopy and 10 astrocytes per animal were traced.

This technique provided data that point out a reduction in astrocyte process length in the dnSNARE animals, when comparing to the respective littermate WT. These astrocytes also display fewer ramifications and thinner processes which result in a less complex arborisation as supported by the sholl analysis.

Altogether the hippocampal impairment described above seems to be correlated with a decrease in astrocytic complexity in the CA1 region. Therefore astrocytes appear as critical elements of the neuroglial network, whose function is required for the normal hippocampal function.

Project final conclusions

Looking at the progress reported here and in previous reports, we do believe that the goals established for this fellowship were fully accomplished. As initially proposed, we have used complementary state-of-the-art techniques such as in vivo electrophysiology, innovative behaviour, anatomical and molecular analysis, to seek a role of astrocytes in the computation of cognitive functions. It is noteworthy that we have made a rigorous management of the available budget, always looking for the best people to help with specific techniques, such as electrophysiology, and the more suitable/optimized protocols and processes. This effort resulted ultimately in an increased consistence of the data obtained, confirmed by the high quality publications obtained so far (other will be submitted soon).

What did we learn from astrocytes?

We employed in this project 2 approaches to affect astrocytes in the brain of living animals and analysed its impact on cognitive function. As described previously, the pharmacological induction of astrocyte pathology, was followed by a progressive neuronal damage observed in the area of lesion. These data pointed out an important role of astrocytes in the neuroglial network, since in the absence of astrocyte the function of network may be severely affected. Interestingly, this reduction of astrocytes obtained with this model mimics the pathological astrocytic decrease observed in several mood disorders (eg. Major depression) in the prefrontal cortex (PFC). For the first time we showed a link between with reduction of astrocytes in the PFC and the cognitive deficits dependent on this brain region. This observation is of great importance if we consider that cognition, namely functions dependent on the PFC, is typically affected in human patients suffering from mood disorders.

Using a second approach that deletes specifically the release of molecules by astrocytes with genetic tools, impairing their cross-talk to neurons we intended a more specific modulation avoiding tissue damage that could affect mice welfare with consequences in behaviour performance. This mice model of gliotransmission impairment allows us to study the impact of neurotransmitter release by astrocytes in the network function. Since we are interested in understanding the cognitive function we have again studied in detail the prefrontal cortex and the hippocampus, areas intimately related with several forms of learning and memory.

The results of the behaviour paradigms used allowed us to verify that these animals do not display locomotor or exploratory deficits, anxious-like neither depressive phenotype. This is very important since these dimensions could interfere with the performance in cognitive tasks. Mice with impaired gliotransmission displayed a level of cognitive dysfunction, in the water maze tests, visible across different sets of animals. The detailed analysis of the electrical activity in the mice brains indicated an impairment in the dorsal hippocampal function, and in the communication of this region with the prefrontal cortex. Since these regions are required for the correct task performance it was expected that the data from both tests would be correlated. Indeed, the correlation between behaviour and electrophysiological data indicated the decrease in PFC-dHIP synchronization supported the worse performance of dnSNARE animals. This data was further supported by a decrease in astrocyte complexity in the hippocampus which suggests that dnSNARE astrocytes in this region are less functional and this may support negative consequences to the neuron-astrocyte communication, ultimately affecting behaviour.

Astrocytes appear as critical elements of the neuroglial network, whose function is required for the normal hippocampal function.

This info accounts for new knowledge on the cognitive computation and this new player may account also for the cognitive decline observed for instance in ageing or pathological processes, arising as a possible therapeutic target for the treatment of prevention of cognitive deficits.

What did we do with this knowledge so far?

We have always sought to share the data with experts in the field either by interacting closely with the project consultants, or by presenting the fresh data in international conferences in the research topic area (such as the congresses of the European Federation or American Neuroscience Societies) or invited talks at expert labs (e.g. CSIC Cajal Institute, Madrid, Spain).

The work produced in the scope of this project resulted so far in two publications in leading peer reviewed journals, namely in Molecular Psychiatry, the most relevant

Journal of Psychiatry, from Nature Publishing Group, (Impact Factor 15.1). We are currently processing the data relative to the dnSNARE model to submit soon to the journal Nature Neuroscience. The close interaction with the consultant Alfonso Araque resulted in the compilation of a comprehensive review on impact of astrocytes in animal behaviour submitted 2 weeks ago to Trends in Neurosciences (Cell Press), after positive feedback from the journal editor-in-chief. The first pages of this manuscript are included below.

We have also made a large number of dissemination activities of the research outcomes to reach not only the scientific community, but also the wider public in multiple modalities:

- workshops for common citizens around the area of Braga;
- press releases and articles in national newspapers;
- communications and workshops at high schools;
- invitation of undergraduate students to the lab;

What to do next?

This project produced the clear message that astrocytes are partners of neurons in computation of forms of cognition. Now it is time to rescue the lost function in astrocytes. We have proposed this idea to the Bial Foundation in a project that was approved for funding. Therefore we will be able to find putative mechanisms that will enlarge our knowledge on the computation of cognitive function and may be used for the design of tools to rescue or prevent of cognitive decline in humans.