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The long-lasting effect of early life stress in mPFC: excitatory and inhibitory balance in a murine model of anxiety

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Introduction and aim of the study

As the interface between one's self and the surrounding environment, our brain is an organ which truly is fundamental for the survival of the individual and its possibility to transmit genes and species' feature throughout generations and time. The features and traits of the encephalon and of the central nervous system have accurately been selected and maintained during the path of evolution, making it one of the most powerful instruments in the struggle for life. This peculiar organ is in fact able to receive, analyze and integrate a wide variety of stimuli and characteristics both from the surroundings and the functions of the body, translate them in a relatively simple informational language and integrate the resulting impulse in an efficient and quick way in order to perform behavioral strategies with the aim to answer to the threats of the outside world, whether they're coming from predators, conspecific, natural events . The system has also the purpose of collecting signals from the body and via them build awareness of its current status and of the maintenance of the homeostasis which is required to sustain life.

Anyway, the recollection and the gathering of information are just a first step in the path of the animals' orientation and action toward the world. The central nervous system in fact didn't just evolve to check and monitor on what's happening. Its purpose is way more complex and useful, and involves different strategies selected by evolution to maximize the possibility of survival. The second step of the process involves the ability of the animal to organize the selected information and coherently integrate them in a significant presentation. This is an ability which is largely shared in the animal kingdom, and tends to emerge in an early stage of its history even if it is differently organized towards the different classes and orders. Different strategies have been experimented to achieve the objective, even if for the sake of this dissertation and the nature of the work hereby described, will be mostly analyzed the structure and circuitry that make this possible for mammals, and in details in rodents.

The phase of the organization of knowledge coming from the internal and external world is not a passive one. This means that every piece of information is not processed and organized in the same way, and does not have the same salience. Some things are considered more important or bringing more advantages or representing an urgent threat that needs a quick answer. So, as long as all the stimuli that are physically perceivable are

processed and translated in neuronal impulses and reach the lower nuclei of the brain, only a portion of them is consciously brought to the attention of the subject and will be evaluated on the base of the necessity for the system to set an appropriate behavioral or cognitive response.

To achieve this purpose, the central nervous system needs to create and maintain a balance between salient and non-salient pieces of information suppressing the latter ones and reinforcing the ones which are significant.

The construction of the state-trait conception of anxiety is an important example of this dynamics of continuous adaption of our brain to its context. In the following study this evolutionary perspective of the emergence, organization and modulation of the psychological events and traits in the brain will be coupled with an experimental animal model of conditional knock out gene alteration and early life experience modification.

Anxiety

Anxiety represents an important and one of the most profoundly studied emotional events in the brain. Since the ethological scientific studies of the classic cognitivism by Skinner and Miller, one of the most powerful motivational engines of animals' action has been considered the emotional process, and in this sense the most important among the others emotions has always been considered fear. This for the importance for the survival of species and the possibility to easily observe its identifying factor and manifestation in the living beings. Soon enough in the research field, it become clear an important relationship between fear and anxiety, as it starts to be conceptualized as a state of fear-related activation in absence of a real or perceived threatening stimulus.

In this fashion, the evolutionary meaning of anxiety becomes clear as a form of activation of the brain to try and anticipate potentially menacing situations, and to focus and maximize attention on the surroundings to identify and prevent any possible harm. In a philological situation the manifestation of anxiety is functional to trigger an enhanced and punctual stress-response to produce a quick adaptation to the unpredictable changes of the environment and it is usually defined as state anxiety. But in a significant proportion of situation, the mechanism starts to work independently from the correct evaluation of the situation, producing a status of continuous activation that hyper stimulate the stress response of the organism, causing the damages and alteration correlated with a long-term elicitation of this state. This is usually referred as trait anxiety, and its correlated with a

variety of symptoms bot in the psychological and psychiatric diagnosis (Marks and Lader 1973).

Identifying the maladaptive and self-sustaining pathways of the anxiety states and correlated disorders in one of the core conceptions and the principal theorical orientation of the treatments of such disease in the contemporary psychotherapeutic approaches and the behavioral cognitive therapy is one of the most recommended choices in the work with patients both in the public health system and in the private practice of the professionals involved in the management and the association with psychopharmacological molecules remains widely recognized as the most effective strategy in the treatment of this important and incapacitating branch of pathologies.

Neuropeptide Y

Neuropeptide Y (NPY) is an important polypeptide produced in the CNS which unrolls its purpose as an hormone-like signal in an abundance of brain districts. Its main functions revolve around the conservation of an homeostatic energetic balance, modulation of stress signaling via its action on the HPA axis, anxiolytic role and influence on learning and memory process (Eva et al., 2006).

Given its abundant presence in limbic neuro-system and in structures such as hippocampus and amygdala it has been hypothesized a role of this compound it the acquisition and consolidation of memory and behavioral abilities. Even given its strict functional association with the GABAergic system and its ability to down-regulate the neuronal activity in the brain, this mnemonic positive effect is evident and it marks an important difference with its homologous, unlike any other anxiolytic compound (Tasan et al., 2015).

The NPY presents a variety of different receptors, the majority present in the brain, even if some of them can also found in the digestive tract (Y4) or in the peripheric nervous system (Y1, Y2).

The most important and studied receptors at a central level are the Y1 and Y5, which are encoded by the same gene from a common promoter, suggesting their evolutionary proximity and common utility, and for the sake of this project the receptor-expression modified was the one related to the version Y1.

Y1 receptor is distributed in the prelimbic (PL) cortex, basolateral amygdala (BLA), Central amygdale (CeA), hippocampus (HC) bed nucleus of the stria terminalis (BNST)

and locus coeruleus (LC, arousal) (Tasan et al., 2015).

It has been demonstrated by (Tan, 2018) that in the NPY receptors the activation is coupled with G-protein coupled receptors (GPCR). Their activation inhibits adenylyl cyclase and cAMP/PKA stimulation of L-TYPE Ca^{2+} current, trough inhibitory subunit of G protein. In neurons, GPCR also inhibit N-type CA^{2+} current and neurotransmission. The G-protein complex can activate the inwardly rectifying potassium (GIRK) current. The activation of Y1 receptor can promote the release of Ca^{2+} from the sarcoplasmic reticulum via inositol triphosphate (IP3). Increase intracellular Ca^{2+} activates Ca^{2+}/calmodulin- dependent kinase (CaMK) and protein kinase C (PKC).

This particular receptor proved particularly interesting (Bertocchi et al, 2011), for its susceptibility to the maternal care effects on the limbic system programming. This opened some interesting perspectives on the research on the interactions and development of the circuitry of this brain area.

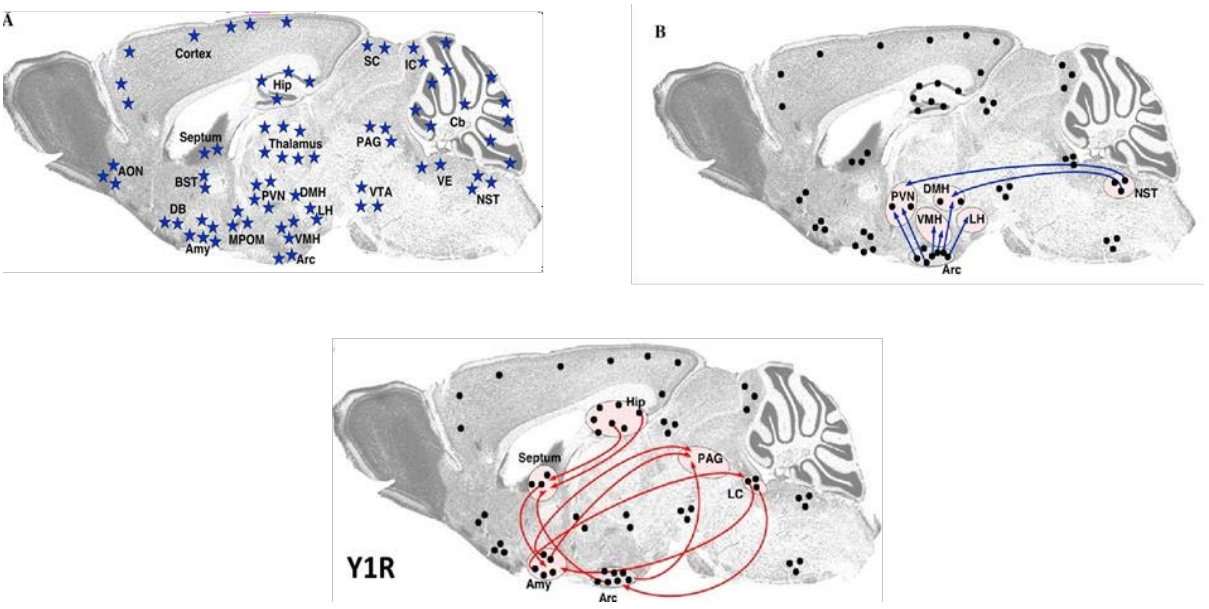


Fig. 1- Representation of NPY system and nucleai in mouse CNS (sagittal view). Adapted from Eva et al. 2006

Excitatory/inhibitory balance in the mPFC

The prefrontal cortex is a structure of the brain which is evolutionary fairly recent in the history of animal species. As such it is strongly interconnected with lower centers of the

nervous systems and mainly received pre-integrated stimuli from them. It does not only serve a purpose of integration, in fact it has been mainly identified as an executive center that as an important role in a number of complex cognitive abilities typical of mammals; its volume dramatically increases in primates and in man, where its full maturation requires long period of time after birth and as such it can be profoundly modified by experiences occurred during the childhood and puberty.

As regarding its executive functions, they are mainly pertaining the ability to select among the different pieces of information recollected from the environment and assigning them an importance which is in accordance with the current pursues and targets. This has to be a fluid and adaptable process, considering the fact that has to mediate to the priming effect of all the stimulation coming from the lower level, select among them the most coherent with the desired output and starting the effective action to reach it sustain the attentive function throughout the whole process.

In addition to the executive process the PFC registers experience, past goals and future planification and considers them as present and effective variable in its decisional process

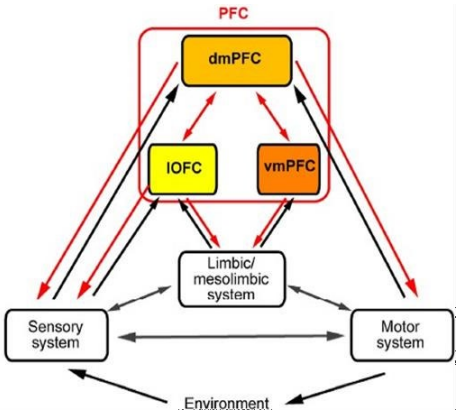


Fig. 2- Sub-connectivity patterns associated with executive functions in rodents. PFC limbic interplay and top-down control of sensory and motor system in rodents. The dorso-medial PFC, shows reciprocal connections to the sensory areas including visual, auditory, somatosensory cortex, and the association areas accessing to exogenous information while influencing motor control. (adapted and modified from Kamigaki., 2018)

The described process is maintained in a dynamic and continuous balance by a constant excitation and inhibition of simultaneous and complex interaction of different internal microcircuits that constantly communicates between them, receives impulses from

integration center of the brain such as hippocampus and amygdala (that basically provide emotional saliency and memory linkage to the past experiences) and also from other sensory-motor and proprioceptive nuclei, principally medial thalamus, ventral tegmental area and locus coeruleus (Ferguson and Gao, 2018).

As described by Griffen and Maffei., 2014 the excitatory and inhibitory balance is constantly maintained by changes in neurons excitability primarily exerted by parvalbumin positive neurons on the pyramidal cells of the deeper layer of mPFC, making more difficult for them to be activated and communicate with the effector centers such as the motor cortex.

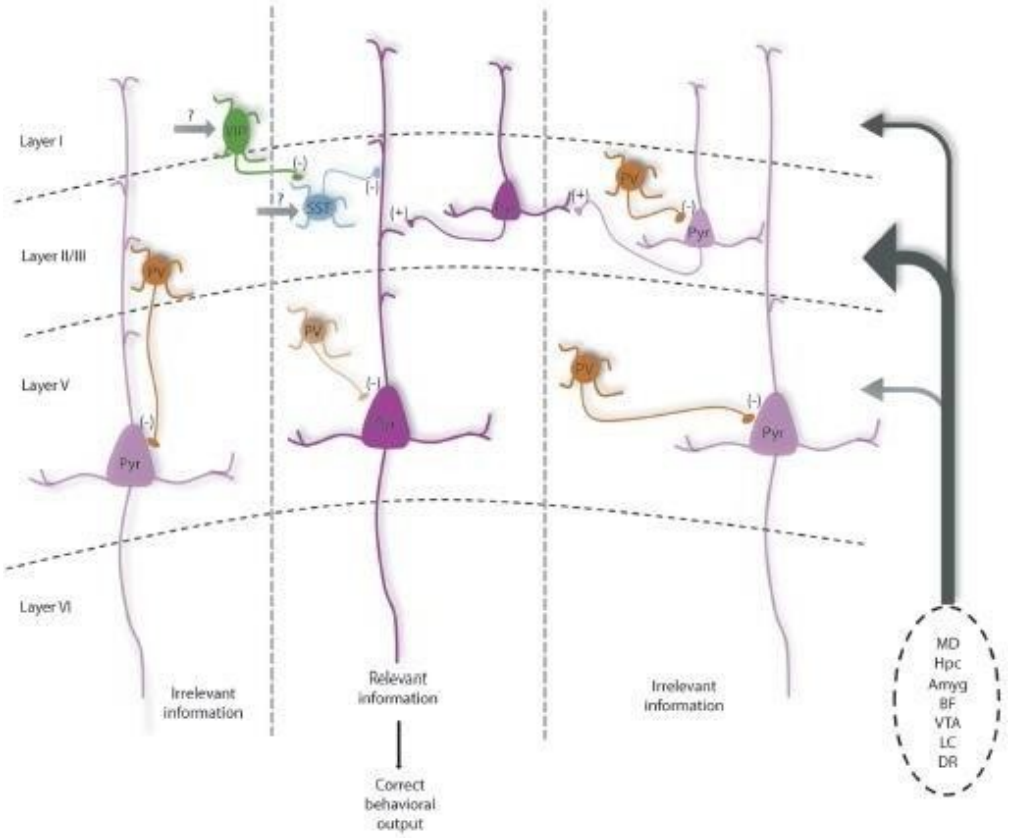


Fig. 3- Maintenance of excitation/ inhibition balance in rodents PFC. Subcortical structures provide the majority of excitatory input to PFC neuronal subtype to regulate their function, acting as a drive force in regulating inhibitory neurons and affecting therefore the E/I balance. Interneurons likely help in regulating spike timing, as well as oscillatory patterns of activity.
Darker and lighter shaded neurons represent high vs. low levels of activity, respectively. Parvalbumin cellular distribution appear both in layer II/III and in layer V. Peri somatic control of principal neuron firing by basket cells PV+ neurons is critical for information processing in cortical microcircuits (model adapted from Ferguson and Gao 2018).

This is an important example of the significant role played in the brain processes by the intracortical excitatory/inhibitory balance which is lately been studied as a mean of

explanation of some maturational aspect of the neurodevelopment. This process is quick and highly adaptable in its potential to become stronger or weaker following the pattern of activation of different neurocircuitry, and as such it can be profoundly altered in a number of different pathologies.

Aim of the study

Basing on the results from previous literature and the important effects that early life experience appears to exert attuning and shaping the areas of the brain that sustain emotional regulation and executive functions, in the present study it has been tried to highlight and look into the neurobiological processes that influence the plasticity and development of mPFC in the adult mice brain according to the external context it was exposed to during the first days of life.

For this investigation, the selected target as a plasticity index were the perineuronal nets (PNNs) and the analysis of their features and number. They have been then correlated with behavioral test performances and in vivo analysis and characterization of a validated animal model described in Bertocchi et al. (2011) as constitutionally subject to anxiety-like behavior both in the case of conditional deletion of Y1 receptor. The same phenotype could be obtained in control animals by exposition to suboptimal maternal cares during their first days of life. The same model was recently used in a submitted work by Mele et al. (2018) in which the PNNs presence in the hippocampus was significantly altered by the genotyping and the early life environment in association with a mild spatial learning impairment.

Considering these starting points, we believed possible a similar effect in mPFC and in specific in Pre-Limbic cortex, given the important connections it has with other limbic centers such as amygdala and hippocampus and the effector action it exerts on motor cortex. This makes it one of the cores of limbic circuitry and an important actor of the behavioral effects of anxiety.

Being the PNNs a structural support and functional support to neurons (Carulli et al. 2010) another hypothesis on their role in shaping plasticity was linked to their ability to modify and modulate the balance between excitatory and inhibitory circuitry and for this reason they have been analyzed in their co-expression with some sub-population of parvalbumin positive neurons (which marks inhibitory interneurons) to test any macroscopic alteration in their presence or in their tendency to be coated by PNNs.

Finally, we experimented a functional confirmation of the role of PNNs by the rescue of the altered phenotype. This was obtained by digestion of nets via surgical injection of chondroitinase and subsequent testing of the treated animal to observe any amelioration in their behavioral vulnerability.

Materials and Methods

The current study was conducted accordingly with the directive 86/EEC 24th November 1986 of the European community council and approved by the Ethical committee for animal research of the University of Turin and Italian Ministry of Health (license no. 139/2014). The number of experimental animals was minimized at its most, and the mice have been cages in group of 2 to 6 in dedicated cages (measures 40x20x20) and hosted in rooms with controlled temperature (22 ± 1 °C) and humidity (50 ± 10%). A regular dark/light cycle was established, with 12 hours of light starting from 8 am and 12 hours of darkness starting from 8 pm. Ad libitum access to standard diet food and beverage was provided in the cages. All the following described behavioral test were performed on p75 to p95 male mice, both conditional knockout and control littermates ($Npy1r^{rfb}$and $Npy1r^{2lox}$, n=24). $Npy1r^{rfb}$ and $Npy1r^{2lox}$ mice (background strain: C57BL/6J). The animal model was generated following the raising scheme described in Bertocchi et al. 2011. $Npy1r^{2lox}/Tg^{\alpha CamKII\text{-}tTA/LC1}$ mice (named herein $Npy1r^{rfb}$) were generated using doxycycline (Tet-Off) dependent control of the Cre-LoxP system (Fig. 4). This conditional knockout allows for the deletion of the Y1 receptor only in neurons expressing the subunit alpha of the calcium calmodulin kinase-type II promoter (αCamKII) in the adult limbic system.

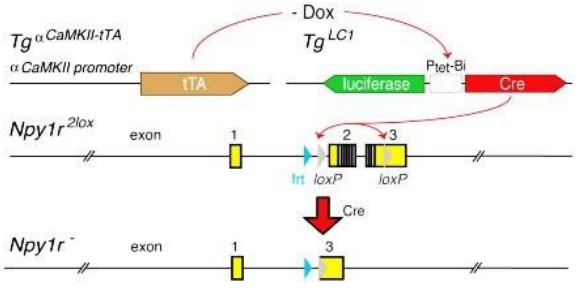


Fig. 4- Schematic representation of transgenic activity in principal neurons of the forebrain in Npy^{rfb} mice. Diagram showing the interaction of the different genetic components: The αCamKII promoter drive the Dox-sensitive transactivator (tTA), which activates transcription of the transgene TgLC1, thereby inducing Cre expression in excitatory neurons of the forebrain. The Cre recombinase interacts with *loxP* sites in the gene-targeted $Npy1r^{2lox}$mice and removes the Npy1r coding regions (adapted from Bertocchi et al., 2011)

1. Maternal Behavior observation.

From P0 to P14 both controls and knockout animals were adopted by naïve foster mothers of two different strains: FVB or C57BL/J6 (Fig.). During the first 7 days of their life, the maternal behavior of pups and dams was observed in their home cage (FVB/J dams n=7 or C57BL/J6 n=5). Since the mouse is an animal which is principally active during the night-time the observation was placed in 2 hours at the end of the dark circadian cycle, using a red light to minimize the disturbance for the animals (lamp power=25W). Every foster mother was observed every for minutes during the 2 hours of the protocol, providing a total of 30 observations. The observations were recorded and classified by the experimenter using a chart (Fig. 5) similar to the one described in Palanza et al. 2002.

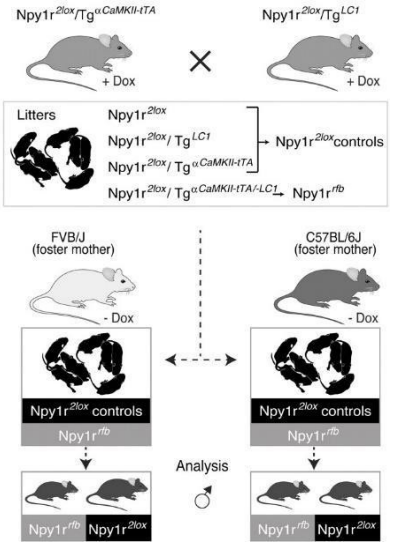


Fig. 5- Breeding protocol and cross-fostering of mutant litters. 4 groups of experimental mice were obtained: $Npy1r^{rfb}$ mutants and $Npy1r2^{2lox}$ control littermates adopted by 2 strains of foster mothers (FVB: High caring and C57: low caring). The comparative analysis of these 4 groups was the substrate of the aim to uncover the links between NPY receptor, early life events and neurodevelopmental aspects of limbic system (Bertocchi et al., 2011).

	Dark															Dark														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	TOT
IN NEST																															
NURSING																															
Arched Bck nursing																															
Forced Nursing																															
LICK PUP																															
NEST BUILD																															
EAT/DRINK																															
SELF-GROOM																															
RESTING																															
ACTIVE																															
																														30	

Fig. 6- Maternal behaviour observation chart. arched back nursing: the female was nursing pups with her body arched over them. In nest: the female was anywhere inside the nest, regardless of the behavior being

exhibited at the moment of observation. Nursing: the female was allowing the pups to suckle; this category did not necessarily imply that the whole litter was nursing. Licking pups: the female was licking or grooming her pups. Nest building: the female was engaged in some aspect of nest building, while she was either inside or outside the nest itself. Eating/drinking: the female was nibbling at a food pellet or drinking from the water bottle. Grooming: The female was grooming her own body. Active: the female was moving in the cage. Resting: the female was lying motionless outside the nest, not involved in any other form of behavior and with no pup attached to her nipples. Out of nest: the female was outside the nest.

2. Behavioral test.

The tested animals were allocated 30 minutes before the starting of the session 30 minutes before the starting of the session in a dedicated testing room, feebly illuminated and completely soundproofed. Every session was set from 9 am to 12 am (except burrowing test). Between trials each instrument was cleaned with 3% ethanol and rinsed with distilled water to eliminate odors and olfactive cues. The order in which the tests are presented reflects the order in which they have been performed and has its reason in the increasing difficulty and stress provoked by them to the animals.

2.1. Burrowing test.

The day before the start of the testing session (P90) mice were isolated into opaque plastic cages, with ad libitum food and water provided (Deacon 2006). The test started at 4.30 pm and consists in placing inside the home cage of the animal a plastic tube (L: 200 mm, Ø:68mm) filled with food pellets (200g). After 2 hours the remaining content of the tube was weighted and then the instrument was placed again in the cage minimizing the interruption and the disturbance for the animal. The tube was then left overnight and the pellets weighted again in the morning to assess the quantity of material burrowed in the two different time windows.

2.2. Open field (OF).

The Open field test is useful to assess locomotor activity and anxious related behaviour. The arena (50x50 cm) is placed under a camera for video recording and further analysis. The test lasted 10 minutes and the total distance and time spent either in the peripheral and central (25x25cm) area were calculated.

2.3. Elevated Plus Maze (EPM).

The EPM instrument is built in a shape of a plus-cross with equal length arms, each 32,5 cm long suspended 60 cm above the floor level. The intersection presents a small 5x5 cm platform. Above the structure a video camera for recording and further analysis Two opposite arms are surrounded by 15 cm high Plexiglas wall (closed arms). The other two are instead open (open arms). At the beginning of the trial the animal was placed in the central platform facing the entrance of an open arm, and then allowed to freely roam the maze for 5 minutes. Time, space and number of entrances in each area were recorded automatically (as for the OF) using the video tracking software Ethovision XT12 licensed by Noldus Information Technology.

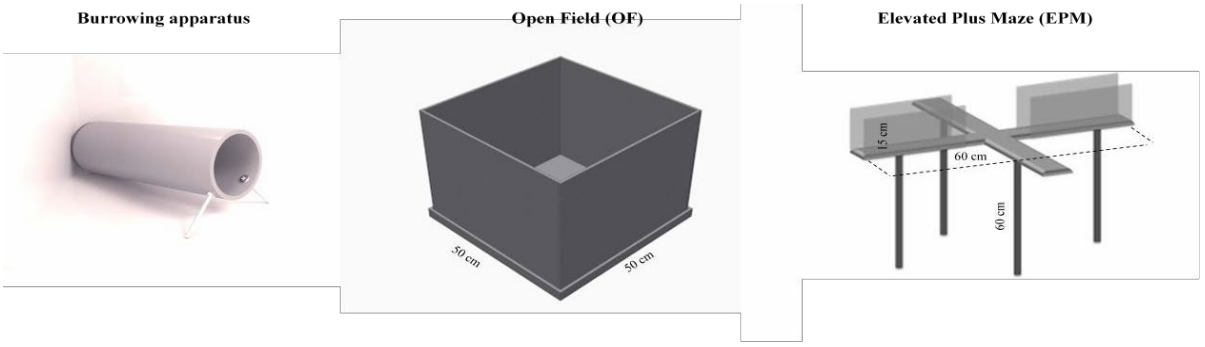


Fig. 7– Apparatus used for behavioral screening. From left to right burrowing tube, Open Field (OF) and Elevated Plus Maze (EPM) lateral view. The batteries of behavioral analysis were performed consecutive.

2.4. Puzzle box Test.

This test, initially described by Ben Abdallah et al. 2011, is designed to observe the problem-solving ability of mice. The animals are required to integrate spatial navigation and planning in a task that requires learning and memory. The test is run in 4 days, each consisting in a 3-trial session in a close succession (every trial lasts 180s, with an equal inter-trial time) save for the last day which only consist in a single recall trial. The arena (40x30 cm) in which the test takes places consists in an illuminated, white covered zone separated from a dark one (defined goal zone). Mice underwent ten trials (T1 to T10), being challenged with increasingly difficult ways to pass from the white to the goal area:

- Day 1 (T1-3): on T1 the passage was unlocked, the barrier presents an open door (task 1). On T2 and T3 the barrier had no doorway and mice entered the goal area via a small tunnel (task 2).
- Day 2 (T4-6): on T4 task 2 is repeated. On T5 and T6 the underpass was filled with sawdust and mice had to dig their way through (task 3).

- Day 3 (T7-9): on T7 task 3 is repeated. On T8 and T9, mice were presented with a puzzle plug, with the underpass obstructed by a cardboard plug that mice had to pull with teeth and paws to enter the goal zone (task 4).
- Day 4 (T10): on T10 task 4 is repeated.

This sequence allowed the evaluation of problem-solving abilities (first presentation of a new task, T5 and T8), and working memory for species-specific or instrumental responses (repetition of the

newly introduced task, T3, T6, and T9). The first repetition of the following day instead gives a measure of long-term memory. When the animal completes the task it was allowed to spend 30s in the dark area as a reward. The same happened for the mice that failed to identify and use the passage; after 180s they were guided by the experimenter in the goal compartment.

Score given according to the latency to enter into the goal box:

From 0 to 29	**0.5**
30 - 59	**1**
60 - 89	**1.5**
90 -119	**2**
120 -149	**2.5**
150 -179	**3**
180	**3.5**
180	**4**

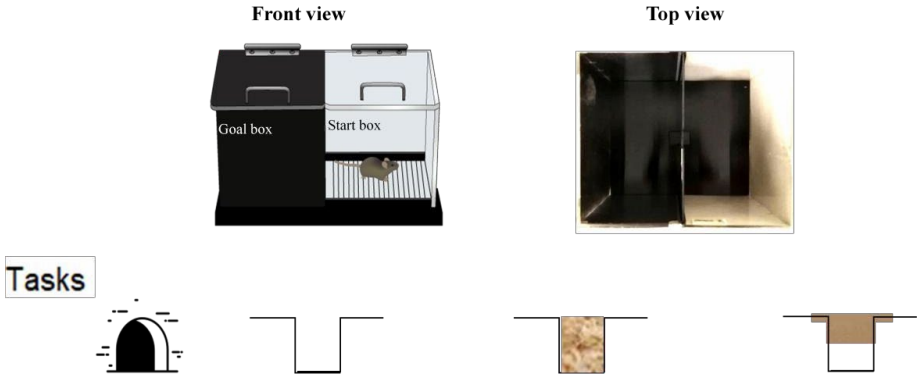


Fig. 8- Puzzle box score, instrument and task per trials. (Up) Frontal and top view of the goal (dark) and start (light) box. Graphical view of the door, tunnel, sawdust and plug tasks.

3. Ex vivo analysis. sacrifice and treatment of the brain tissues. Animals were trans-cardiac perfused with a 4% solution of paraformaldehyde in phosphate buffer saline (PBS), the brain taken, cryo-conserved and frozen at -80°C. Then with a Leica Microsystem cryostat, 25□m coronal brain sections were cut and the series corresponding to the medial prefrontal cortex were collected

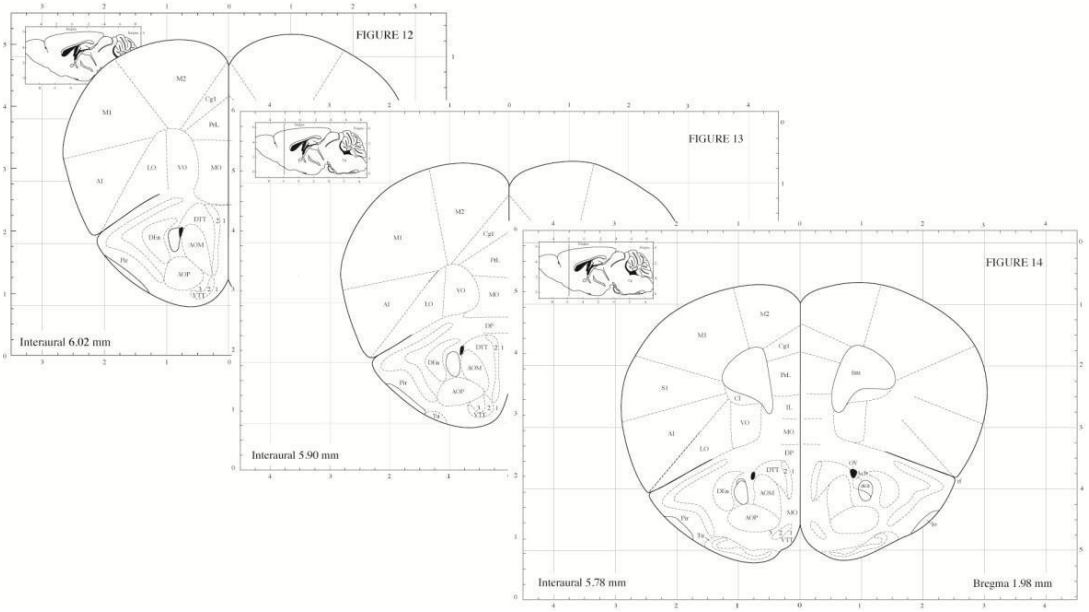


Fig. 9- Schematic of a central fields. Coronal and a sagittal section of the same field. Rostro-caudal collection. *Image were designed consulting the Mouse Brain Atlas.*

3. Immunofluorescence staining.

Free-floating sections from the cryostat were washed in 0.1M PBS and incubated with a blocking solution (10% normal goat serum in 0.1M PBS in 0.2% Triton X-100) at room temperature for 1h. Perineuronal nets (PNNs) were detected by mean of biotinylated Wisteria floribunda agglutinin (WFA, Sigma -Aldrich, Milano, Italy) in a proportion of 1:200 in 0.1 M PBS for 2 h at room temperature. After washing in PBS 0,2% Triton X-100, rabbit α-Parvalbumin (PV) (Swant 340 Bellinzona, Switzerland) and mouse α-NeuN (Merck Millipore, Milano, Italy) primary antibodies were diluted on a proportion of 1:5000 and 1:1000 in 0,1 M PBS with 0,2% Triton X-100 that was applied to the sections overnight at 4 °C. The following day the slices were washed with PBS then to bind biotinylated WFA and primary antibodies they were incubated 1h at room temperature with Texas Red streptavidin (1:500, Jackson Immuno Research, West Grove, USA), anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 647 secondary antibodies (1:800 and 1:400, Life Technologies, Milano, Italy). After two final washings in 0,1 M PBS and one in 20% Tris HCl, slices were mounted on glass slides and fixed with cover slips with 70 ul of Mowiol (SigmaAldrich).

4. Immunohistochemistry.

Immunostaining was carried out on free floating coronal brain sections (25 um thick). Day I. After endogenous peroxidase blocking by 0.5% H2O2 in 0.1 M PBS (PT buffer) for 15 min, sections were blocked with 10% normal goat serum (NGS) in PBS containing 0.02% Triton X-100 and 1% bovine serum albumin for 1 h and incubated over- night at 4 C with biotinylated Wisteria floribunda agglutinin (WFA, Sigma-Aldrich, Milano, Italy) at 1:1000 dilution with 1% NGS in PT buffer to detect PNNs. Day II. After three washing with PBS 0.1 M, sections were incubated with the ABC Vectastain kit (Vector Laboratories). Peroxidase was reacted with 0.04% DAB and 0.003% hydrogen peroxide, mounted on slides and air-dried overnight. The slides with DAB-developed sections were then cover slipped with DPX mounting medium and analyzed with a Leica microscope**.**

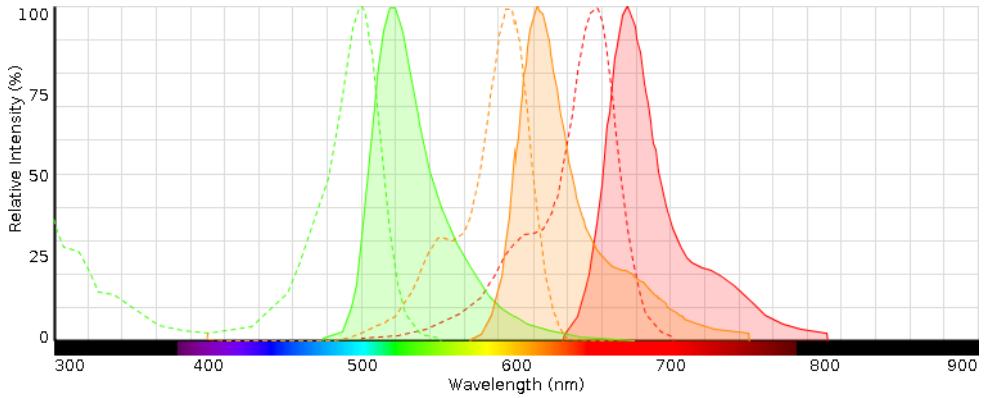


Fig. 10 - Merged excitation/emission spectra of Alexa Fluor® 488-/ 647-/ Texas red 543: Excitation (dotted line)/emission (continuous line) spectra of Alexa Fluor® 488- conjugated antibodies (green), Texas red Streptavidin (orange) and Alexa Fluor® 647- conjugated antibodies (red). Imaged edited with Fluorescence SpectraViewer, available online at *https*://w*ww*.thermofisher.com

5. Image acquisition and analysis.

Confocal Images were acquired with a TCS SP5 confocal microscope (Leica) at a resolution of 1024x1024 dpi at 100 Hz and each plan was 1 um thick. 14 to 18 microns stacks were collected with a 40x objective. Four images per animal were collected. Two fields for each PL region were acquired to cover both superficial and deep layers. Laser power, gain and offset were maintained constant during the acquisition of each experiment. For the analysis of WFA intensity acquired confocal images were loaded into ImageJ software for both qualitative and quantitative analysis.

6. Quantification of labelled neurons.

To measure PNNs intensity, the slice of the stack in which the PNN signal reached the maximum brightest was chosen. The area was selected by hand-drawing with ImageJ a ring along the borders of the WFA-positive signal around the neuronal soma, and the mean brightness intensity (range 0–255) of about 3–12 PNNs/animal was measured. Intensity measurements taken from four unstained circles fixed in size in the surrounding background area were subtracted from the PNN intensity. Each net was then assigned to one of four categories of staining intensity, ranging from the lowest to the highest detected value of WFA: weak = 1,54–26,16%, intermediate-weak = 26,17-50,77%, intermediate-strong = 50,78–75,39% , strong = 75,40-100% of maximum staining intensity.

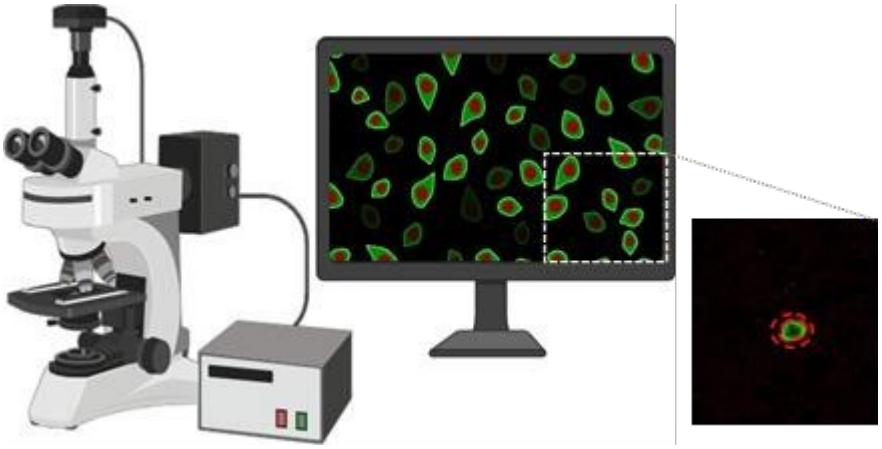


Fig. 11- Schematic drawing of confocal images collection and images analysis workflow. Confocal Images were acquired with TCS SP5 confocal microscope (Leica) and analysed with ImageJ software free available.

7. PNN colocalization.

To measure the percentage of colocalization of WFA with PV and NeuN, sections were analyzed throughout the stack by calculating the percentage of co-labeling markers over the total WFA-positive and PV-positive neurons and the total number of NeuN-positive mature neurons, counted with ImageJ Cell Counter Plugin in both superficial and deep layers of the mPFC prelimbic area (PL). The density of PV^+ neurons were calculated over NeuN-positive mature neurons.

Perineuronal nets positive for Parvalbumin marker (PV)

(WFA PV+/WFA_{tot}) x 100

Parvalbumin neurons positive for Perineuronal Nets (WFA)

(WFA PV+/PV_{tot}) x 100

Perineuronal Nets density

(WFA/NeuN) x 100

Parvalbumin density

(PV/NeuN) x 100

9. Stereotaxic chondroitinase ABC (ChABC) injection into the mPFC.

Gas anesthesia was induced in a chamber in which 3% isoflurane was dissolved in a mixture of N_2O / O_2 (30:70). The animals were then fixed on a stereotaxic apparatus and

the anesthesia maintained (1.5% isoflurane in the same mixture) through a nose mask. Chondroitinase ABC (ChABC, 25 U/ml) (AMSBIO, Abingdon, UK) or vehicle (saline, 0.9% NaCl) was pressure-injected bilaterally (0.2µl/side) in the mPFC (from Bregma: AP+1.8 mm; ML ± 0.3 mm; DV–1.5 mm) by a glass capillary. Each injection was delivered in about 5 min, afterwards the capillary was left for two minutes *in situ* to allow solution diffusion. Finally, the mice were sutured and received common post-operative care and then allowed to recover and rest for 3 days. All the procedures were done in a Biosafety Cabinet (BSC) Class II room for manipulating viruses. On the fourth day they were subjected to the Puzzle Box test

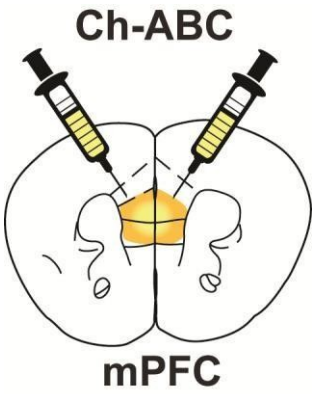


Fig. 12– Graphical representation of Ch-ABC bilateral injection. Coronal section of mPFC mouse brain. Yellow area indicates the putative target area of injection into the mPFC.

8. Statistical analysis.

Results are presented as mean± standard error of the mean. Two tailed Student t test was used for comparison between two independent groups. For comparison of more than two groups one-way or two-way analysis of variance (ANOVA) was performed, followed by *Bonferroni* multiple comparison post-test. Pearson's correlation analysis between behavioral measures and cellular and molecular outcomes were also conducted. When significant Pearson's correlations were found, follow-up correlation analyses were conducted on each group separately to identify the source of the relationship. Given that the sample size of molecular data was of 5-7 subjects, the non-parametric Spearman's

coefficient (q) was used. All the statistical analyses were conducted using GraphPad Prism 5.01 (GraphPad Software Inc., La Jolla, California).

Results

1. Behavior

1.1 Maternal behavior and body weight.

The protocol for maternal behavior observation revealed and confirmed the previous Bertocchi et al. results between FVB/J and C57BL/J6 foster mothers. In the overall view, the difference in the total amount of time spent in the nest with the pups was statistically significant between the two groups as emerged from the one-way ANOVA analysis (P=0.0606). As regarding the single types of behavior instead, the arched-back nursing and the licking (which are commonly considered to be the most informative signals of a good dam/pup bonding), again the FVB/J dams showed a significantly higher presence of it if confronted with the C57BL/J6 (P>0.01 and P=0.0167, Fig.).

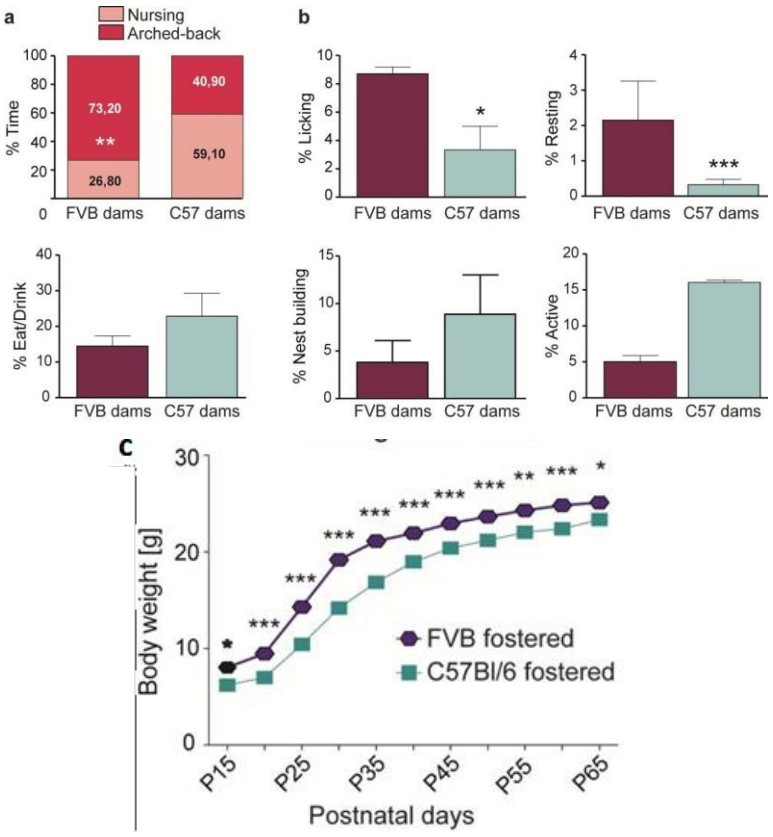


Fig. 13- Maternal scoring and weight gaining curve. Maternal behavior of FVB/J and C57BL/6J foster mothers. Average percent time spent by FVB/J and C57/6J dams on total nursing (A) and on maternal behavior variables (B) during postnatal P1-P8. Data are expressed as mean ± SEM. n = 4-3 *P< 0,05; **P < 0,01; ***P < 0,001 by unpaired t test for independent samples. **c)** Average body weight from P15 to P65 of pups (6-8 litters) fostered to either C57BL/6J or FVB/J mothers. Subjects N=41. Two-way ANOVA revealed a significant effect of foster mother strain: [$F_{(1,410)}$=37,84; P <0,0001] and foster mother strain-days interaction: [$F_{(10,410)}$=9,023; P< 0,0001]. * P < 0,05; ** P <0,01 and***P<0,001 by Bonferroni post test (adapted from a master thesis work by Canicatti 2019)

Moreover, the total time of activity of the C57BL/J6 mothers was higher (P=0.0002). Furthermore, considering the weight of the pups during time as an health index and performing a two way ANOVA for repeated measures, the animals exposed to high quality cares from the dams showed a significantly higher one (P<0.001), showing an influence of maternal cares in the growth curve of the litters.

1.2 Burrowing

The burrowing test has the aim to assess the general well-being of the animals and to highlight possible abnormalities in their behavior specifically associated with the activity of their medial prefrontal cortex (mPFC). It is a relatively simple and effective method to do so and it also allows to observe the mice in its innate behaviors. As previously described the test was performed to be as ecological and effortless as possible for the animals, giving the following behavioral paradigms. A two-way ANOVA was performed on the result, highlighting no significant differences between the groups (N=12, for each group P=0,0682). For each group the burrowing activity grew normally with the two measures, even if slightly less evident for the animals exposed to low quality cares during their first day of life.

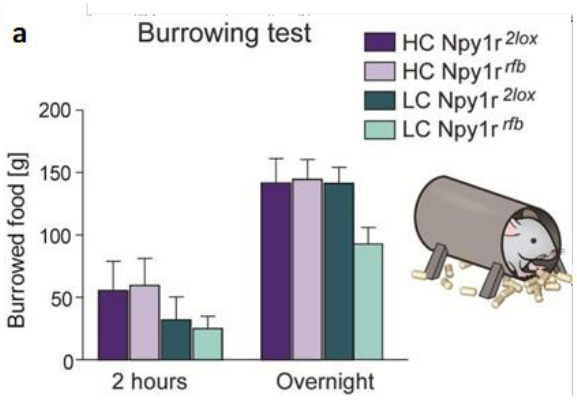


Fig. 14– Burrowing activity graph.. *a)* Burrowing behavior. Two way ANOVA revealed not significantly difference between groups in burrowing behavior after 2h and overnight [$F_{(3,88)}$= 2,457; *P* = 0,0682], and

significant effect of the time in the burrowing behavior (P < 0,001). Data are expressed as the mean ± SEM (adapted from a master thesis work by Canicatti 2019)

1.3 Evaluation of anxious phenotype

To confirm the phenotype of the conditional KO model and to link it with the different type of early life cares received by the pups we used as described the Open field and the Elevated Plus Maze test. On the data obtained by these tests we performed a two-way ANOVA which revealed a statistically significant difference linked to the strain of the foster mother on the time and space spent in the center of the OF arena (Fig.). In detail, high care ko mice and both low care controls and ko showed higher levels of anxiety (as interpreted by the avoidance of the center part of the arena) if confronted with the control high cared littermates. This difference doesn't regard the latency of the first entrance in the center of the box.

Likewise, in the EPM, where the two-way ANOVA showed a difference for both genotype and strain of foster mother in the percentage of the entries on the open arm on the total of entries. Here the HC ko tend to stay less in the open arms if confronted with their control littermates. Moreover, their number of entries is similar to both the control and ko raised with a low level of cares (Fig.)

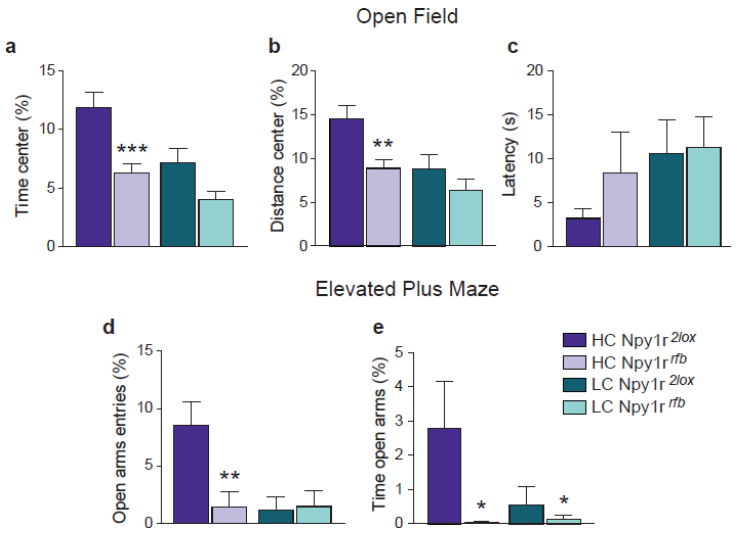


Fig. 15. – Analysis of anxiety-like behavior in the OF and EPM paradigms. a) Percentage of time spent in the center of the OF. Two-way ANOVA revealed a significant effect of genotype and foster mother strain [$F_{(1,38)}$ = 17.31, P = 0,0002; $F_{(1,38)}$ = 10,90, P = 0,0021, respectively]. $^{***}P < 0,001$ versus HC Npy1r^{2lox} mice. N=7-10. **b)** Percentage of distance travelled in the center of the OF. Two-way ANOVA revealed a significant effect of genotype and foster mother strain [$F_{(1,38)}$ = 8.530, P = 0,0058; $F_{(1,38)}$ = 8.940, P = 0,0049 respectively]. $^{**}P< 0,01$ versus HC Npy1r^{2lox} mice. **c)**Latency to enter in the center of arena. Two way-ANOVA revealed no significant differences of genotype and foster mother strain [$F_{(1,38)}$ = 0,6726; $F_{(1,38)}$ = 2,046, respectively,] **d)** Percentage of entries into the open arms of the EPM. Two-way ANOVA revealed a

significant effect of genotype, foster mother strain and foster mother strain interaction [$F_{(1,30)}$ = 5.099, P = 0,0314; $F_{(1,30)}$ = 5.881, P = 0.0215; $F_{(1,30)}$ = 6.011, P = 0.0203, respectively].P < 0,01 versus HC Npy1r^{2lox} mice revealed by *Bonferroni posttest*. *n=7-11*. **e)** Percentage of time spent in the open arms. Two-way ANOVA revealed a significant effect only for genotype [$F_{(1,30)}$ = 6.721, P = 0.0144]$^{**}P$ < 0,01 versus HC Npy1r^{2lox} mice. Data are expressed as the mean ± SEM. On the x axis is indicated the strain of the foster mother (adapted from a master thesis work by Canicatti 2019).

1.4 Puzzle box test

The puzzle box paradigm was useful to evaluate possible differences in the executive abilities of the groups of animals. A two-way ANOVA analysis was performed on the results of this test to cross-evaluate effects of both the genotype and the maternal care style and it did emerged a significant effect for genotype and strain in the overall score, highlighting the significantly better performance of HC controls if confronted with the other groups [$F_{(1,32)}$=10,19, P=0,0032*;* $F_{(1,32)}$=4,424,P=0,0434*,* respectively].

Furthermore, a two-way ANOVA for repeated measure was applied to the single trials of the test to better evaluate and explain the executive and memory task of the paradigm and their differences among the groups. A significant effect of groups (P < 0.001), trials (P < 0.001) and group-trials interaction (P = 0.0190) was found. In detail, the task from habituation (T1 and T2) to the executive (T8, T9) proved to be achievable for the HC controls within the time window (as described in Ben Abdallah, 2011). The other three groups showed a similarly good performance till T6 when the difficulty starts to become significant. In these groups at this point the score starts to diverge from the HC cared control mice, particularly reflected in an augmented latency of the time required to complete the task. In particular, *Bonferroni post tests* showed a significant difference between HC controls and ko littermates in the latencies to escape from the start box at trials 6 and 9, when animals have to repeat the executive tasks on the same day. In a similar fashion, an impairment emerged in LC ko suggesting a possible impairment in memory and executive functions due to the conditional inactivation of the NPY1r, an effect independent from the maternal care provided. The effect of impairment was also present in the LC controls, which is probably due to the ability of the maternal style to make evident the phenotypical differences produced by the inactivation of the Y1 receptor. The evolution in the performance of the different group appeared to be fairly peculiar according to the different style of maternal care to which the mice were exposed. For example, the controls adopted by C57BL/J6 dams showed a longer latency in finding the right solution to the task when it becomes more difficult (T8, T9) whilst the recall of the same task appears quite not impaired, suggesting an executive deficit in the problem-solving strategy.

Another interesting result is the significant improvement of HC ko mice at the T10 task, which requires long term memory abilities, suggesting an intact mnemonic ability which is not present for the LC animals.

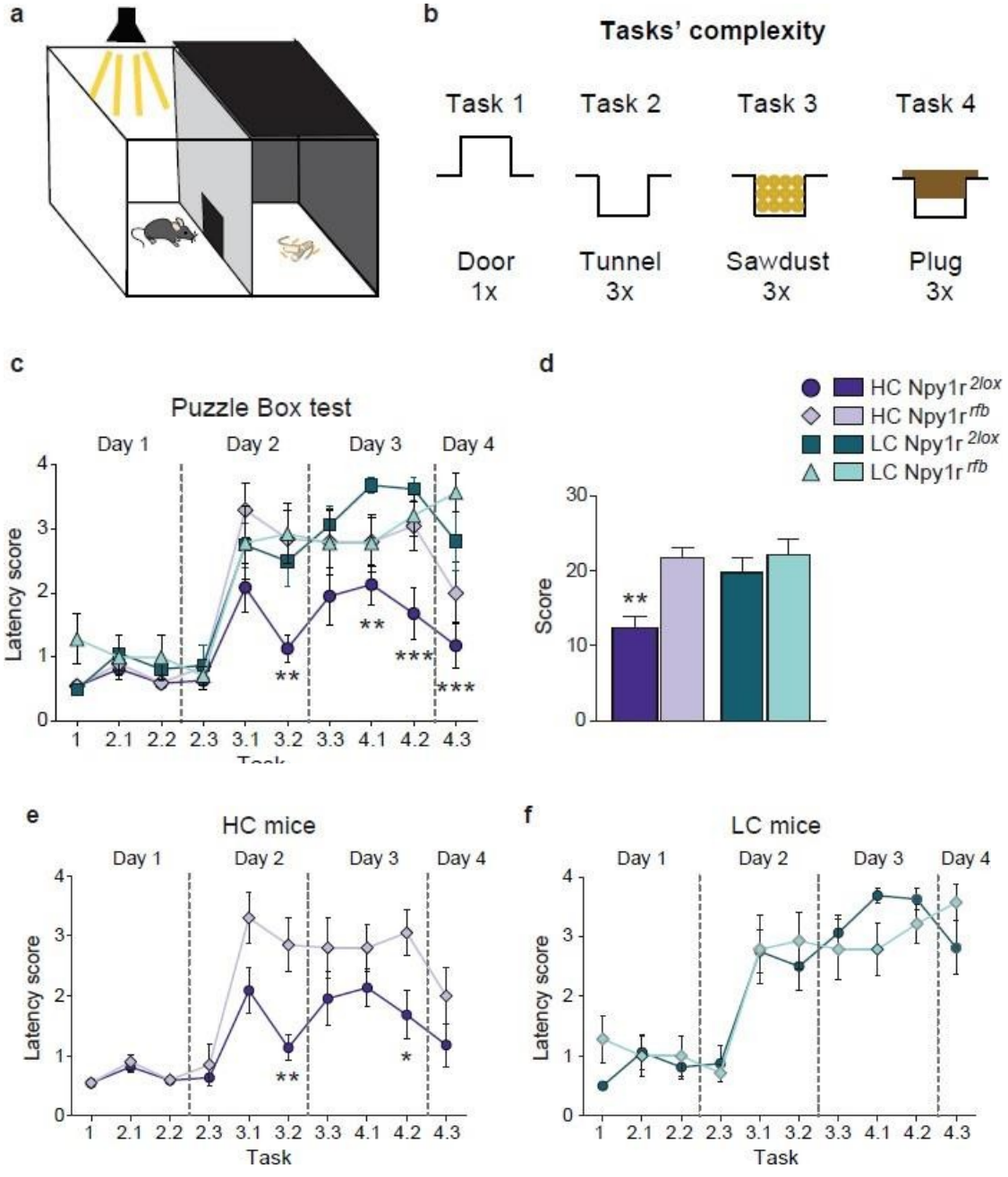


Fig. 16 - Evaluation of executive functions in the puzzle box test. a, b) Experimental setup of the Puzzle Box test. **c)** Performances of HC and LC reared control and mutants' mice along trials. Two-way ANOVA revealed a significant effect of groups, trials and their interaction [$F_{(27,288)}$=2.209, P = 0,0007]. Particularly, mutant mice showed impairments in trials 6, 9, 10, whereas LC Npy1r^{2lox}controls showed impairments in trials 6,8,9,10 compared to HC control mice. **d)** General score of performance in the puzzle box test. Two - way ANOVA revealed significant effect of genotype and strain [$F_{(1,32)}$=10,19, P=0,0032; $F_{(1,32)}$= 4,424,P=0,0434,respectively]. **e)** Puzzle box performance comparison between controls and mutant mice adopted by FVB dams. Two way-ANOVA analysis showed a significant effect of genotype, trials and their interaction between HC Npy1r^{2lox} and Npy1r^{rfb} mice /$F_{(1,171)}$= 6,919, P= 0,0165; F (9,171)=19,18, P< 0,01,

$F_{(9,171)}$=2,400, P=0,138 respectively. **f)** Puzzle box performance of LG controls and mutant mice was not significantly influenced by the genotype, as emerged by two- way ANOVA analysis (P = 0,8815). $^{***}P$ < 0,001, $^{**}P$ < 0,01, $^{*}P$ < 0,05. Data are expressed as the mean ± SEM and the score is expressed as the function of latency to enter into the goal compartment (adapted from a master thesis work by Canicatti 2019)**.**

2 Molecular analysis

2.1 Analysis of Perineuronal nets expression in the superficial and deep layers of the Pre-Limbic cortex

To provide a possible neurobiological link to the early maternal cares and the behavioral results on the anxiety levels and the impairment of executive functions that we observed in our animals we decided to evaluate the presence and the intensity of the perineuronal in an area of the limbic brain which in strongly associated with the expression and regulation of this behaviors; the Prelimbic area of the medial prefrontal cortex. We performed here an immunofluorescence staining and confocal imaging analysis as described above to identify the structural compounds that constitute this structure of the brain matrix.

The variables analyzed are the density of PNN and their intensity. The first one is expressed as the percentage of cells positive for the WFA (a lectin that binds the N- acetyl galactosamine of chondroitin sulfate chains) on the total number of mature neurons marked with NeuN. This analysis was clustered in two different areas of PrL cortex, since it is possible to identify two different neuronal layers in its anatomy (Saffari et al., 2016; Shrestha et al., 2015; Santana et al., 2017). The superficial layer (II/III) and the deep layers (V/VI) have different patterns of connectivity and different neuronal populations and are quite distinguishable during the confocal selection of the field acquired. The aspect of PNNs greatly differs in the two different layers, as supported by previous literature (Ueno et al., 2017). In the superficial layers they tend to appear more protruded and extended, while in the deep layers they tend to be rounder and less extended to the connection of the neurons. The counts were obtained as previously described and analyzed with a two-way ANOVA and confirmed a significant difference in the distribution both between the different groups of animals and the layers within they are analyzed. Specifically, if confronted with the LC mice, the HC control animals have significantly less PNNs in their superficial layers (P=0.0113), and this is even more evident for the HC ko animals (P=0.0008). As regard the deep layers of the PrL cortex, this effect doesn't emerge and they have not been furthermore analyzed in the following investigations.

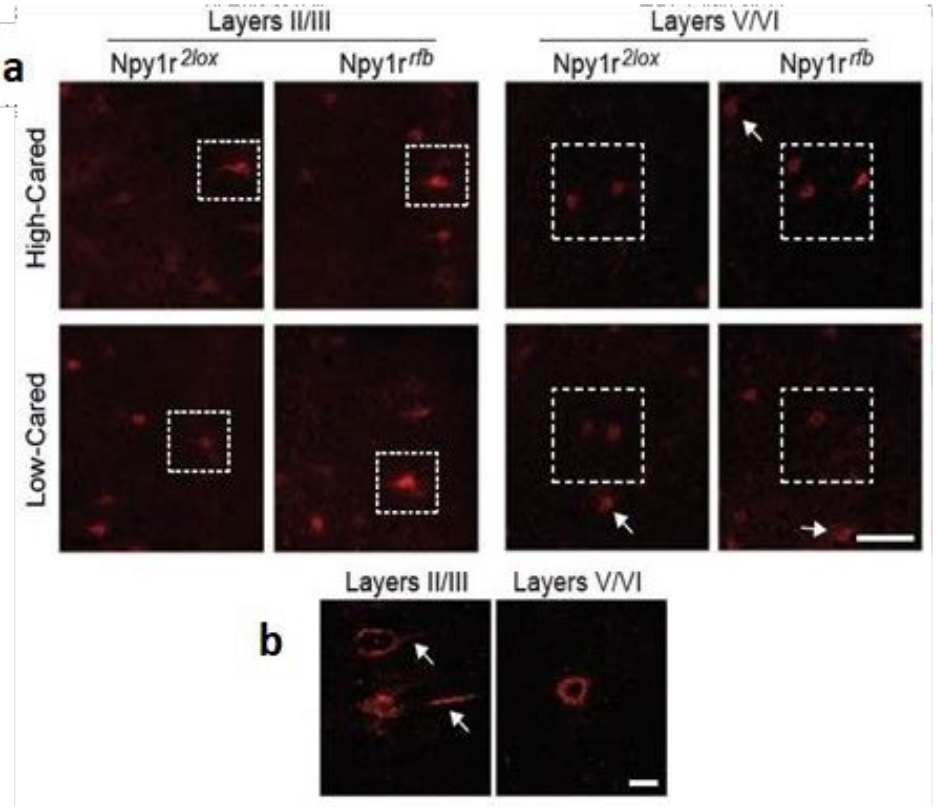
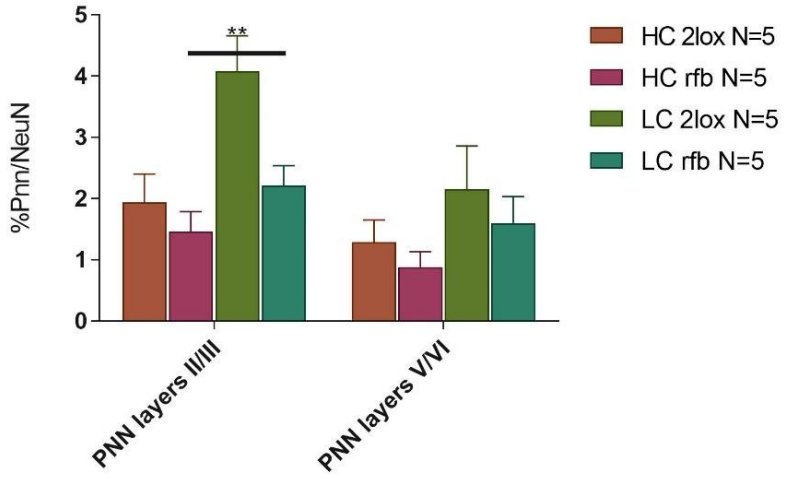


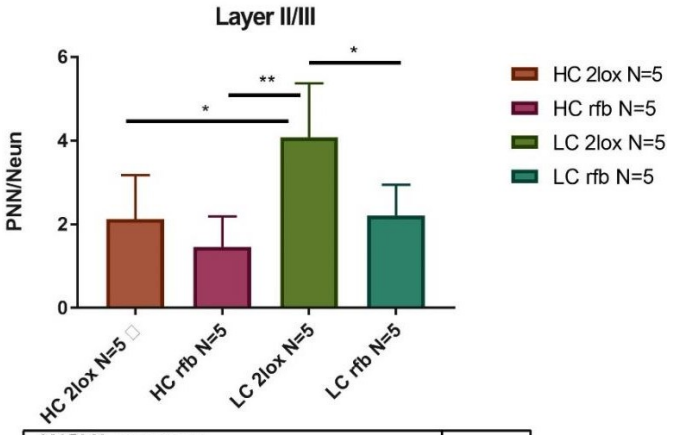
Fig. 17– Analysis of PNNs in the PL cortex. a) Representative immunofluorescence stack image of WFA-positive PNNs in superficial (left) and deep (right) PL layers of Npy1r^{2lox} and Npy1r^{rfb} mice reared by FVB/J and C57BL/6J foster mother**. b)** Morphological features of WFA- positive PNNs around neurons in the different mPFC layers. Confocal images of WFA-positive PNNs in individual layers of the PL Scale bar=20 um



Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	4.937	0.3807	ns	No
Row Factor	13.55	0.0059	**	Yes
Column Factor	31.73	0.0011	**	Yes

Fig. Percentage of WFA-positive neurons for all mouse groups in both superficial and deep PrL cortex layers. The distribution of PNNs in the PL cortex is significantly different between layers. Scale bar=100 um

To further analyze the distribution pattern of the PNNs between groups, a one-way ANOVA was performed only on the data coming from superficial layers. This allowed to highlight a peculiar situation in which the HC ko and control mice significantly differs in the number of PNNs if confronted with the LC controls (P=0,0278 P=0,0032). This also applies to the difference between LC controls and ko (P=0.0365).



ANOVA summary	
F	6.62
P value	0.0041
P value summary	**
Significant diff. among means (P < 0.05)?	Yes
R square	0.5538

Fig. 18- Percentage of WFA-positive neurons for all mouse groups in superficial PrL cortex layers. The distribution of PNNs in the PL cortex is significantly different between groups. Scale bar=100 um

Given the particular nature in the distributions of PNNs in the PrL we then proceeded to evaluate if only this is the only features that varies accordingly with the genotype and the early life treatment. So we also analyzed as described their intensity and the overall number of high intensity PNNs in the ko and control group, finding a significant difference calculated using unpaired t-test. Further investigations would be needed to find out if this difference also applies considering maternal care.

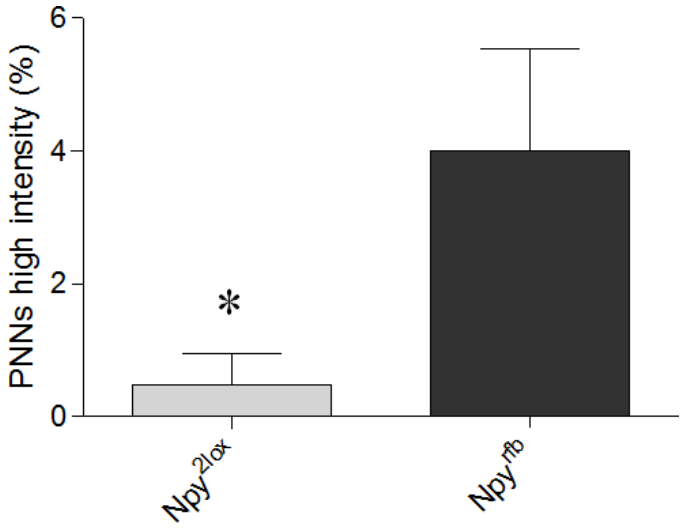


Fig. 19 Unpaired t-test revealed a significant effect of conditional inactivation of Npy1r on the expression of high-intense PNNs around PV- neurons (P = 0,0483).

2.2 Parvalbumin analysis

Given the interesting distribution of PNNs among the four groups and the important structural and regulatory role that they have on neurons (Ulrich-Lai and Herman, 2009), it became interesting to further analyze which population of neurons are involved in the modification of the overall balance of excitatory and inhibitory signaling in the PrL. To do so, immunohistochemistry was performed to highlight one of the most important markers of the inhibitory neurons: parvalbumin (PAV). An analysis of their presence and their distribution among the four experimental group was then performed to evaluate if their number and intensity was somehow perturbed by the ko model or the maternal care.

What emerged, as regards the overall number of PAV+ neurons in percentage on the mature NeuN marked ones, is that in the superficial layers the animals who received low maternal cares present a significantly higher percentage if confronted with the high cared ko ones (P=0.0010; 0.0009). This effect is not present in the deep layers, confirming the previous results on PNNs.

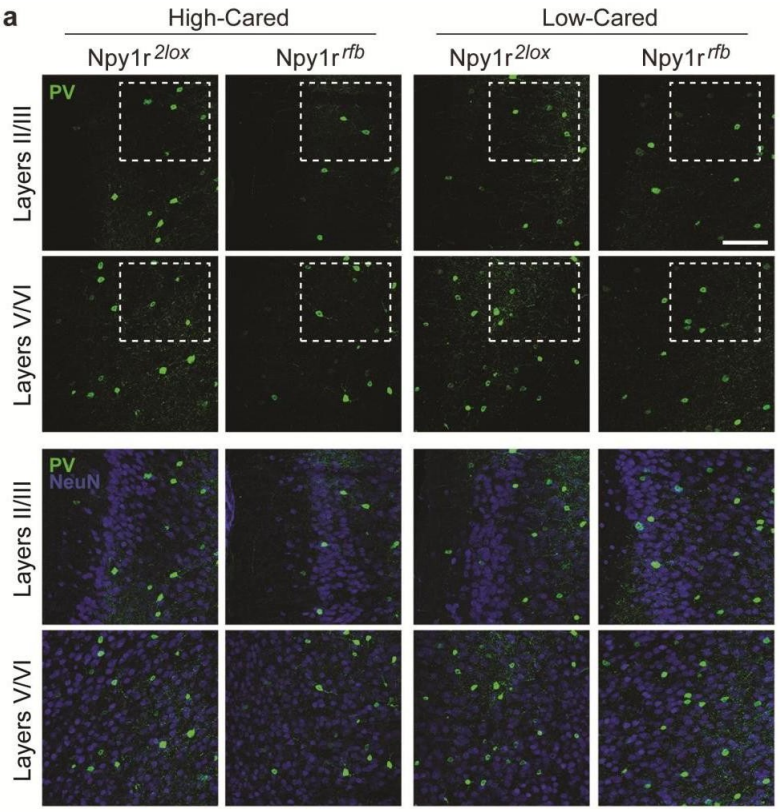
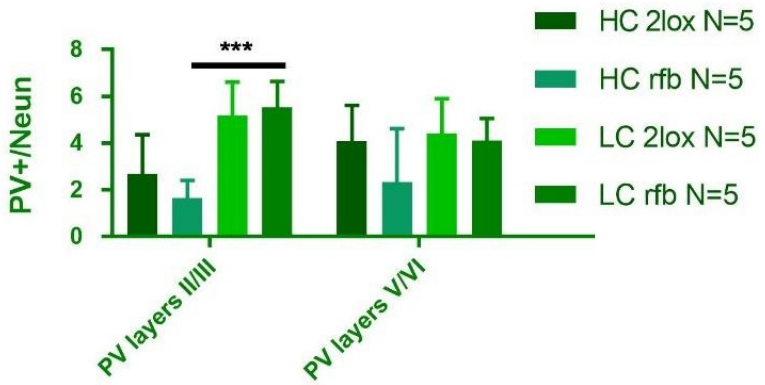


Fig. 20--Co-localization image in different groups and layers of NeuN and Pav+ neurons

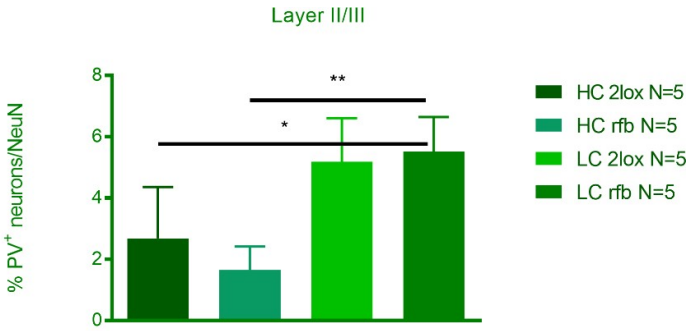


Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	9.153	0.1470	ns	No
Row Factor	0.002931	0.9661	ns	No
Column Factor	39.86	0.0003	***	Yes

Fig. 21- Two-way ANOVA of the percentage of PAV+ neurons on the total number of Neun positive cells. The number of inhibitory neurons appears higher in the LC groups.

A further One-way ANOVA analysis limited to the superficial layers showed the important result that both the ko and controls raised by good dams show a diminished number of

PAV+ neurons in their PrL if confronted with the animals raised by low care dams (which don't statistically differ between them.



ANOVA summary	
F	10.62
P value	0.0004
P value summary	***
Significant diff. among means (P < 0.05)?	Yes
R square	0.6657

HC 2lox N=5 vs. HC rfb N=5	1.024	-1.443 to 3.491	No	ns
HC 2lox N=5 vs. LC 2lox N=5	-2.498	-4.965 to -0.03148	Yes	*
HC 2lox N=5 vs. LC rfb N=5	-2.842	-5.309 to -0.3755	Yes	*
HC rfb N=5 vs. LC 2lox N=5	-3.522	-5.989 to -1.055	Yes	**
HC rfb N=5 vs. LC rfb N=5	-3.866	-6.333 to -1.399	Yes	**
LC 2lox N=5 vs. LC rfb N=5	-0.344	-2.811 to 2.123	No	ns

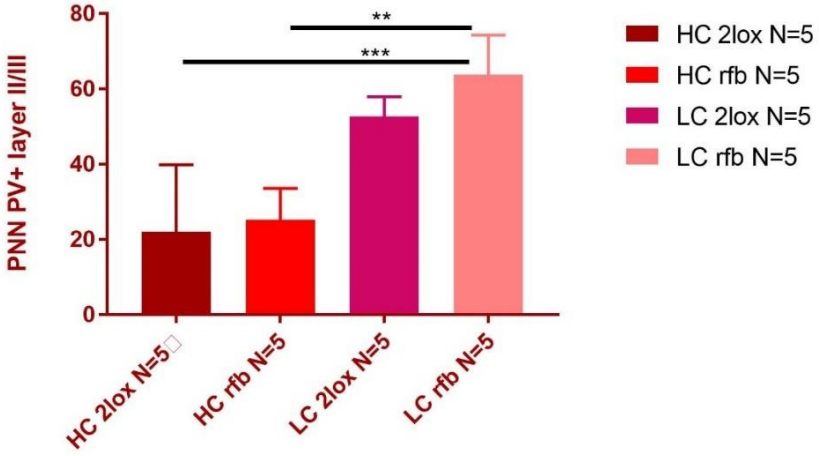
Fig. 22-One-way ANOVA of PAV+ neurons in the different groups. The analysis reveals a difference between both ko and controls from high care animals and the low cared ones.

These results suggest the possible presence of an excitatory/inhibitory imbalance due to the exposition to an adverse environment during the development associated with the effects of a genetic vulnerability.

2.3 Pnns and Parvalbumin neurons co-localization

Once determined the presence of an augmented number of PNNs and parvalbumin positive neurons in the PrL cortex, a final molecular analysis was performed to assess their co-presence in the area. To do so we counted the number of PNNs and evaluated if the net was enveloping a PAV positive or negative neuron, reporting the total amount to the total of NeuN positive cells in the analyzed area.

We performed a two-way ANOVA on these counts to highlight possible differences in the distribution of PNNs around different population of neurons and what appears is that HC controls present a significant difference regarding the distribution if confronted with the LC ko (P=0,0025;0.0002). This is true also for the HC ko animals, which present a lesser percentage of PNNs forming around PAV+ neurons confronted with LC controls and ko (P=0.0048; 0.0002) In detail, the PNNs tend to form more around the PAV+ neurons in the ko animals if they have been exposed to a poor maternal environment.



ANOVA summary	
F	16.03
P value	<0.0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0.7503

Fig. 23-Two-way ANOVA of PNNs enveloping PAV+ neurons

2.4 Neuronal link between executive functions and PrL

Finally, in order to find a possible correlation between the molecular results presented above and the executive functions results provided by the Puzzle Box test we performed an analysis considering the number of cells Pav+ surrounded by PNNs and the scores obtained by the animals in the puzzle box. The analysis was limited to the layers II/III of the PrL cortex, given the less defined situation found in layers V/VI. The statistical instrument chose was the Pearson correlation, which highlighted a particular pattern suggesting a correlation (r = -0. 6489; *n* = 14) between the score obtained at the Puzzle box (which indicates a poorer performance as it become higher), and the percentage of Pav+ surrounded by PNNs.

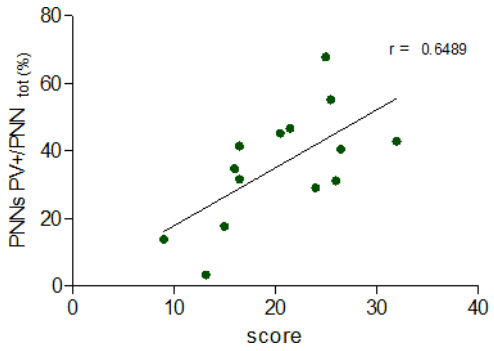


Fig. 24 – Correlation analysis. A) Pearson correlation between the percentage of PNNs PV+ over the total PNNs density in superficial layers of PL cortex and the latency to enter the goal box in the puzzle box test (*P* = 0,0120).

To further test the hypothesis of the PNNs playing a functional role in the functional deficit of animals at the Puzzle box paradigm we experimented a procedure of digestion of PNNs in the PrL of test-naïve mice (the procedure was conducting administrating Ch-ABC for nets digestion or saline to provide a control sample). After the surgical procedure and recovery, the animals were tested at the Puzzle box to see if the performance was affected between groups and to evaluate if the digestion of WFA was useful to improve the score of groups which presented executive functions impairment. The procedure proved interesting, providing a significant improvement in the scores of both LC control and ko mice, as analyzed with two-way ANOVA ([$F_{(1,50)}$ = 13,70, *P* = 0,0005]. *n =6-8).* Also, one-way ANOVA on the score of the saline treated animals confirmed the group and genotype effect obtained in the original protocol (P=0,0026).

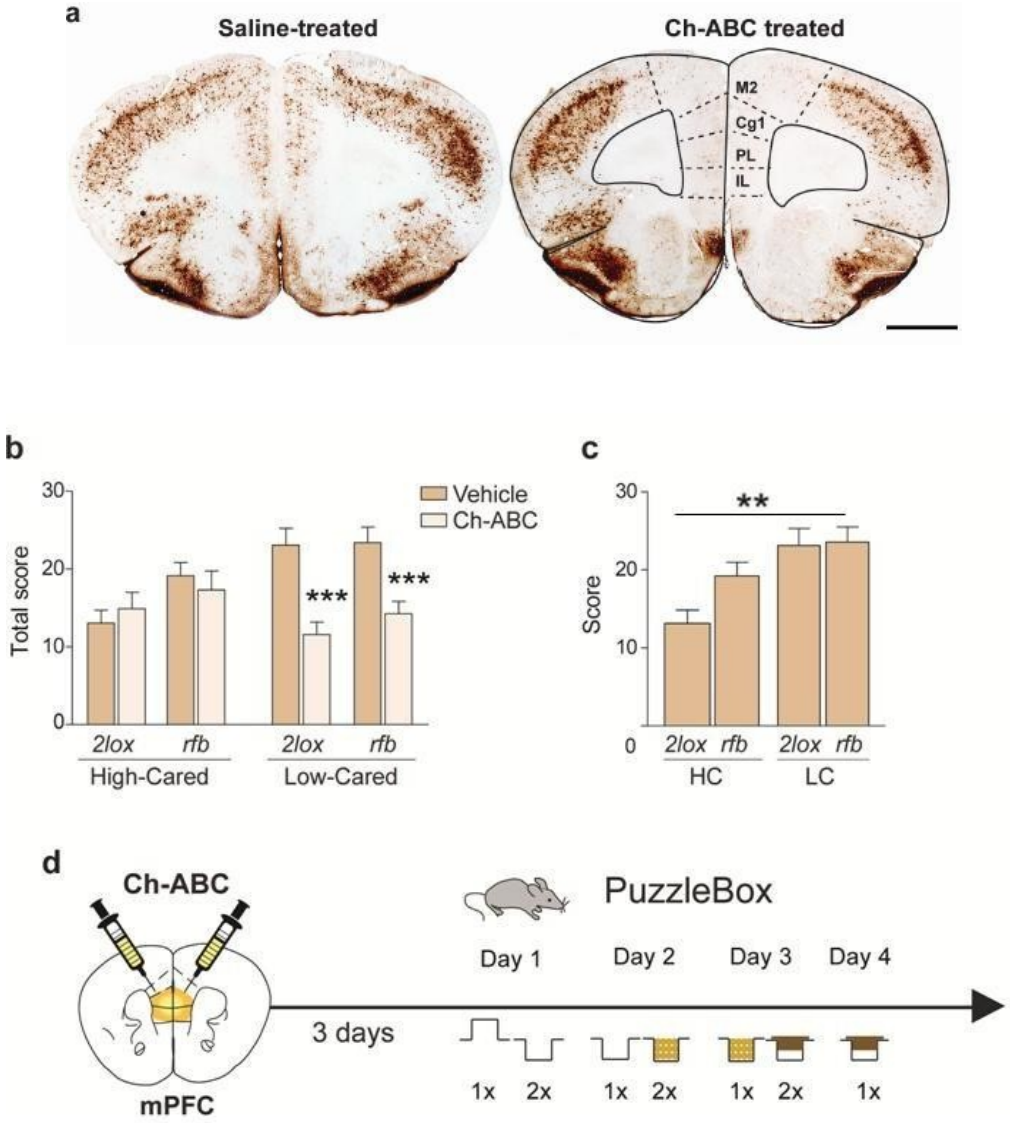


Fig. 25 – Puzzle box analysis after PNNs digestion. a) WFA staining of brain coronal sections from saline or Ch-ABC-treated HC ko mice. Scale bar, 1mm. **b)** Two- way ANOVA analysis showing the rescue in the performance of LC mice. **c)** One-way ANOVA analysis of variances showed a significant difference between groups injected with saline. **d)** Experimental procedure (adapted from a master thesis work by Canicatti 2019).

Discussions

The current conceptualization of the brain as our interface with our surrounding environment is a powerful tool to better understand the role and the relationship between our behavior and the neuronal architecture of our central nervous system. In this paradigm the structure of the brain is fluid and adaptable, always capable of adaptation and redesigning basing its modifications on different stimuli which are collected both from the inside and the outside of the body. The internal communication of the different brain areas is reassured by different types and modalities of connections, and their strength and structural stability can change and transform via several events and time windows. Thus given, the pathology is no more conceived as a disruption of an ideal formation of the nervous system inscribed in the genetic code. Instead, given the dynamic interplay between genes, environment, internal signaling and autoregulation, the psychological pathologies become a form of specific adaptation to a very peculiar situation present in the early stages of the formation of the overall system. This is currently described as a bio-psychological-social approach (Engel, 1977) to mental health and is largely accepted in the medical and clinical field, representing the official position of World Health Organization on the intervention and promotion of psychological well-being and treatment of related disabilities (World Health Organization, 2002).

For example, psychiatric diseases such as schizophrenia, major depression, and anxiety disorders are lately associated with an imbalance of the crucial dynamic equilibrium existing between excitatory and inhibitory neurotransmissions within the prefrontal cortex and related brain circuits (Nuss et., 2015). Emerging evidences show that chronic environmental stress exacerbates the susceptibility and severity of pathological conditions, and triggers alterations in favor of an arousal state. So, in this paradigm what becomes interesting is to investigate in particular how the formation and development of our brain is influenced by the dynamic interaction between environment and genetic predisposition and in which way from these variables the behavior emerges. As described, in our experimental model this has been researched by trying to establish a strong enough link between genetical set (obtained by the manipulation of the Y1 receptor) and behavioral model encountered during the early stages of life. A possible causal co-factor of the process in medial prefrontal cortex that we tried to investigate was the particular microenvironment which creates and maintains a structural stability in this important area of the limbic system, working on the model of the quad partite synapse (Schafer 2013). This particular

functional model of the synapse, modifies the historical conception of the connection between neuron as a mere matter of pre and post synaptic cell and their chemical communication. It highlights instead the important role of support and balancing in the excitability and structural stability that is played in the synapse by other actors of the surrounding environment, such as the microglia and the extracellular matrix i.e. the perineuronal nets system.

The validated murine model we utilized in the present study was useful to provide a substrate for the analysis of the effect and the role of PNNs in the sustenance and modifications of neuronal circuits that plays a role in the activation of anxiety and via its interconnection with the observation of the different maternal cares provided during the perinatal period.

First of all the important effect of maternal style was confirmed by both the observation of dams' behavior and the monitoring of the weight-gain curve of the pups after the weaning. This is an important result and is consistent not only because represents a further validation of the conditional KO model associated and its ability to modify in a tangible way the phenotype of the animals, but also because it's the starting point of a reproducible and significative behavioral and physiological difference to work on when searching for an external signaling that could be potent in the shaping and organizing a brain area such as the limbic system during a sensitive time window such as the pre-weaning period. In fact observing that there is a strong difference in the quality and quantity of cares provided by the different strains of adoptive mother and observing its medium-term consequence on the ability of the pups to gain and maintain weight probably indicates a vulnerability in the animals raised by C57BL/J6 which is evident in confront with the animals raised by FVB/J dams. Moreover, this vulnerability evens the phenotypical diversity between KO and controls, which is instead exalted by the situation in which the pups are raised in a correct and protective way. The final indication we argue from this is that we have provided a strong, observable and significant difference in our four experimental groups, which is the very basis of the following result.

Subsequently from the analysis of the behavior of the grown-up mice we were able to observe that the difference in the treatment of the pups evolves and becomes a behavioral trait well recognizable in the young adult mice as a susceptibility to anxiety and to paradigm designed to evaluate and reveal an avoidant and fearful phenotype. The distribution of their performances follows in a similar one the one of the maternal styles

received, seeing the ko and controls raised in a poor relational environment, and the ko raised in a good relational environment to develop a behavioral disadvantage in their ability to explore and exploit their surroundings, being too fearful to balance their natural tendency to roam and find novelties with their evaluation of risks and potential threats. Even if this effect is well-known in literature (Burghy et al., 2012) and previously published in Bertocchi et al (2011), remains important for it confirms the narrow relationships with our conditional model and their behavioral phenotype. Their overall wellbeing and general low level of contextual stress and state-anxiety were considered not to be relevant in their behavior during the test, having been evaluated via Burrowing test showing no affections in their daily life routine or perturbances in their behavior due to perturbances in their context of life. This allows us to reasonably exclude the presence of major medial prefrontal cortex impairments ad well as to assess an even level of overall functionality which can be useful as a confrontational point for more complex and cognitive challenging task such as the Puzzle box.

This test was in fact able to focus the attention on a possible more subtle cognitive and planning deficit in both the animals raised by bad mothers and conditional ko raised by good mothers.

This impairment manifested in different ways in the groups according with the presence of conditional inactivation of Y1 receptor. In fact, here the genotype seems to negatively affect the test as regarding the abilities more related to working memory and attention (thus having problems in the repetition of the task for example). On the other hand, instead the maternal care style seems to affect more the ability to solve the more difficult problems. This could be interpreted considering that the conditional ko animal carries a specific deletion of Y1r in limbic areas, incapacitating the response to the anxiolytic effects of NPY only in this area, whereas in the low care paradigm the insensibility to the effect of NPY is due to hyper activation of HPA axis and amygdala caused by the behavioral imprinting during the early life experience, resulting in a wider effect in terms of brain areas affected and cognitive impairment caused.

It is also possible that some of the characteristic of the experimental paradigm could cause a confusion between structural and anxiety related cognitive impairment of the animal. The puzzle box is a useful and pretty simple experimental procedure to evaluate cognitive functionality in rodents, and it doesn’t expose animals to major stress and requires no particular training to be performed. This surely makes it versatile and ecological for the

mice, but it can also be difficult to maintain under control some factors of confusion. This is due to the fact that the recording of the scores is manual, and even if the procedure is standardized, no videos of the sessions were available for further analysis of some behavioral cues that could provide an identification of trait anxiety during the test. Surely it would be interesting to repeat the procedure recording the sessions and analyzing the micro-behaviors of the animals during the trials to better evaluate this factor.

The molecular analysis of the Prelimbic cortex was principally concentrated on the counting and co-localization of perineuronal nets in relation with the neuronal sub-population they tend to form around to. This is for sure an important hint on the molecular mechanism that regulates the activity and excitability of neuronal pathways in a crucial area of the limbic brain, but it also leaves open different questions on the details of the process of their formation and on the signals that orientate their presence around specific types of neurons or their morphological variety or their peculiarities is thickness, intensity, composition. Also, their effective role in the structural stabilization and connectivity potentiation or limitation remains quite unexplored and requires further analysis and different experimental approach to be better understood in the several implications and co-factors involved in the phenomenon.

The mechanisms involved in the differentiation of perineuronal nets remains largely unknow and not so researched in literature and working on them could clearly provide a better comprehension of their functional link for example with the PV+ neuronal circuit and its inhibitory effect in the different layers of the PrL cortex.

Based on the result of this study still, is it possible to reach some conclusions on the role that perineuronal nets exerts on the limbic brain, specifically on the Prelimbic cortex, and to establish an initial connection between parvalbumin network and extracellular matrix. In the first place the evaluation of a not-significant difference in the density of PNNs in the experimental groups exposed to a good maternal environment seems to corroborate the hypothesis that the changes in the expression and sensitivity to the regulatory effect of NPY doesn't affect the overall synthetization of proteoglycan-based extracellular matrix. The neuropeptide in fact, being secreted by GABAergic neurons, plays a role in the regulation of the excitability of neurocircuitry, in the limbic system especially in the hippocampal region. The effects of the conditional ko in this area has been more accurately described in a 2018 paper by Mele et al. (submitted) and unveils that also in this area of the brain the perineuronal nets are heavily influenced (even more than in the mPFC) by the maternal cares in association with the selective deletion of Y1r.

The observation on the significantly augmented density of PNNs in the controls and ko exposed to bad maternal environment corroborates this assumption; the genetic alterations is not responsible alone for the increasing in the number of nets. Here the role of early life experience is predominant. This could be probably due to the role of PNNs as an epigenetic regulator of plasticity period (Bosiack et al. 2019) and it is in some way interesting to note that in a situation of hostile emotional environment during early life the limbic system adapts to it by upregulating the presence of extracellular matrix. This is even more interesting in comparison with the LC conditional ko animals, in which the upregulation is still present but it's lower. This could be due to their natural susceptibility to the development of an overactive circuitry that does not require such a strong structural response to develop the same anxious phenotype as the mistreated controls.

The question then becomes: how the PNNs augmented density translates in an anxious phenotype as we observed in our mice? The possible answer could be provided by looking at which type of neurons the PNNs develop around.

As we chose to focus our attention on the parvalbumin positive neurons we had to consider their role in the construction and maintenance of inhibitory/excitatory balance in the area we selected for the analysis.

In the superior cortex neurons positive to the antibody parvalbumin are strongly associated with fast-spiking inhibitory interneurons, and in this area of the brain the modification in their number and connectivity is associated with aberrant alteration of behavior and changes in physiological changes in different psychiatric syndromes and correlates with a constellation of symptoms usually associated with emotional dysregulations (Nuss et al., 2015; Panthi, 2019). Their effect on inhibiting the excitatory output of the area in which they exert their function can potentially be disruptive whenever they meet an imbalance in the proportion with other population. In our animal model we witnessed an increasing in the proportion of parvalbumin positive cells in the mice exposed to low quality in the early life experiences with no appreciable differences between ko and controls. This allows to hypothesize an important change in the balance between excitatory and inhibitory inputs.

This tendency is as well present when considering the ratio of parvalbumin positive neurons wrapped by perineuronal nets (in this latter case the LC ko mice present an increased percentage of PNNs/pav+ neurons even in confront with the control ones). Considering the structural role and the potentiation and stabilization in the connectivity provided by perineuronal nets, this peculiar distribution could mean an even further

potentiation of the inhibitory role of interneurons leading to a poor activity of Pre limbic cortex which is associated with an anxiety-like behavior in rodents (Wang et al. 2015) which is exactly the situation that occurs in our mice.

The hypothesis is strengthened by the direct correlation between the score at the Puzzle box test and the percentage of parvalbumin positive neurons coated by PNNs and more noticeably by the rescue of the scores in the low cared animals when they are treated in prelimbic cortex with ch-ABC to dissolve PNNs.

The presented results should be considered a starting point in the evaluation and elaboration of new models of neurobiological functionality of connectivity of the limbic brain and indeed shed some light on the links between behavioral influences during early life, excitatory and inhibitory balance in the superior cortex and the role of extracellular matrix.

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