AgroParisTech
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RAPPORT DE STAGE DE RECHERCHE

Spatial reorganization of proopiomelanocortin (POMC)-expressing neurons in the arcuate nucleus of POMC-EGFP mice resistant or prone to obesity

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NON CONFIDENTIEL

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Contents

Abstract

The substantial increase in adult obesity prevalence has been linked to a growing weight promoting environment and sugar sweetened beverages such as sodas. However, not all people exposed to such an environment become obese. Obesity can be linked to altered biological processes of energy homeostasis, and the arcuate nucleus (ARC) of the hypothalamus is the main brain area of such processes. One of the neurons originating from the ARC expresses proopiomelanocortin (POMC), a precursor of various polypeptides, whose effect is mainly anorexigenic.

An altered food-intake regulation could be linked to a modified recruitment of POMC expressing neuronal populations, and spatial organisation of a neuronal network can be modulated by its level of activation. A previous study has shown that there were some differences between spatial organisation of POMC expressing neurons in the arcuate nucleus between obesity-prone (OP) and obesity-resistant (OR) mice when submitted to high-fat high-sucrose (HFHS) diet. In order to extend this study, we examined POMC neurons of the ARC in 6 mice of the same strain, C57BL/6J POMC-EGFP mice (which express the Green Fluorescent Protein (GFP) concomitantly with POMC) without submitting them to an HFHS diet and thus without an obesogenic factor (Control).

The brains of OR, OP and control mice were fixed and cut into coronal sections covering the whole hypothalamic area of the ARC. These sections were digitized and analysed to obtain three-dimensional reconstructions of the ARC area, using the Free-D software. A statistical analysis of these reconstructions showed no significant differences so far between the three groups. However, this analysis is still to be completed, and could eventually enable us to conclude more thoroughly concerning differences in the spatial organization of POMC-expressing neurons in the ARC.

Chapter 1. Introduction

1.1 Control of food intake

Despite frequent fluctuations in food intake and in physical activity, most healthy adult mammals are able to maintain a stable body weight and body fat for many years. This indicates that energy intake and energy expenditure are matched with great precision. Such processes can however be disrupted and lead to obesity. The substantial increase in adult obesity prevalence has been linked to a growing weight promoting environment (rising consumption of energy-dense, high-calorie foods (Slimani et al, 2009) and sugar sweetened beverages such as sodas (Popkinet al, 2006)). However, not all people exposed to such an environment become obese. Today we know that in mammals, when submitted to identical obesogenic diets, some tend to be resistant (obesity-resistant, OR) and others more prone (obesity-prone, OP) to obesity (Even et al, 2011).

This regulation of energy homeostasis is due to numerous circulating peptides and steroids circulating in the body which influence appetite through their actions on the hypothalamus, the brain stem and the automatic nervous system (Anthony and al, 2007). These are of three major types; adiposity signals, nutrient-related signals and satiety signals (Fig 1).

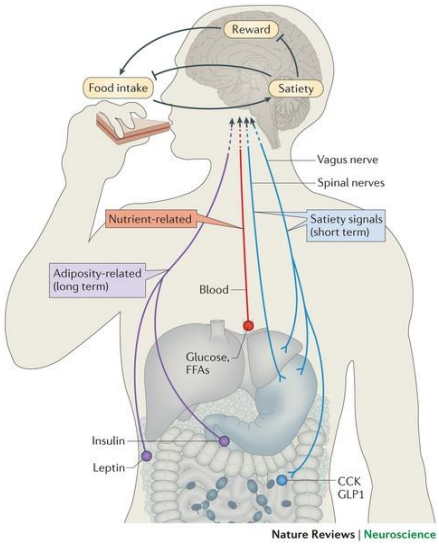


Fig1*: Control mechanisms of food intake (Adapted from (Morton et al, 2014*). The central nervous system (CNS) integrates input from long term energy stores such as leptin, as well as short term satiety or nutrient related signals to regulate food and energy intake.

- **Adiposity signals** circulate in proportions mirroring fat cell stores. Leptin, insulin, positively correlated with body fat, plays an important role in reducing food intake. Other adipocyte derived hormones such as adiponectin are negatively correlated with body fat and seems to be protective against the development of insulin resistance or glucose intolerance.(Nawrocki et al, 2006)
- Several **nutrient-related signals** are implicated in the homeostatic control of feeding. Among these are free fatty acids which exert insulin-like effects in key brain areas for energy homeostasis (Morton et al, 2006), intravenous glucose and amino acids which help reduce food intake.
- **Satiety signals** such as Cholecytokinin (CCK), glucagon-like peptide 1 (GLP-1), peptide YY (PYY) and ghrelin generated in the gastro-intestinal tract provide postprandial information to the hypothalamus and the brain stem through activation of the vagal afferents and sympathetic pathways. Activated neurons in the nucleus of the solidary tract (NTS) participates to satiation.

Satiation and satiety are two distinct notions. Satiation corresponds to the processes which ends the meal, while satiety corresponds to the time interval until the next eating episode.

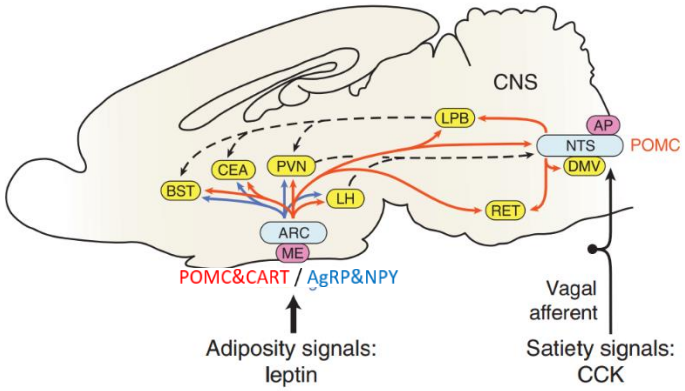


***Fig 2**: Schematic of the central melanocortin system (Adapted from (Cone et al, 2005)) Blue, nuclei containing POMC neurons; yellow, nuclei containing MC4R neurons; red arrows, representive POMC projections; blue arrows, representive AgRP projections; CNS, central nervous system, AP, area postrema; ARC, arcuate nucleus; BST, bed nucleus of the stria terminalus; CEA, central nucleus of the amygdala; DMV, dorsal motor nucleus of the vagus; LH, lateral hypothalamic area; LPB, lateral parabranchial nucleus; ME, median eminence; NTS, nucleus tractus solitaries; PVN, paraventricular nucleus of the hypothalamus; RET, reticular nucleus*

Such signals are incorporated in the central melanocortin system, one of the key neural pathways involved in the regulation of energy homeostasis. This system is defined as a collection of CNS circuits that include (i) neurons originating in the arcuate nucleus (ARC) that express hypothalamic neuropeptide Y and agouti gene-related protein (NPY/AgRP) or proopiomelanocortin (POMC) and cocaine and amphetamine related transcript (CART), (ii) brainstem POMC neurons originating in the commissural nucleus of the solidary tract (NTS) and (iii) downstream targets of these POMC and AgRP neurons expressing melanocortin receptors (MC3R and MC4R) (fig 2, Cone et al, 2005). Neurons that co-express NPY and AgRP are known to stimulate food intake and neurons expressing POMC and CART which are anorexigenic neuropeptides.

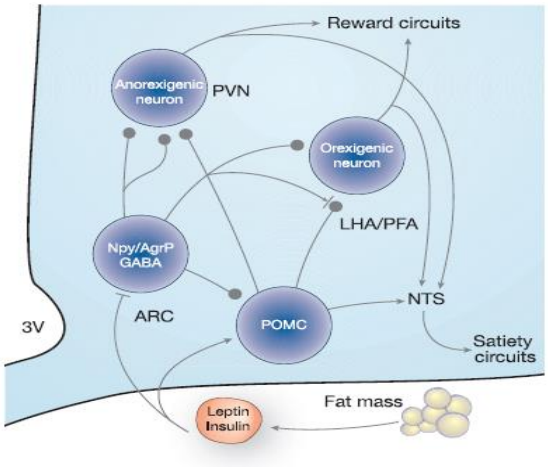


***Fig3**: Hypothalamic neurocircuits involved in energy homeostasis. (Morton et al 2006) PVN, paraventricular nucleus; GABA, gamma amino-butyric acid; LHA, lateral hypothalamic area; PFA perifornical area*

POMC and AgRP neurons are referred to as "first order" neurons. Their axons can project to "second order" neurons located mostly in two other brain areas: the paraventricular nucleus (PVN), which produces anorexigenic compounds (such as oxytocin), and the perifornical area (PFA) (fig 3 (Morton et al 2006)), which secrets orexigenic substances (melanin-concentrating hormones (MCH) and orexins) (Valassi et al, 2008).

1.2 Hypotheses

Studies have shown that obesity is often a result of change in food intake originated from an altered regulation of appetite (Savastano et al, 2005). It has been shown that a change in the level of activation of a network of neurons could imply a shift in this network's density and

spatial distribution (Maguire et al, 2000), and this may be the case with neuron populations involved in food intake regulation.

Other publications reported that an increased population of newly generated neurons in the ARC expressed a POMC phenotype when submitted to a high-fat diet, as an adaptive anorectic function to adjust future energy intake and prevent further weight gain (Gouazé et al, 2013).

The results of the study preceding this project has shown some evidence of difference in spatial organisation of POMC neurons in the ARC between OP and OR mice. However the mice were aged 8 weeks (young adults) at the beginning of the procedure. Thus the observed changes in spatial distribution of POMC neurons could either be a cause or a consequence of mice susceptibility to obesity. Furthermore the data did not allow to conclude whether these differences existed prior to the administration of the diet.

1.3 Project aims

To continue the works of the previous study, we will use the C57BL/6J POMC-EGFP mouse strain, which has the particularity to express the green fluorescent protein (GFP) concomitantly with POMC and whose POMC neurons are therefore fluorescent. Moreover, C57BL/6J mice have a natural tendency to develop obesity when given an obesogenic diet, and they show a great variability in response to these diets. We will investigate the spatial distribution of POMC neurons in the ARC of these mice, using three-dimensional (3D) reconstruction and statistical mapping of POMC-neuron densities.

The main objective in this project is to determine whether the spatial redistributions of POMC neurons occur in OP mice or mostly in OR mice, which could develop a “protective system” against weight gain when fed a HF diet, and if spatial reorganization is mainly a cause or a consequence of susceptibility to weight gain.

Chapter 2. Methods and Materials

2.1 Animals

Six male C57BL6 – POMC-EGFP mice (Jackson Laboratory, Bar Harbor, Maine, USA; Annexes 2 and 3) received at the age of 6 weeks were housed in a common cage under controlled temperature (~22℃) and under a 12-hour light/ dark cycle (light time: 9.30 P.M. to 9.30 A.M.). Annexes 2 and 3 give a more detailed explanation on how these mice, whose particularity is to have green fluorescent POMC neurons, were obtained. All experiments were carried out according to the guidelines of the French National Committee for Animal Care and the European Convention of Vertebrate Animals Used for Experimentation, under European Council Directive 86/609/EEC dated November, 1986.

2.2 Brain fixation, collection and slicing

These mice were sacrificed 3 weeks after their arrival. On the day of sacrifice, mice were fed at the usual time (9:30). Mice received a lethal i.p. injection of sodium pentobarbital (2 mL/ kg, Pentobarbital® Sodium, pentobarbital 54.7 mg/ mL, Ceva) and were transcardially perfused (21G needle placed in the left cardiac ventricle) with 50 mL of DPBS (with Ca^{2+} and Mg^{2+}, Lonza, Belgium) supplemented with 0.05% of $NaNO_2$ (Merck, Schuchardt). They were then immediately perfused with 100 mL of 4% formaldehyde (Neutral Buffered Formalin, formaldehyde 4%, DiaPath Microstain Division, Italy). The brains were removed and placed in 25 mL of a 4% formaldehyde solution for 3 to 4 days at 4℃. They were then transferred for dehydration in 25 mL of a solution containing 15%w/ v of sucrose for 24 hours, followed by a 30% sucrose solution for 2 days. The brains were then frozen at -80℃ in optimal cutting temperature medium (OCT) (Tissue-Tek, Sakura Finetek). Embedded brains were stored at -20℃ until sectioning. Approximately 140 coronal 20 µm-thick sections were cut at -19 to -21 ℃ using a cryostat (CM1520, Leica, Germany) from bregma -0.26 mm to the third ventricle (bregma -2.84 mm). These sections covered the entire extent of the arcuate hypothalamic nucleus (Figure 4) Brain positions were localized using a stereotaxic atlas (Paxinos et Franklin, 2001) (Figure 5). Every other section was collected on glass slides (Superfrost ultra plus, Menzel-Gläser). The slides were then mounted under cover glasses with Vectashield® Hard set with 4',6'-diamidino-2-phénylindole (DAPI) (Vector Laboratories, Inc. Burlingame, CA 94010), which stains the nuclei of cells, then were digitized by the platform team of laboratory UMR GABI (Panoramic Scan 150, 3D Histech)

2.3 Three-dimensional model construction

Three dimensional reconstructions of the third ventricle (Figure 4) and of fluorescent POMC neuronal populations will be made using Free-D, a reconstruction and modelling software (Andrey et al, 2005). It will consist in performing the following steps: image segmentation, image registration and model rendering.

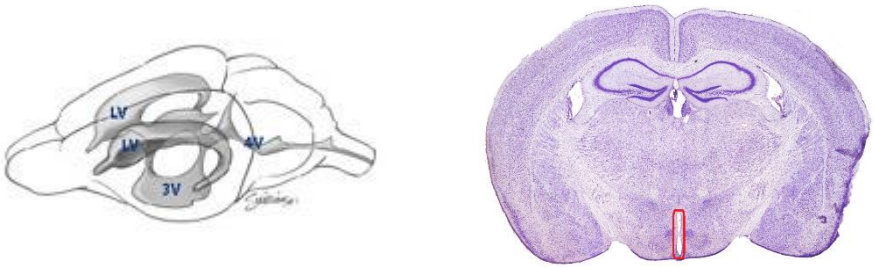


***Fig 4a**: ventricles in the mouse brain. LV, lateral ventricle; 3V, third ventricle; 4V, 'fourth ventricle (http://jaxmice.jax.org/images/jaxnotes/490f.jpg)*
***Fig 4b**: Coronal slice of mice brain at Bregma -1.34mm, 3rd ventricle in red (Extracted from (Paxinos et Franklin, 2001)).*

Image segmentation. Each structure of interest was manually delineated on the digitized image of each coronal section of each mouse. The contours of the 3rd ventricle were registered using the DAPI-stained images

The fluorescent POMC neurons were registered on the images scanned in green fluorescence (Figures 5 A and B) and then transferred on top of the DAPI -stained images.

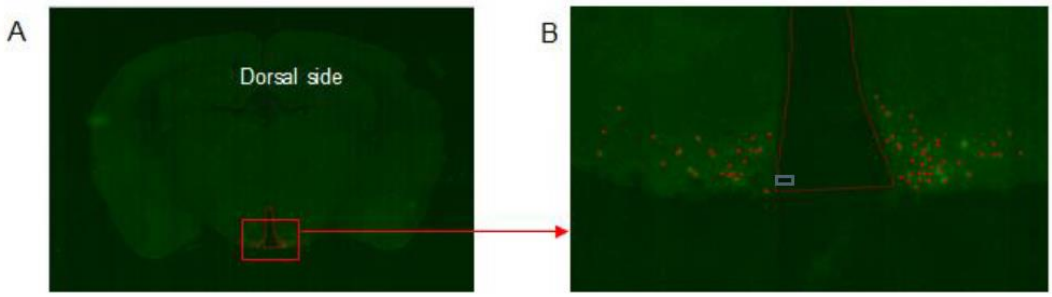


***Fig 5**: Registration of fluorescent POMC neurons using the images scanned in green fluorescence.(A) View of the whole slice. (B) Zoom on the area of interest (ARC)*

Image registration and model rendering. After this first step of segmentation, each section was adjusted on top of the previous one (image registration) in order to get a coherent 3D model (model rendering) (Andrey P., 2014).

Once 3D reconstruction models had been done for each mouse, we found a common landmark that would enable us to align with better accuracy the models together. This step was necessary before considering the possibility to create averaged brain models for each group. The landmark which was chosen is the moment when the third ventricle disappears, at bregma -2.82 mm.

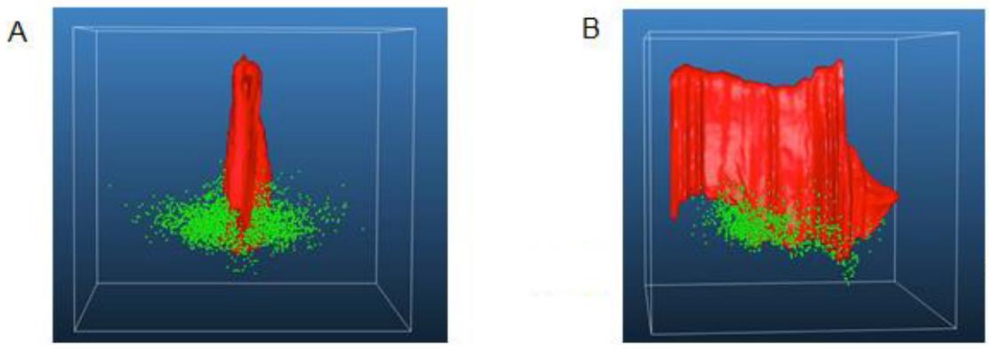


***Figure 6**: 3D reconstructions of the 3rd ventricle and of the POMC neuronal population of a mice brain using Free-D software (A) Frontal view (B) Lateral view.*
In red: the 3rd ventricle, in green: POMC neurons.

A maximized common area to all models, and which contained all POMC neurons for all mice, was then kept. This maximum area began at bregma -0.96 mm and ended with the third ventricle (bregma -2.82 mm), containing 48 brain sections.

2.4 statistical analysis

Analyses of our 3D reconstructions will first be performed on Free-D, in collaboration with the team who built this software at the Institut Jean-Pierre Bourgin (UMR 1318, INRA-AgroParisTech, Versailles) and with whom our laboratory had already worked on a previous project (Schwarz et al, 2010).

Reframing. The 6 ventricles will be reframed and aligned together thanks to rotations and translations only. An average ventricle will then inferred from this re-alignment.

Normalization. The next step will consist in normalizing all the ventricles and POMC neurons next to it. It will consist in applying to each of the 6 3D models affine transformations (we chose 1st degree transformations only). These transformations will enable to fit as accurately as possible each model with the average ventricle constructed in the first step (reframing). The neurons follow the same transformations as the ventricle.

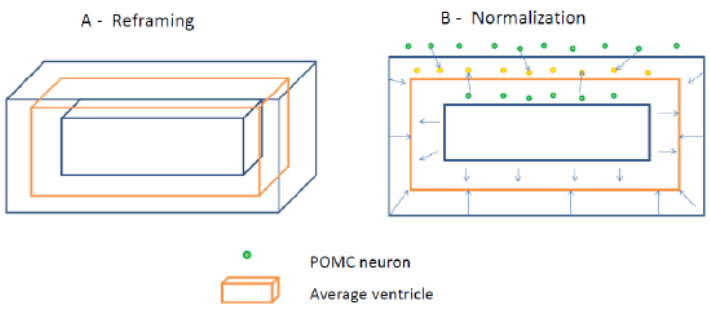


***Fig 7**: Sketches of reframing (A) and normalization (B) steps.*
Ventricles are in blue, the average ventricle in orange and POMC neurons in green

Figure 7 illustrates reframing and normalization steps, supposing we have two parallelepiped ventricles, with one slightly smaller than the other.

Generation of surfaces representing the organization of POMC neurons. Once the 6 ventricles have been normalized, different kinds of surfaces can be drawn to represent the spatial organization of the POMC neurons. So far, two types of representation have been explored:

- **Percentile method**: A surface including 50% of the neurons, according to the following logic: the point of the 3D space whose local density is the highest is included in the surface, with the neurons around it, the point with the second highest density is then included, and so on, until 50% of the neurons are inside the surface. This method may end up with a surface with some detached blebs.

- **Density method**: A surface which includes all points whose local density is superior to a chosen threshold.

Generation of maps. P-value maps will be created from density maps, and maps of iso-p-values will be extracted from p-value maps.

Chapter 3 results

3.1 results of the previous study

3.1.1 Selection of OP and OR mice

OP and OR mice were selected on the 9th week according to their weight gain. Among the 16 mice, 4 mice with the most gained weight were selected as OP and 4 with the least gained weight were selected as OR.

Figure 8 and 9 show that OP mice gained more weight (10.275g in 9 weeks) and fat (10.14g) compared to OR mice (8.19g in weight and 6.24g in fat). When averaged by group, the weight gain was significantly different from the 4th week, and fat gain at the 9th week.

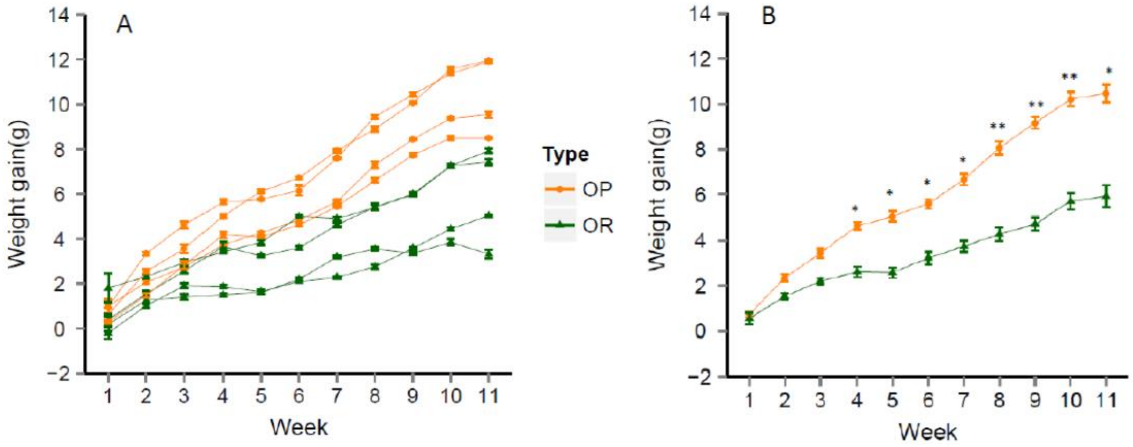


***Fig 8**: Body weight gain of OP and OR mice individually (A) or averaged by group (B)*

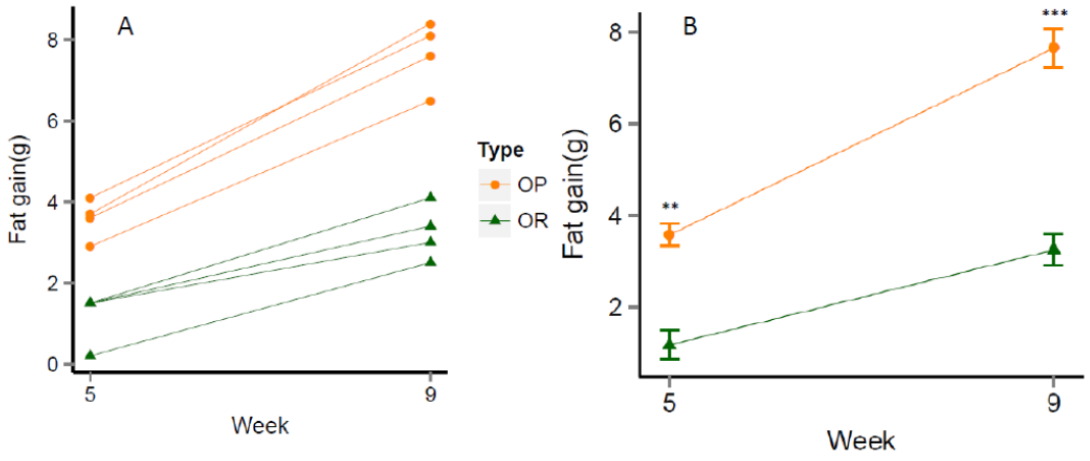


***Fig 9**: Fat mass gain of all OP and OR mice individually (A) or averaged by group (B)*

3.1.2 Energy intake between the two groups.

As shown in figure 10 below, OP mice consumed greater amounts of HFHS food than OR mice leading to greater total energy intake (Fig 9, p-value=0.03 at week 11). From week 8, the cumulated food intake was significantly higher for OP mice. (Fig 10 B, p-value=0.05 at week 8).

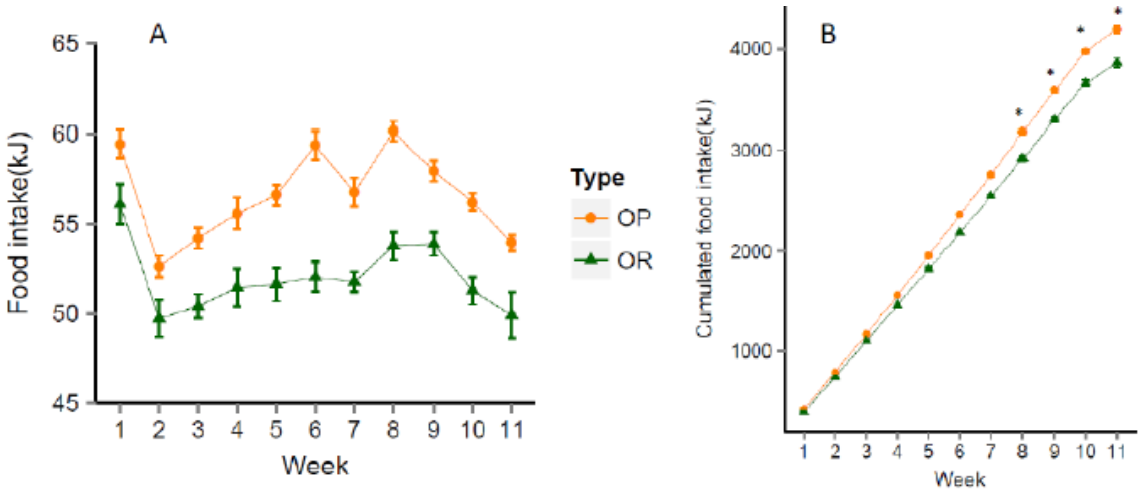


***Fig 10**: Average daily (A) or cumulated (B) food intake of OP and OR mice*

3.2 Spatial organisation and density of the POMC neuronal population

The total number of POMC neurons was recorded for each group of mice (fig 11). We found no significant difference in the total number of POMC neurons between OP, OR and control mice.

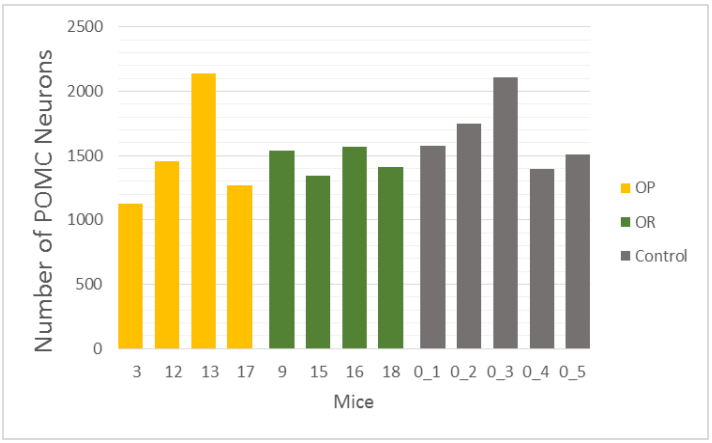


***Fig 11**: Total number of POMC neurons recorded in the ARC for each mouse*

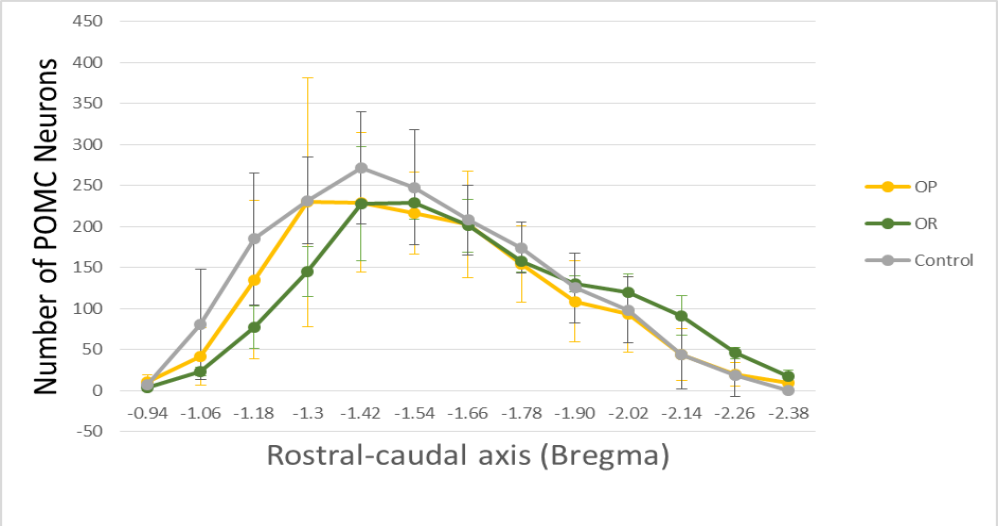


***Fig 12**: Number of POMC neurons in each slice (1 slice= 3 added sections) averaged for each group*

The ANOVA tests performed from this data (Fig 12) gave a significant difference in the total number of POMC neurons according to the position of the slices (p-value less than 0.05), but none according to the type of mice or to the "type of mice & position of slice".

Also, in the previous study we obtained an average ventricle model, with surfaces covered by either POMC neurons of the OP group (in orange) or those of the OR group (blue).(Fig 13) In the same way, we have obtained an average model for the control group.

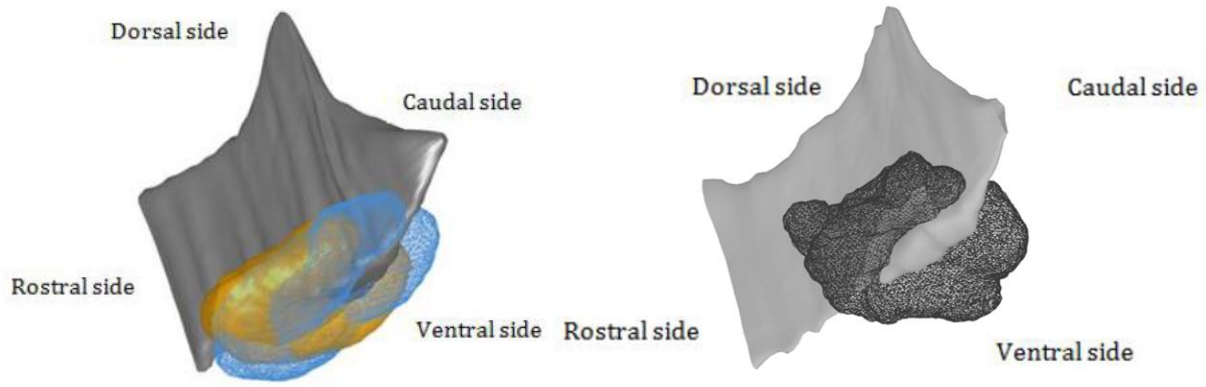


***Fig13**: Average ventricle (in grey) and surfaces.*
In orange: surface of the OP group, in blue: surface of the OR group,
in black : surface of the Control group

Chapter 4: Discussion and perspectives

The spatial distribution and density of POMC expressing neurons in the ARC within the control group had no significant differences. The variability in obesity susceptibility can thus be explained by epigenetic, and not genetic, factors induced by behavioural factors, which could lead to altered physiological and cellular mechanisms (signalling pathways and angiogenesis, for instance) (Azzout et al, 2014). The control model not submitted to the HFHS regime were different from the OP model, but were similar to the OR model. This would imply the existence of local plasticity in the brains of young adult mice in response to external stimuli, eventually constructing a protective system against excessive food intake in OP mice, but not in OR mice. Comparing the three models in detail will give us insight on the different evolution of neural populations during early adulthood. To explain these different evolutions, we will investigate the potential mechanisms:

-The group whose density is higher in some places of the arcuate nucleus could have higher transcription rate of POMC expressing neurons, or have increased neurogenesis of such neurons.

-A particular group of POMC neurons could also be subject to increased cellular death, such as apoptosis, in the group where there are locally less neurons.

Some articles have studied that a high fat diet did in fact induce increased proliferation of POMC expressing neurons in the arcuate nucleus of the hypothalamus as a short term response (5 to 11 days) (Gouazé et al, 2013).

Also, spatial distribution changes could be linked to regional functions within the arcuate nucleus. If a given region gained more POMC neurons in OP mice than in OR, knowledge of its role and connectivity to other regions could give us a functional insight on spatial distribution of this area. Such an in-depth knowledge of the functionalization of the neuronal populations in the ARC is not well known yet.

Chapter 5: Conclusion

Although we anticipate partial outcome of our results, there are few that can be said before having all the raw data. Parts of the data of previous studies were not accessible. Detailed statistical analyses between the three groups (OP, OR, Control) were thus not possible.

Further research on differences in this same population of neurons, but at different locations in the brain, such as the nucleus of the solitary tract, or of other neurons also implicated in the regulation of food intake could also prove discerning and fruitful. Although this wasn't analysed, such neurons were digitized in the previous project, and we will compare with neurons of the current project.

Supposing that the differential spatial distribution of POMC neurons in OP and OR mice are induced by an obesogenic diet, we still don't know what sets off the mechanisms. There are therefore many questions which are still unresolved, but which open a path to many more experimental projects in the field of nutrition and neurobiology.

This research internship was a very enriching experience, which furthered the scientific knowledge I have learnt at AgroParisTech by giving it a more practical and experimental approach. It also helped me learn what it was to be in a laboratory, and understand those who worked in the field of research in biology.

Annex

1 Structure of the POMC gene

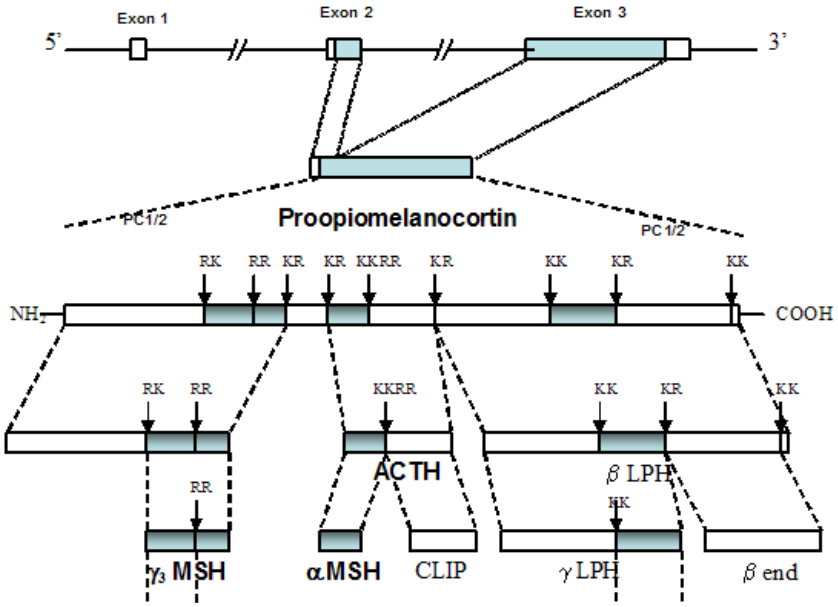


Fig12 :Gene structure and post-translational processing of proopiomelanocortin (POMC). (Extracted from (Millington, 2007)).

2 Construction of the cosmid used to obtain the gene of interest (POMC-EGFP)

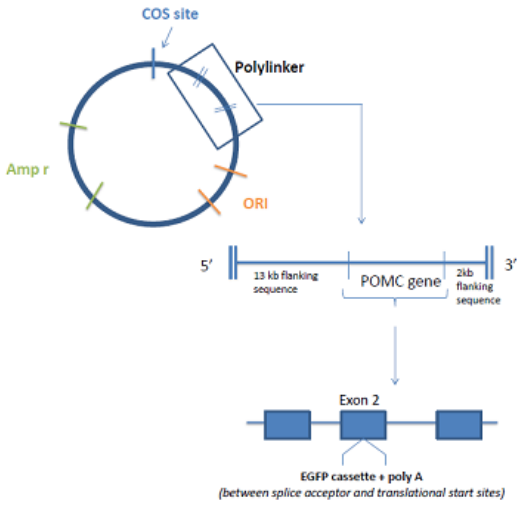
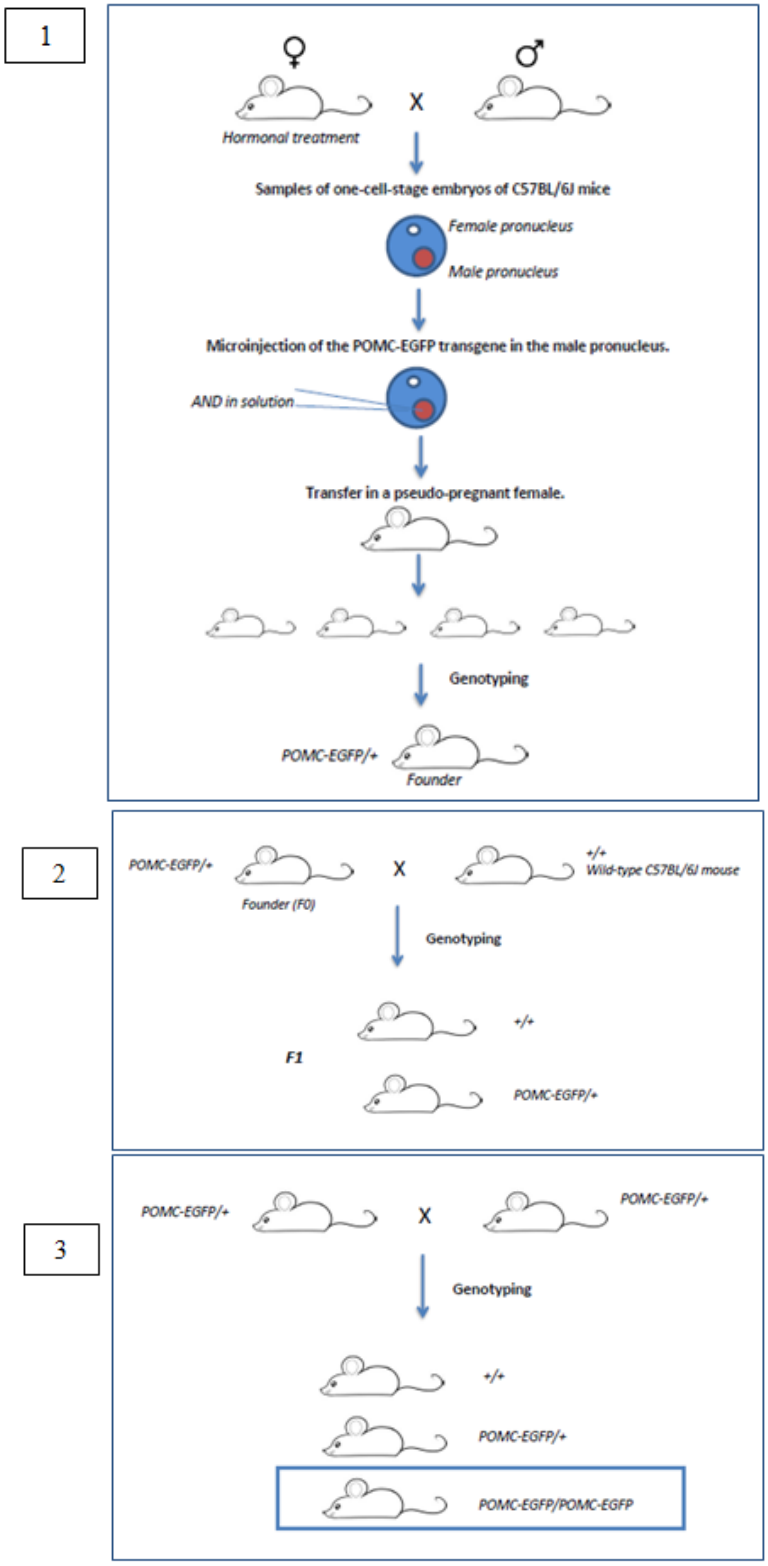


Fig 13:Cosmid used to obtain the gene of interest to introduce in mice strains.

3 Crossings between strains to obtain transgenic C57BL/6J POMC-EGFP mice



POMC-EGFP mice were first developed by Dr Malcolm J Low of University of Michigan Medical School

POMC-EGFP mice were bred to C57BL/6J wild-type mice for approximately 12 generations before their arrival at the Jackson Laboratory.

After arriving at the Jackson Laboratory, mice were bred with C57BL/6J inbred mice for one generation or more, to establish the colony.

Fig 14: Obtaining transgenic mice. http://jaxmice.jax.org/strain/009593.html

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