UNIVERSITÀ DEGLI STUDI DI MILANO

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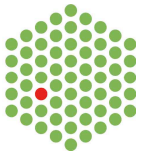

UNIVERSITÀ DEGLI STUDI DI MILANO

Scuola di Dottorato in Scienze biologiche e molecolari

Dipartimento di Scienze Biomolecolari e Biotecnologie

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Synaptic pruning by microglia is essential for normal brain development

A new juvenile chronic social stress paradigm as a model for early maltreatment in mice

(BIO-06)

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Project 1

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1. Introduction

1.1 Microglia: origin and function

Microglia are the immune cells of the brain and were described for the first time by del Rio Hortega (1932), a student of Ramòn y Cajal. They constitute a distinct population of non neuronal-derived glia cells and their developmental origin is still largely debated. The earliest appearance of microglial precursor cells into the brain has been reported at embryonic day 8.5 (E8.5) (Ashwell, 1991; Chan et al., 2007), likely originated from macrophages of the yolk sac. Myeloid cells expressing the hematopoietic marker CD45 and adult macrophages/microglia markers F4/80, CD11b and Cx3cr1 were first seen in the brain at E9.5 (Alliot et al., 1999). During the early post-natal days (P0-P11) a massive increment of microglia cells number is registered, however it is still not completely clear whether it is due to proliferation of embryonic cells or to recruitment of new circulating monocytes. Several studies using bone marrow transplantation of EGFP-monocytes from transgenic mice showed recruitment of these cells into the brain parenchyma (Hess et al., 2004, Ono et al., 1999).

However, it could be that the irradiation techniques are compromising the physiological process of macrophages replenishment. More recent experiments, in fact, based on parabiosis (when two animals are joined surgically to create a common vascular compartment) suggest that infiltration of circulating monocytes is absent under non-traumatic conditions and that bone marrow-derived cells are recruited only

upon irradiation-induced brain injuries (Ajami et al., 2007, Ginhoux et al., 2010). It needs to be considered that all these studies have been conducted on adult animals, and the hypothesis that monocytic cells can be recruited during a specific developmental stage to settle and take residence into the parenchyma brain still cannot be excluded. A piece of data supporting this latter possibility comes from the study of Hristova et al., 2010, where, upon intravenous injections of fluospheres in newborn mice, a subpopulation of ramified cortical microglia resulted to be positively labeled. These results indeed provide evidence for the recruitment of circulating monocytes to the unperturbed neonatal brain parenchyma. Actually, the blood brain barrier at that developmental stage could still be immature, letting the fluosphere reaching the parenchyma; alternatively, recruitment of macrophages to neonatal parenchyma could be interpreted as a physiological mechanism restricted to a particular developmental window, occurring during the first postnatal weeks. Work from the laboratory of M. Capecchi is also supporting this idea: by investigating Hoxb8 positive cells into the brain (with Hoxb8 being a transcription factor specific for myeloid lineage) they infer that about 40% of microglia are bone marrow-derived (Chen et al., 2010). However, recent in vivo lineage tracing studies established that a consistent proportion of adult microglia derive from primitive myeloid progenitors that arise in the extra embryonic yalk sac just before E8.0 and invade the embryo after E9.0, when blood vessels are formed; apparently these cells are maintained throughout life and replenishment from circulating monocytes is minimum, if any (Ghinoux et al., 2010).

For a long time, microglia cells have been considered to switch themselves on and off according to their functional phenotype: they were believed to become ‘activated’

in response to pathogens or injuries and to stay ‘inactive’ for the rest of the time. Now it is becoming more and more clear that these cells, even when not activated, are not ‘resting’ and very far from being ‘inactive’. Hanisch and Kettenmann have recently introduced a new term for defining the status of physiological microglia: ‘surveying’ cells, responsible for constant surveillance of neighbors (Hanisch and Kettenmann, 2007). The majority of studies on microglia in the past were attempted to characterize the function of these cells in pathological conditions, mainly in animal models of disease or in culture, but not much is known about their function during physiological development. It is well known that microglia can sense and rapidly respond to threats to the CNS integrity by expressing a broad pattern of cytokines, either pro- or anti-inflammatory, and by changing their MHC expression profile. They can exacerbate inflammatory processes by releasing reactive oxygen species, nitric oxide (NO) or TNF-α, or, on the other side, they can confer neuroprotection and promote neurogenesis. Some evidence shows that fully activated microglia can have neurotoxic effects. In some models of neurodegenerative disorders such as Parkinson, microglial activation mediated by INF-ϒ has been clearly shown to induce neuronal death (Block and Hong, 2007; Mount et al., 2007). At the same time, in different models, microglia can also release neurotrophic factors that can confer neuroprotection, promote neurogenesis and glia development (Kitamura et al., 2004; Monje et al, 2003). This dual aspect of microglia cells make their function complex, controversial and even detrimental, especially in regarding to pathogenesis of autoimmune disease, the etiology of which could reside sometimes in over-triggering an inflammatory response. Microglia react to dangerous or inflammatory signals by changing their cell

morphology: retracting their long processes, transforming into an ameboid cell shape and migrating to the site of injury.

In the healthy brain microglia show a ramified morphology. Recent time-lapse studies have shown that thin microglia processes are highly motile and in continuous and rapid movement, scanning the surrounding parenchymal space. They do not overlap, but rather repulse and avoid each other, and they appear to scan a certain volume of competence (Davalos et al., 2005, Nimmerjahn et al., 2005). By using in vivo two-photon imaging, Wake and co-workers showed that resting microglia processes make brief contacts (~ 5 min) with synapses, at a frequency of about once per hour, suggesting a direct role for microglia in monitoring the functional state of synapses (Wake et al., 2009). The interaction between microglia and axonal button or dendritic spines could be driven by many possible different factors, such as chemoattractant molecules released from synapses (glutamate, brain-derived growth factors or chemokines). Purinoreceptor stimulation has been shown to drive directional guidance of microglial processes toward sites of microlesions; so ATP is considered another possible signal in promoting microglia-synapses contacts (Haynes et al., 2006). A role for microglia in maintaining synaptic integrity has been proposed, based on the observations that dysfunction in microglial activity can lead to synaptic impairment (Graeber, 2010). Wake proposed that microglia can detect the functional state of synapses and respond to it by making longer contacts upon pathological conditions. In his study it has been observed that occasionally these prolonged contacts can lead to the disappearance of synapses, suggesting that microglia might take part in synapse elimination (Wake et al., 2009). A recent study by Tremblay and

co-workers used a double transgenic mouse line (Thy1::YFP;Cx3cr1$^{GFP/+}$) which allows the time-lapse scanning of microglia-neuron contacts. These data show that synapses are contacted by distal microglial processes and that occasionally, upon visual deprivation, dendritic spines of visual cortex neurons disappear after being contacted by microglia, supporting a role for microglia in synapse elimination (Tremblay et al., 2010).

1.2 Fractalkine chemokine and its receptor Cx3cr1

Fractalkine is a member of the ∂-chemokine subfamily and it is also known as neurotactin or Cx3cl1 because of its unique motif with a disulfide bridge separated by 3 residues Cys-X-X-X-Cys. This chemokine has a size of 395 amino acids (aa) in mouse (with the gene located on chromosome 8) and 397 aa in human (with the gene located on chromosome 16q13). Fractalkine contains a signal peptide (24 aa), an N-terminal Cx3C-chemokine domain (76 aa) attached to a highly o-glycosylated mucin-like stalk region (239/241 aa), a transmembrane domain (21 aa) and a C-terminal cytoplasmic tail (35 aa). It is expressed in many peripheral tissues such as heart, lungs, kidneys, but it is predominantly found in the brain (Pan et al., 1997). During the first postnatal weeks mRNA levels of fractalkine are upregulated compared to embryo (Mody et al., 2000) and then it remains constitutively expressed. This chemokine is present in two different forms: it can be membrane-bound (functioning

as a cell adhesion molecule) or it can be soluble, released by the cleavage of the membrane-bound form upon proteases activity, such as ADAM10 or TACE/ADAM17 (Garton et al., 2001; Tsou et al., 2001). Tarozzo and colleagues carried out a gene expression analysis to evaluate the level of fractalkine in normal mice and to map cells expressing fractalkine by both in situ hybridization and immunocytochemistry. The results from these studies indicated high levels of fractalkine protein in cortex, hippocampus, basal ganglia, and olfactory bulb. In particular, the presence of abundant immunoreactive neurons was observed in layers II, III, V, and VI of the cortex. In the hippocampus, the CA1 region was the most intensely labeled, but immunoreactive neurons were present also in CA2 and CA3, whereas in the basal ganglia, immunoreactive cells were observed in the caudate putamen. This work not only confirmed that fractalkine is exclusively expressed by neurons, but also nicely showed that its protein expression follows a clear rostro-caudal gradient with the rostral regions of the CNS (olfactory bulb, cortex, striatum, and hippocampus) expressing high levels of fractalkine and the caudal regions displaying low levels (diencephalon and brainstem) or no immunoreactivity (cerebellum) (Tarozzo et al., 2003).

The unique receptor for Cx3cl1 (either for the membrane-bound or for the soluble form) is Cx3cr1 (Combadiere et al., 1998). Like all chemokine receptors, Cx3Cr1 is a G-protein coupled seven transmembrane-spanning domain receptor. The gene contains 4 exons and it is localized on the human chromosome 3p21.3 and the mouse chromosome 9. It is expressed by circulating monocytes, T cells, NK-cells and

dendritic cells, and throughout the brain uniquely by microglial cells.

1.3 Role of Cx3cr1 in microglia

Cx3cl1-Cx3cr1 signaling has been extensively studied in pathological condition. Cardona and coworkers showed that Cx3cr1 deficient microglia, when activated, exhibit impairment in their ability to migrate towards the site of injury, supporting an important role for fractalkine as chemoattractant factor (Cardona et al., 2006). Fractalkine is also considered to have a constitutive 'calming' influence on microglia, in maintaining low their status of activation (Hanish and Kettenmann, 2007).

Interestingly, a role for Cx3cl1 signaling has been proposed also in driving microglia migration during development. By using Cx3cr1 null mutant mice, Ruitenberg showed that fractalkine regulates normal branching and migration of monocyte-derived cells in the olfactory epithelium (Ruitenberg et al., 2008). Another recent study showed that Cx3cl1 signaling from retinal neurons and endothelial cells likely modulates dynamic microglia behavior and influences the level of surveillance either under basal conditions or in response to tissue injury (Liang et al, 2009). All together, these data contribute to address the possibility that Cx3cl1-Cx3cr1 represents an important signaling pathway between neurons and microglial cells, not only necessary in pathology but also during normal brain development.

1.4 Synaptic pruning process during development

The term ‘pruning’ refers to a neurological regulatory process, that facilitates a productive change in neural structure by reducing the overall number of connections, leaving a different configuration. More specifically, synaptic pruning is defined as the physiological elimination process of unwanted or not functional synapses during development. In rodent brain it takes place during the first post-natal weeks, strictly matching synaptogenesis. Early in development, in fact, neurons extend supernumerary connections to neighbouring cells; these exuberant inputs are then removed through a specific and ordered developmental programme (Hua and Smith, 2004). The highest peak of turnover has been shown to occur around P8, with dendritic spine density increasing between the first and the third post-natal week (Cruz-Martìn et al., 2010).

Molecular mechanisms such as retraction have been proposed to explain dendritic spine disappearance. However, synaptic stripping, a process of synaptic elimination by microglia during pathological condition, has been suggested as a possible mechanism occurring also during a particular developmental window. This study is aimed at demonstrating that microglia can directly engulf synaptic terminals during synaptogenesis, selectively removing inappropriate synapses, thus performing an important role in refinement of brain circuits.

1.5 C1q as candidate opsonizing signal

Complement cascade components have been recently suggested to mediate synaptic pruning. C1q is one of the most interesting candidates at present to be under investigation. It is the initiator of the classic pathway of the complement cascade and can act as a soluble molecule alone or in association with serine proteases molecules (C1r and C1s) that then trigger the cleavage of C3 and the consequent steps of the cascade. Stevens and colleagues have shown that C1q is highly expressed in the developing brain, when neural circuits refinement takes place, and that it is highly associated with incomplete synapses, being frequently apposed to pre- or post-synaptic terminals missing their partners (Stevens et al., 2007). C1q null mutant mice show clear deficits in synapse elimination in *lateral geniculate nucleus* (LGN) and, interestingly, C3 knock-out mice show a similar phenotype, suggesting that the complement cascade –and not only the initiator component C1q- is involved in the process of synaptic pruning. A recent study has shown that C1q knockout mice exhibit an increase in cortical excitatory connections, revealing a deficit in axonal pruning compared to littermates wild-type (Chu et al., 2010). All these data, taken together, support a role for complement cascade components as good candidates in mediating synaptic pruning. Ideally, they could act as opsonizing factors for unwanted synapses. Microglial cells, moreover, express receptors for both C1q and C3 factors, suggesting a possible direct involvement in recognizing them and eliminating synapses.

1.6 Electrophysiological correlate of circuit maturation: sEPSC and LTD

The postnatal development of excitatory circuits in mammalian brain reflects a progressive maturation of dendritc spine functionality, as it is revealed by temporal changes in electrophysiological properties. Spontaneous excitatory post-synaptic currents (sEPSC) represent both action potential and non-action potential dependent neurotransmitter release and are know to increase during development. Miniature EPSCs (mEPSCs) recorded in the presence of the sodium channel blocker, tetrodotoxin (TTX) reveal only non-action potential dependent vesicle release. The sEPSC/mEPSC amplitude ratio increase is considered a feature of development, reflecting increased connectivity and redundancy of afferent synaptic inputs (Hsia et al., 1998). All these parameters must be taken into account in the evaluation of a proper developmental refinement of excitatory connections.

Along with these basic electrophysiological parameters, also the weakening of synapses after a short series of pre-synaptic action potentials, known as Long Term Depression (LTD), represents an important landmark for brain maturation (Wagner et al., 1995). Plasticity of neural circuits is higher, in fact, in immature brain (Dudeck and Bear, 1992; Mulkey and Malenka, 1992; Ducdeck and Bear, 1993; Shinoda et al., 2010).

1.7 Phenotypic consequences of overnumerary dendritic spines

Dendritic spines are the sites of postsynaptic specializations and as a result their density can be considered a direct measure of the quantity and distribution of connections onto individual neurons in the developing brain. The spines present on dendritic arbors of pyramidal cells, in particular, represent the post-synaptic terminals of excitatory connections and, thus, have been widely used as an index of excitatory connectivity onto these neurons (Fiala et al., 2002). Altered spine densities are often associated with cognitive developmental disorders, with changes present especially in the superficial layers of the cortex, which are the last to develop and undergo significant pruning during the postnatal period.

In Fragile X Syndrome (FXS), the most common form of inherited mental retardation, altered spine density and morphology in cortical regions represents a consistent phenotype (Irwin et al., 2001; Churchill et al., 2002) and similar deficits have been also observed in dentate gyrus of *Fmr1* knockout mice, a genetic mouse model for FXS (Grossman et al., 2010). In a recent study, a significant spine density increase in cortical areas of subjects with autism spectrum disorder (ASD) has been shown, providing structural support for connectional changes that may result in altered cortical computations (Hutsler and Zhang, 2009).

2. Aim of the project

The role of microglia in the brain has been extensively studied in pathological conditions, but little is known about microglial activity in healthy brain, in particular during early development. This work is aimed at understanding the function of these phagocytic cells in synaptic pruning, during the first postnatal weeks, when synaptogenesis occurs. We suggest that microglia processes, continuously moving and contacting synapses, can sense and detect unwanted dendritic spines and ultimately proceed to their engulfment. In order to demonstrate our hypothesis we first needed to show anecdotal evidence that engulfment of synaptic material by microglia can be observed. This first part of the project was focused on identifying colocalization between microglia and synaptic material (confocal microscopy technique). Then, by employing mice with microglia lacking the expression of the fractalkine receptor (Cx3cr1) as a model of microglial dysfunction, we wanted to verify if the process of synaptic pruning was impaired. In order to elucidate this aspect, we first characterized morphological deficits in microglia $Cx3cr1^{KO/KO}$ mice, confirming our model for compromised microglial function; then we compared these animals with wild-type (WT) littermates, to investigate differences in synaptic pruning. Experiments aimed at testing susceptibility to seizures have been performed, to study the excitability of brain circuits in these animals. Electrophysiological properties were also investigated to assess the maturation and connectivity of neural circuits in presence or absence of Cx3cr1. Finally a battery of behavioural tests has been performed, in order to correlate the phenotype of supernumerary spines and

delay in brain circuit maturation with a possible developmental deficit in social behaviour, a key feature of developmental disorders like autism associated spectrum with altered pruning. All these experiments, taken together, are part of a project aimed at showing an important, previously unknown, function of microglial cells during brain development.

3. Material and methods

3.1 Animals

Animals were group housed and kept on a 12 h light/dark cycle (lights on at 7:00) with constant ambient temperature (21.5 ± 1°C) and humidity (55 ± 8%) with food and water available *ad libitum*. *Cx3cr1*^{KO} and *Cx3cr1*^{GFP} mice were kindly provided by Israel Charo (Haskell et al., 2001) and Littman (Jung et al., 2000), respectively. In experiments comparing wild-type and homozygous *Cx3cr1*^{KO} littermates, mice were produced by intercrossing *Thy1::GFP-M/Thy1::GFP-M*;*Cx3cr1*$^{KO/+}$ mice (Feng et al., 2000). In experiments comparing heterozygous and homozygous *Cx3cr1*^{KO} littermates, mice were produced by crossing *Cx3cr1*$^{GFP/GFP}$ females with *Cx3cr1*^{KO}/+ males. All mice were on a C57BL/6J congenic background. Mice were bred and genotyped at EMBL following protocols approved by the Italian Ministry of Health.

3.2 Morphological reconstruction of microglial cells

For morphometric analysis, single microglial cells were filled with biocytin and processed. Control (GFP/+) mice and Cx3cr1 homozygous knockout (GFP/GFP) littermates were deeply anesthetized with alothan and sacrificed by decapitation, at

two different time points: P7-9 and P40-43. Brains were removed and cut by using a Leica Vibratome (Leica Microsystems, Wetzlar, Germany). Coronal brain slices 150 μm thick were then fixed ON at 4°C in 4% PFA, then permeabilized with PBS 0.5 % Triton-X 100 (Tx-100) and incubated with blocking solution (PBS 0.5 Tx-100 + 1% BSA) 1hr RT. Slices were incubated ON RT with Chicken anti-GFP antibody (1:800; Aves Labs, Inc., Portland, Oregon), then incubated with secondary antibodies anti-Chicken IgY conjugated to Fluorescein (Aves Lab. 1:800) and with Streptavidin conjugated to fluorophor 594 (Alexa, 1:800). All antibodies were diluited in blocking solution (PBS 0.5 Tx-100 + 1% bovine serum albumin (BSA)). Slices were finally mounted on slides with Mowiol 20% and let dry ON RT in the dark. Morphology of single microglia cell was analysed by using confocal laser microscopy, at 40x magnification. Acquisition files were then processed with ImageJ Software (NIH) for two-dimensional analysis and Imaris Software (Bitplane, Zurich, Switzerland) for three-dimensional reconstruction and morphometry.

3.3 Microglia density analysis

For analysis of microglia cell density, mice were obtained from breeding of females $Cx3cr1^{GFP/GFP}$ and males $Cx3cr1^{KO/+}$ in order to have Cx3cr1GFP/+ and Cx3cr1GFP/KO littermates: heterozygous or homozygous for the CX3cr1 receptor, but all expressing only one GFP copy. Mice at post-natal day P8 and P40 were deeply

anesthetized with Avertin (Sigma-Aldrich, St Louis) and perfused transcardially with 4% PFA in 0.1 M PB, pH 7.4. Quickly the brains were removed and immersed in cold PFA and post-fixed overnight at 4 °C. Coronal slices 150 µm thick were cut by using a Vibratome Leica, then permeabilized for 1hr RT with PBS 0.5 % Triton-X 100 (Tx-100) and incubated with blocking solution (PBS 0.5 Tx-100 + 1% BSA) 1hr RT. Slices were first incubated ON 4°C with primary antibodies directed against GFP (Aves Lab. 1:800) diluited in blocking solution, then with secondary antibodies conjugated to Fluorescein, 1hr RT. Slices were stained with DAPI for 30min, then mounted on slides with Mowiol 20% and let dry in the dark ON RT.

3.4 Colocalization analysis: microglia and synaptic markers

Mice were deeply anesthetized intraperitoneally with Avertin (Sigma-Aldrich, St Louis) and perfused transcardially with 4% PFA in 0.1 M PB, pH 7.4. Brains were postfixed in PFA4% overnight at 4°C and then cryoprotected in 30% sucrose PBS. For colocalization experiments, horizontal sections were cut on a cryostat (30 µm, Leica Microsystems), permeabilized in 0.5% Triton X-PBS for 1hr and blocked with 2% BSA+ 0.5% Triton X in PBS for 1 hr at room temperature. Primary antibodies were diluted in blocking solution as follows: GFP, chicken 1:800 (Aves Labs) and PSD95, mouse 1:200 (Millipore, Billerica, MA). Sections were incubated with primary antibodies overnight at 4°C and stained with secondary antibodies at 1:800 and

1:1000, respectively, for 2 hr at RT. Slices were then stained with DAPI and mounted in Mowiol mounting solution. Confocal microscopy was performed with a TCS-SP5 (Leica) Laser Scanning System.

Quantifications. For quantitative analysis of PSD95 IR puncta and colocalization studies, 4 serial optical sections were acquired (0.4 mm Z-step size) from hippocampal CA1 *stratum radiatum* of $Cx3r1^{KO/+}$ and $Cx3cr1^{KO/KO}$ mice, using 40X magnification. Colocalized puncta between PSD95 and microglia processes were quantified using the ImageJ software plug-in Colocalization Finder and 3D Counting Objects (NIH). Three-dimensional reconstructions of microglia engulfing PSD95 were generated with Imaris Software (Bitplane AG, Zurich Switzerland).

STimulate Emission Depletion Microscopy (STED). For STED microscopy, brains were processed as described for conventional microscopy; sections were incubated overnight at 4°C with primary antibodies (rabbit anti-GFP, 1:800, Invitrogen, Carlsbad, CA; mouse anti-PSD95, 1:200, Millipore) and incubated with secondary antibodies (2 hours at room temperature; anti-rabbit Chromeo494, anti-mouse Atto647N, Active Motif, Carlsbad, CA). Sections were imaged on a TCS-STED System (Leica).

Electron Microscopy. Brains were perfused and were fixed with 4 % (w/v) PFA, in 0.1 M sodium phosphate buffer pH 7.1. Hippocampal *stratum radiatum* areas were selected from thick sections, cut in 0.5 mm^3 squares, embedded in 12% gelatin, and infused in 2.3 M sucrose. Mounted gelatin blocks were frozen in liquid N_2 and ultrathin (50 nm) cryosections were cut at -120˚C with an Ultracryo-microtome (Leica). Sections were retrieved in 1.15 M (w/v) sucrose with 2 % (v/v)

methylcellulose solution. Immuno-labelling was performed using rabbit anti-GFP (Invitrogen) and mouse anti-PSD95 (BD Biosciences, *Franklin Lakes*, NJ) as primary antibodies, and protein A coupled to 10 and 15 nm diameter gold particles, respectively. Electron microscopy images were acquired using a Philips Morgagni 268D Transmission Electron Microscope (FEI, Hillsboro, OR).

3.5 Dendritic spine density analysis

For dendritic spine density experiments mice were produced by intercrossing Thy1::GFP/GFP;Cx3cr1KO/+, in order to have littermates that were all homozygous for GFP mosaic expression in neurons and, at the same time, WT, heterozygous or knockout for Cx3cr1. Animals, 15 days o 3 months old, were perfused and brains were removed (as previously described). After brain post-fixation, coronal slices 100 μm thick were cut on a vibratome (Leica Microsystems, Wetzlar, Germany), permeabilized and incubated in blocking solution as already described. Antibodies anti-GFP (chicken, Aves Labs. 1:800) were used for overnight incubation at 4°C; slices were then incubated for 2hr RT with secondary antibodies (anti-Chiken IgY-cojugated to Fluorescin, Aves Lab. 1:800), then incubated with DAPI for 30' RT and finally mounted on slides with Mowiol 20%. Confocal microscopy was performed with a TCS-SP5 Laser Scanning System (Leica). Images of pyramidal neurons were acquired from *stratum radiatum* of hippocampal CA1. Analysis of dendritic spine

density was performed on secondary dendritic shafts of apical branches, starting 5 μm far from the branching point. Average of spine density was measured per 10 μm of dendritic length.

3.6 PTZ-induced seizures

Seizures were induced by intraperitoneal injections of 70 mg/kg pentylenetetrazol (PTZ, Sigma-Aldrich, St Louis) in WT and Cx3cr1$^{KO/KO}$ littermates at P17-18. Animal behaviour was videoscored for 10 minutes after injection: myoclonic and tonic-clonic convulsions were analysed offline by using The Observer XT Software (Noldus Information Technology, Wageningen, Netherlands). Latency to the first myoclonic jerk and to the first tonic-clonic seizure was scored, and duration of both responses was measured.

3.7 Behavioral Investigations

During juvenile period, WT and littermates Cx3cr1 homozygous knockout mice were tested for social behaviour and cognitive tests. All the behavioural experiments were performed before weaning and litters were housed with their dams. After each

test, mice were returned to their nest. Experiments were carried out in light cycle, at random time of the day between 10am and 6pm.

3.7.1 Homing Test

The homing test (see as reference Scattoni et al., 2010) exploits the tendency of juvenile pups to maintain body contact with the dam and their siblings, and requires adequate sensory (olfactory) and motor capabilities. It can also be considered a cognitive test because discriminative capabilities are necessary for the pup in order to recognize the mother's odor and discriminate it from others.

On P18, WT and homozygous littermates were separated from the dam for at least 30 min. Individual pups were then transferred to a Plexiglas arena (50 cm X 50 cm, walls 30 cm high), containing fresh bedding spread out overall the floor, except for one corner (defined as 'nest corner') which was covered with nest bedding. Each pup was initially placed on one corner, defined as 'starting corner' and for 3 minutes locomotion was videoscored. The arena was virtually subdivided into three areas (starting corner, central area and nest corner; Fig.1). The latency to leave the starting corner, the latency to reach the nest corner and the overall locomotion were measured offline by using the tracking Videomot Software. After 3 min of exploration, pups were exposed to two mesh tubes introduced in the arena and placed in two opposed corner: one empty tube and one other containing the dam. Each session, lasting 5

additional minutes, was videorecorded and manually scored offline. Pup behavior was analyzed and number of visits to the tubes, time spent sniffing each tube and number of entries in the nest corner were counted. Mice were exposed only once to the homing test.

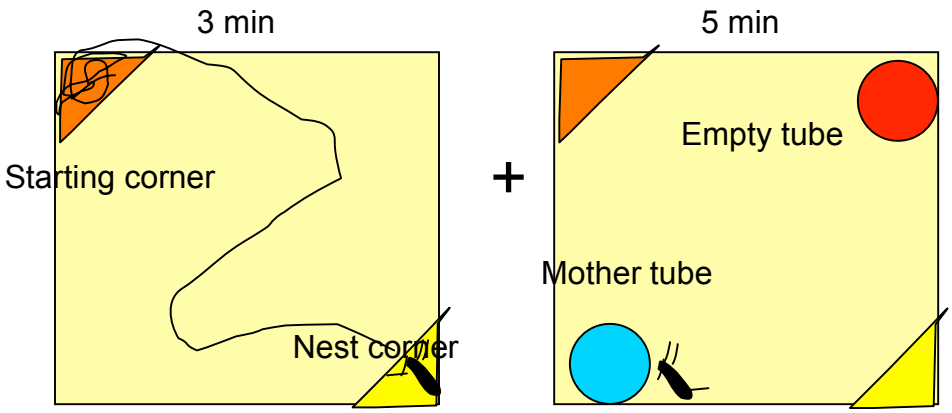


Fig.1 Apparatus for Homing Test: (left) habituation session for the first 3 min followed by (right) 5 min of social preference in presence of an empty plastic tube and a tube containing the mother.

3.7.2 Open Field and Object/odour Memory Test

For cognitive abilities and social memory investigation we set an 'Object/Odor Memory Test' (Fig.2), in which mice were tested for their ability to remember objects and social odours through consecutive sessions in an open field arena (50 cm x 50 cm). The test was made of five consecutive sessions, with the first one being a 5 minutes habituation to the context, thereby considered as an Open Field Test session. Mice were exposed then to two identical objects stained with adult male urine, for

three consecutive training sessions of 3 min each. In the fifth and last session, one familiar object was replaced with a novel one, different in shape and stained with novel male urine. Intervals between sessions lasted about 2 min, during which mice were kept isolated in a cage with fresh bedding. Arena and objects were cleaned at the end of each session. Parameters such as 'latency to the center', 'time in the center', 'total distance' 'distance in the center' and 'time spent grooming' were scored for the Open Field Test. Time spent exploring the objects across sections was scored in the Memory Test.

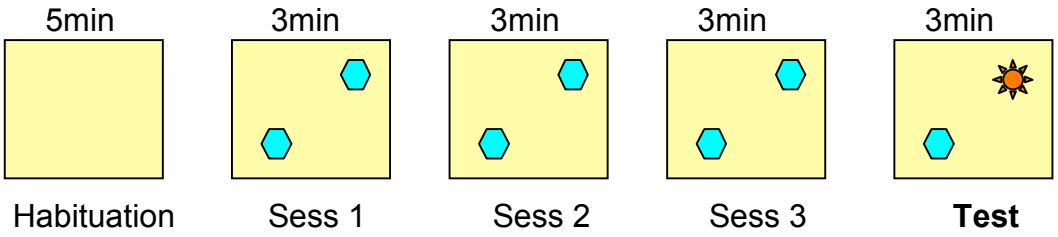


Fig.2 Object/Odor Memory Test, made of one habituation session (Open Field Test lasting 5 minutes), three training sessions of exposure to 2 objects identical in shape and odor, and a final Test session, where one familiar object was replaced with a novel one.

3.8 Statistical Analysis

All data are shown as mean ± SEM. Effects of genotype were analyzed by t-test, or Mann-Whitney test in cases of non-normal distribution. Survival curve comparison for PTZ experiments was analysed by Gehan-Breslow-Wilcoxon Test.

4. Results

4.1 Morphological reconstruction of microglial cells

Morphometric analysis of single microglial cells from *in vitro* fixed slices revealed a significant effect of genotype on surface area and volume measurements. Two-dimensional projection of microglia by using Imaris Software (Fig.3) shows that $Cx3cr1^{KO/KO}$ cells have, in fact, reduced size compared to controls.

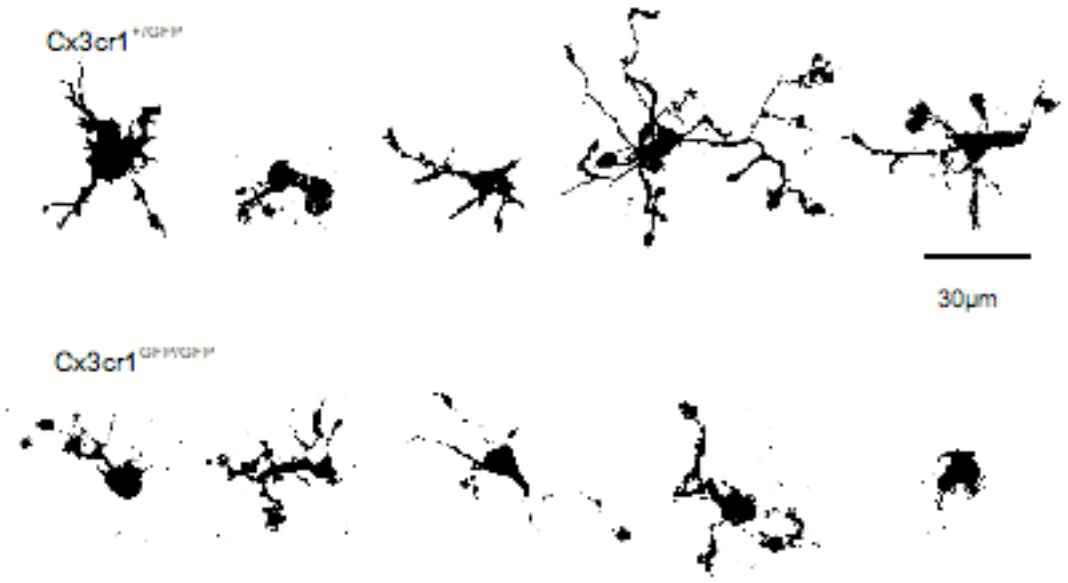


Fig.3 Representative confocal z-stack projection of microglial cells from control and *Cx3cr1* homozygous knockout mice, in the CA1 of hippocampal *stratum radiatum* at P7-9, processed with ImageJ Software.

Three-dimensional reconstructions of these cells and relative morphometric analysis revealed that Cx3cr1$^{KO/KO}$ cells have reduced surface area and decreased volume compared to control, either at P7-9 (Fig. 4) and at a later time point P40-43 (Fig.5).

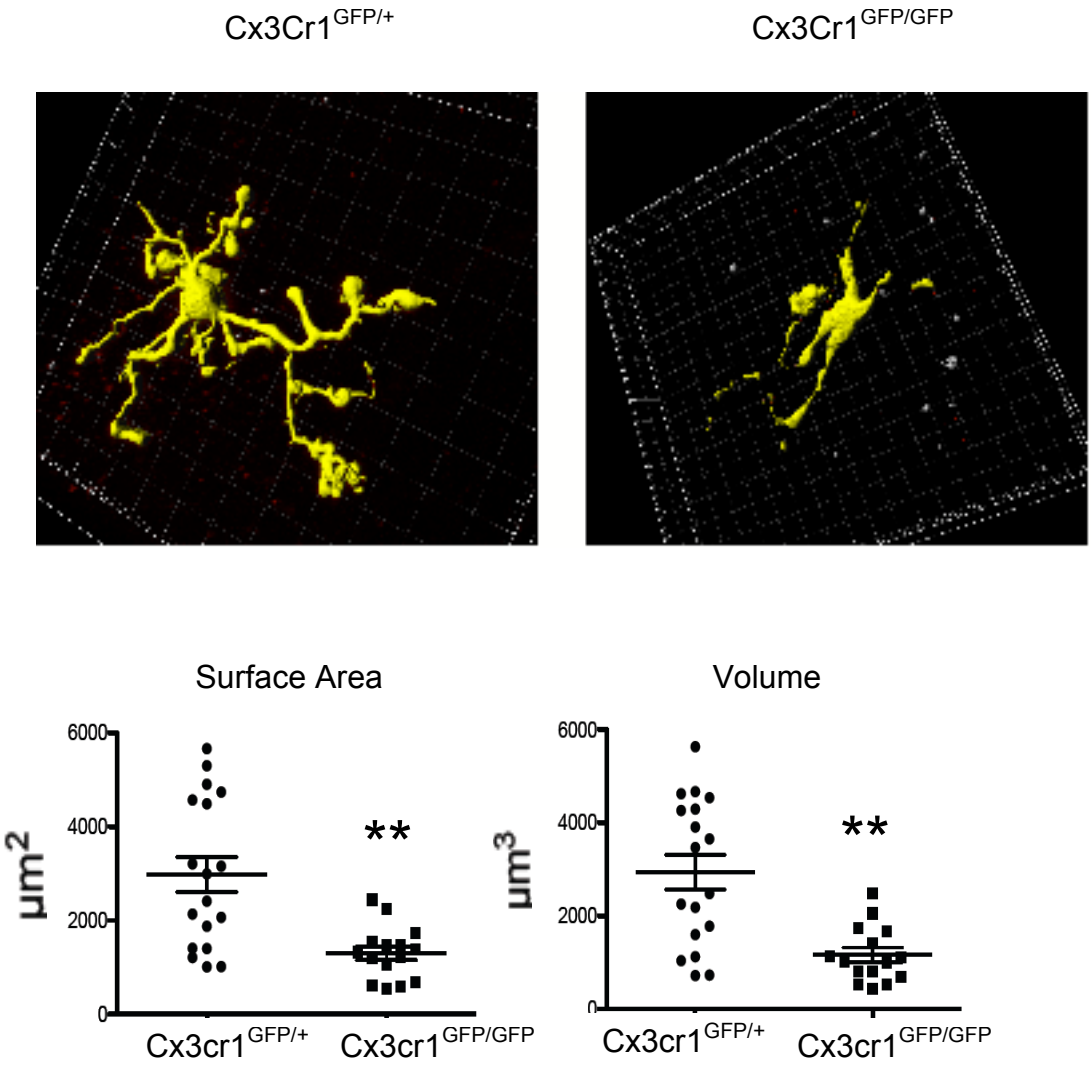


Fig.4 P7-9, 3D reconstruction and morphometric analysis of microglial cells in the *stratum radiatum* of hippocampal CA1 by Imaris Software. Area Surface Control: 2976 ± 375.2 µm^2 vs. Cx3cr1$^{GFP/GFP}$: 1303 ± 146.5 µm^2; Volume Control: 2941 ± 370.9 µm^3 vs. Cx3cr1$^{GFP/GFP}$: 1161 ± 154.8 µm^3; Control N=18; Cx3cr1$^{GFP/GFP}$ N=15. ***, P<0.0005

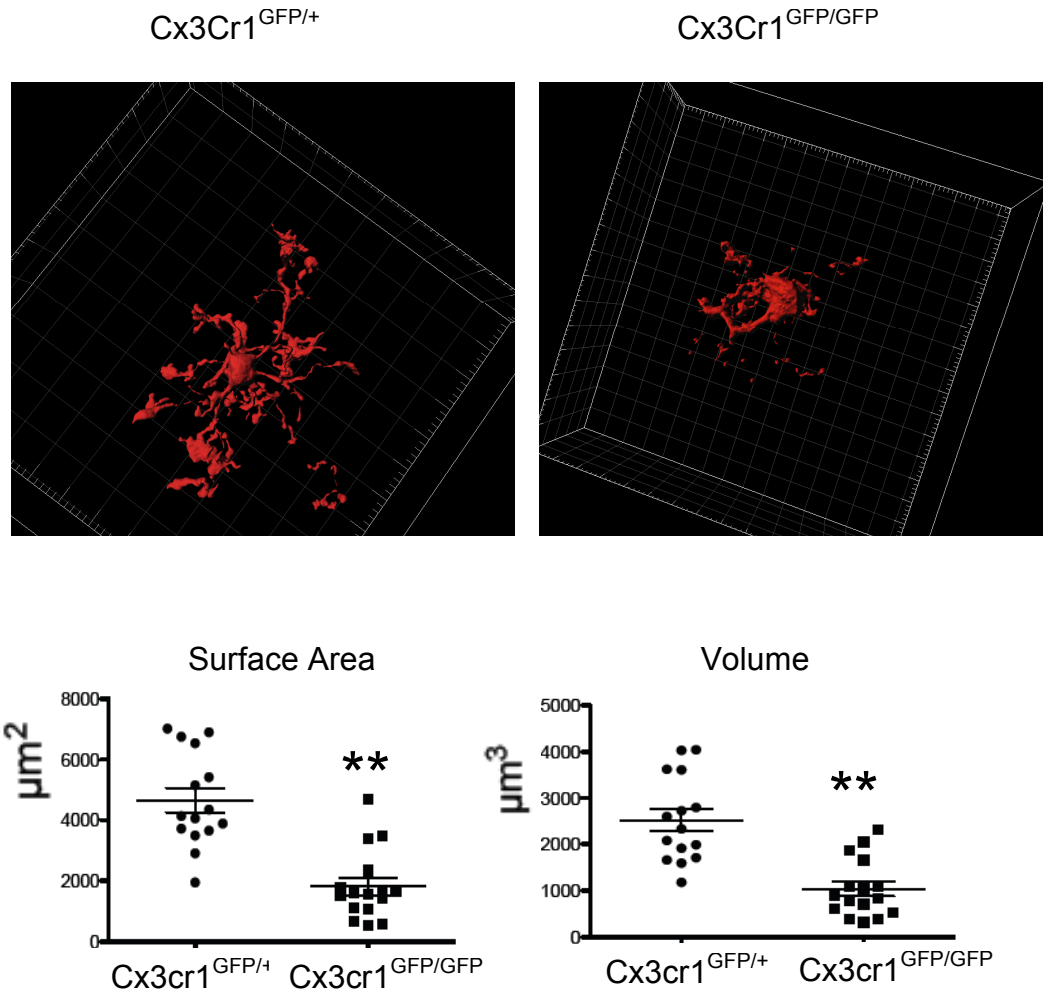


Fig.5 P40-43, 3D reconstruction and morphometric analysis of microglial cells in the *stratum radiatum* of hippocampal CA1 by Imaris Software. het N=18; homo N=15. ***, P<0,0001

4.2 Microglia density analysis

Investigating microglial distribution in the *stratum radiatum* of CA1 hippocampus, we observed a significant reduction in density of these cells in

Cx3cr1$^{KO/KO}$ mice compared to their littermates control, at P8 (Fig. 6A-C). However, this deficit was not any longer observed at a later time point; at P40, in fact, the density of microglia in knockout mice was comparable to control (fig.6D).

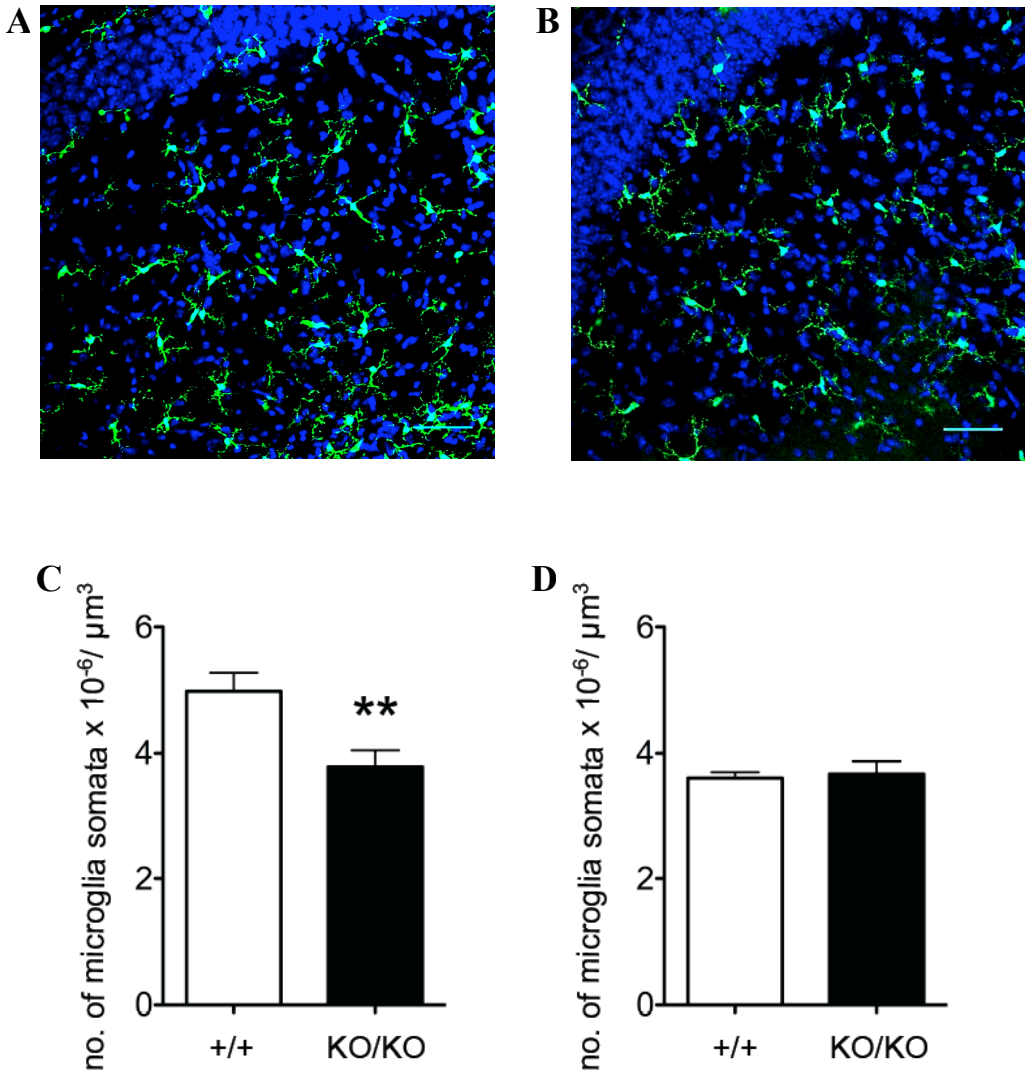


Fig.6 Representative low resolution confocal images of CA1 stratum radiatum taken from the hippocampus of (**A**) a *Cx3cr1*$^{GFP/+}$ reporter mouse (KO/+) and (**B**) a *Cx3cr1*^{GFP}/*Cx3cr1*^{KO} reporter knockout mouse (KO/KO) at P8 revealed extensive arborization of microglia (GFP-immunoreactivity) across the brain parenchyma. (**C**) Quantification of microglia nuclei (DAPI$^+$, GFP$^+$) at P8 revealed a significant decrease in

microglia density in *Cx3cr1* knockout mice ($3.79 \pm 2.56 \times 10^{-6}$, $n = 7$ mice, sections = 28) compared to control littermates ($4.98 \pm 0.31 \times 10^{-6}$, $n = 8$ mice, sections = 25) ($P = 0.0044$). Data are shown as number of microglia somata per μm^3 (** $P < 0.005$). (**D**) Microglia density in *Cx3cr1* knockout mice was then normalized at postnatal day 40 (P40), $P = 0.7$.

4.3 Microglia contact and engulf synaptic material: anecdotal evidence

4.3.1 Conventional Confocal Microscopy

We have performed different experiments in order to show how microglia processes get in contact with synaptic terminals. In first place, we analysed hippocampal slices from mice expressing GFP both in neurons and microglia (Thy1::GFP/+;Cx3cr1GFP/+). By using confocal microscopy and consequent image processing, we provided anecdotal evidence showing points of contacts between microglial branches and synapses at P15 (Fig.7). Interestingly, microglial tips preferentially make contacts with synaptic terminals rather than with the dendritic shaft: dendritic spines and axonal butouns, in fact, appear to be specifically touched by microglia.

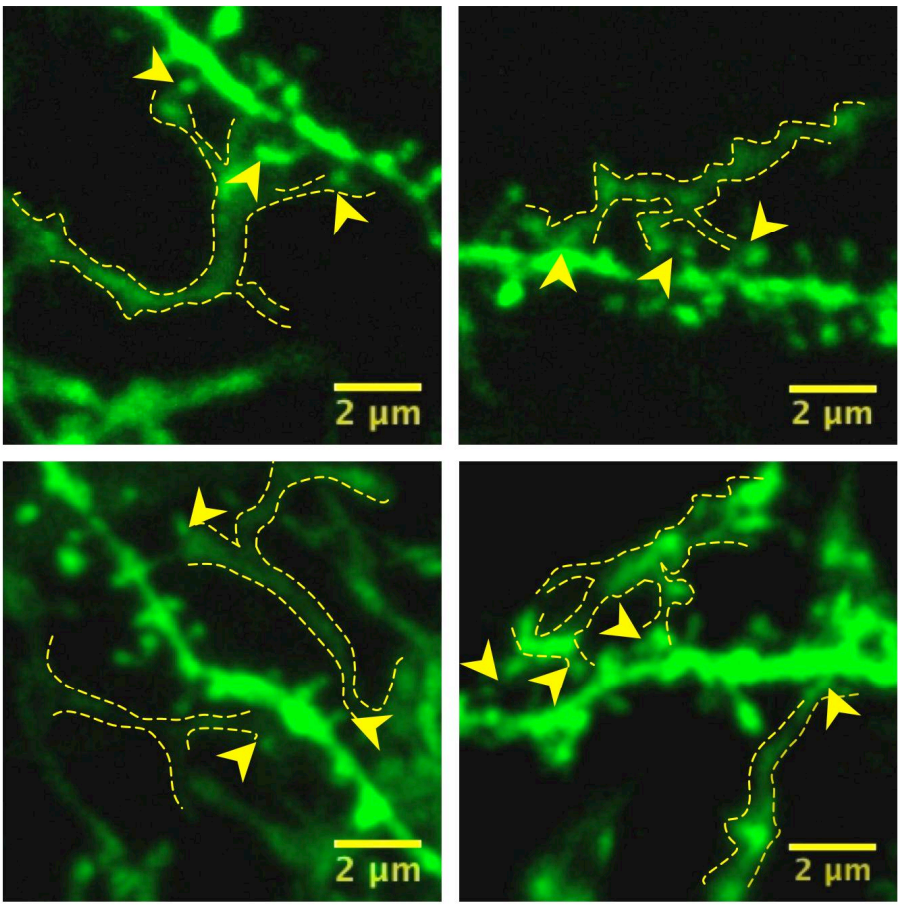


Fig.7 Representative example of microglia and neurons GFP-positive; yellow arrows indicate point of contacts between microglia and post-synaptic dendritic spines.

Double staining of hippocampal slices with GFP antibodies and synaptic markers, either pre-synaptic (Synapsin and SNAP25) or post-synaptic (PSD95), have been performed, in order to investigate contacts and possible event of engulfment.

Synapsin

Synapsins are a family of presynaptic proteins involved in regulating vesicles release at synaptic sites. Our staining in CA1 and CA3 area of hippocampus appears to be punctate and particularly intense in the hippocampal *stratum lucidum*, where big

mossy fibers of dentate gyrus granule cells project onto pyramidal neurons of CA3 (Fig.8A). Three-dimensional reconstruction shows that microglial processes can tightly contact pre-synaptic material stained with synapsin (Fig.8B) and can occasionally engulf it (Fig.8C).

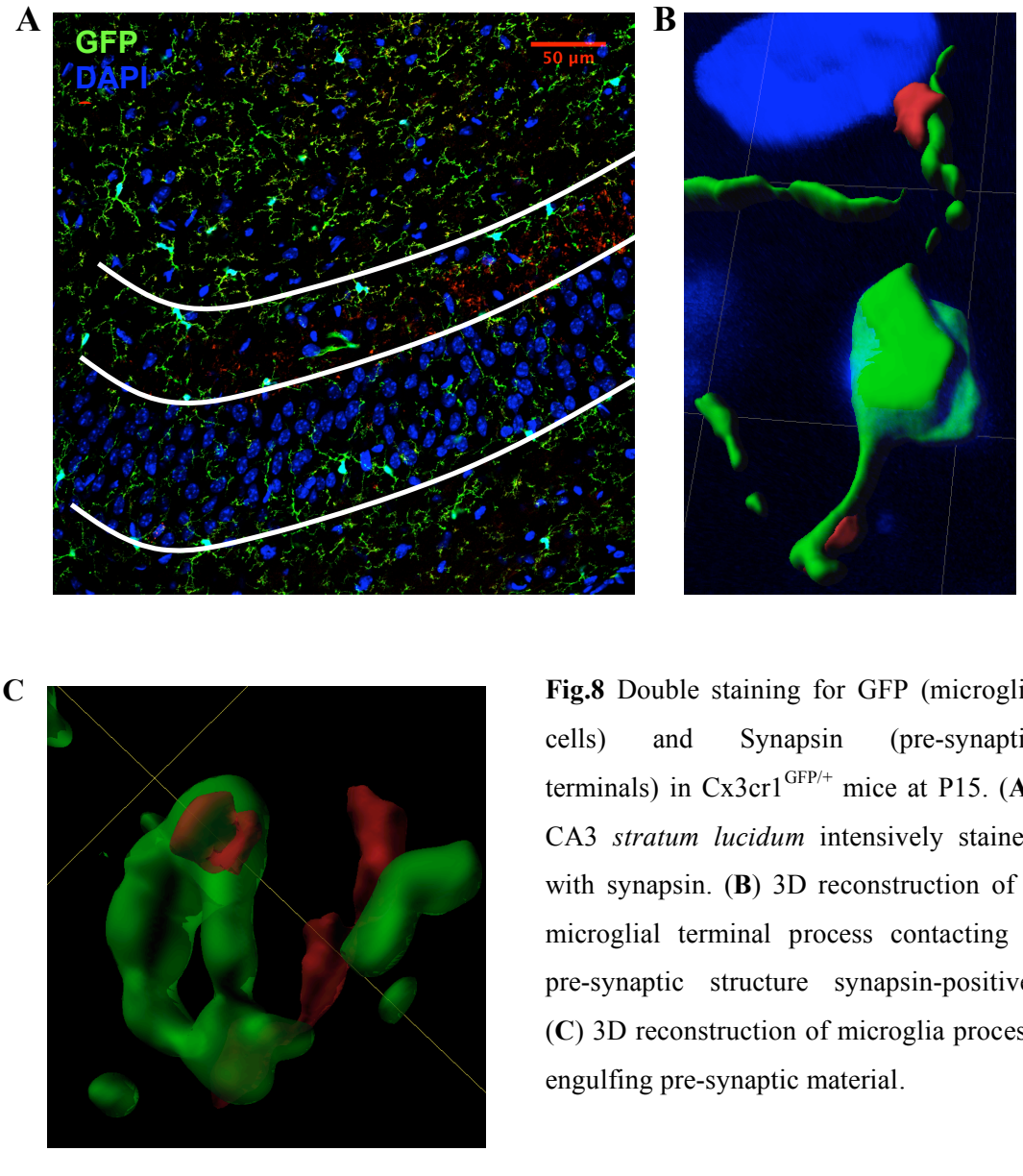


Fig.8 Double staining for GFP (microglia cells) and Synapsin (pre-synaptic terminals) in Cx3cr1$^{GFP/+}$ mice at P15. (**A**) CA3 *stratum lucidum* intensively stained with synapsin. (**B**) 3D reconstruction of a microglial terminal process contacting a pre-synaptic structure synapsin-positive. (**C**) 3D reconstruction of microglia process engulfing pre-synaptic material.

PSD95

Post-synaptic density 95 (PSD95) is a scaffold protein located at dendritic spines of excitatory synapses. We observed frequent contacts between microglia processes and PSD95 positive puncta in *stratum radiatum* of hippocampal CA1. Occasionally we observed also that a small but significant number of PSD95 puncta were colocalized with GFP (Fig.9A). Importantly, 3D reconstruction demonstrated that many PSD95 puncta that colocalized with GFP were entirely surrounded by microglial material (Fig.9B) consistent with the intracytoplasmic localization of PSD95, possibly in an endosomal or lysosomal compartment. These data show that in the resting brain microglia actively engulf synaptic material.

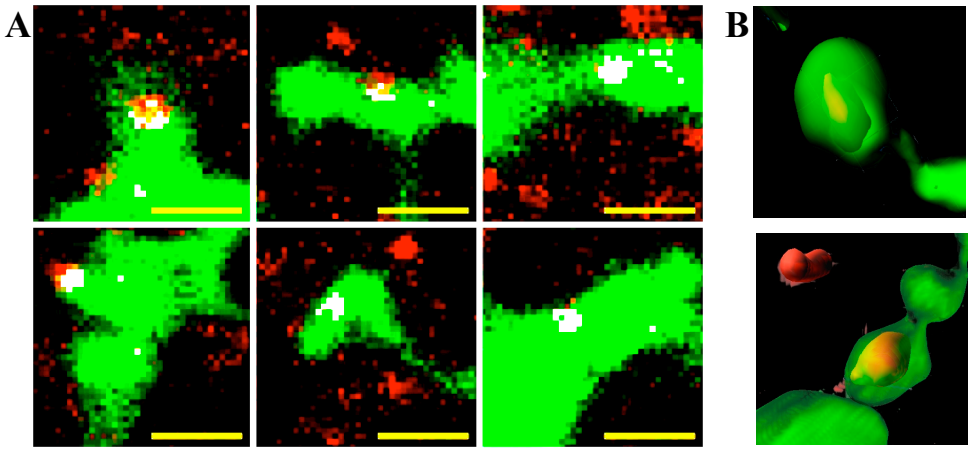


Fig.9 A) Representative high magnification images of single colocalization puncta (white color) between PSD95-immunoreactivity (red) and GFP fluorescence, determined in single consecutive confocal planes (0.4µm Z step-size), with each volume scanned (sections 1.2 µm thick); scale bar: 1µm; **B**) three dimensional reconstructions showing PSD95-immunoreactivity completely inside small diameter microglia processes.

4.3.2 Evidence of engulfment by STED Microscopy

Anecdotal evidence of engulfment obtained by using conventional confocal microscopy was confirmed by STED microscopy. This technique, first proposed by Stephan Hell in the nineties (Hell and Wichmann, 1994) and based on the employment of a scanning fluorescence microscope, allows us to dramatically increase the resolution by inhibiting the fluorescence in the outer region of the point-spread function (Nägerl and Bonhoeffer, 2010). By literally switching off the fluorophors, only the innermost region of the main maximum of the point-spread function contributes to the fluorescence signal. In our brain sections, a single PSD95 immunoreactive puncta, that in confocal images had a diameter of about 400 nm, could be resolved up to 80 nm by STED microscopy (Fig.10).

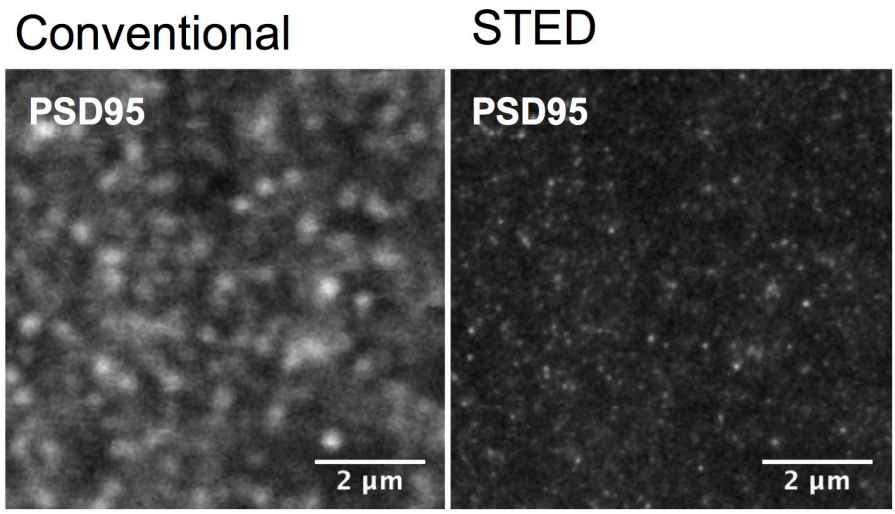


Fig.10 Comparison of conventional (left) and STED (right) scansion of the same single-plane image of P15 CA1 *stratum radiatum* showing localization of PSD95-immunoreactive puncta (scale bar: 2 μm).

Examples of colocalization between PSD95 immunoreactive puncta and GFP-positive microglial processes were observed in *stratum radiatum* of CA1 hippocampal slices of P15 mice, also by using STED microscopy (Fig11).

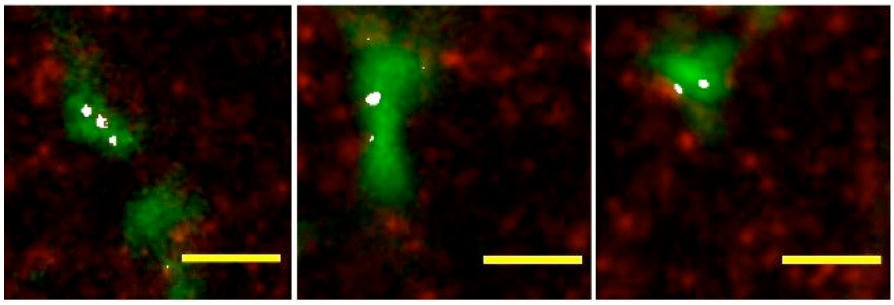


Fig.11 Representative stimulated emission depletion (STED) microscopic single plane images confirming colocalization of PSD95 puncta and GFP immunoreactivity in *stratum radiatum* of control mice (scale bar: 1 μm).

Colocalization between PSD95 puncta and GFP-positive microglia was investigated in single confocal planes and consecutive planes were analyzed, in order to show that in the planes above and below the colocalization only the GFP signal was present, but not the PSD95. This confirms that the PSD95 immunoreactive puncta is actually inside the microglia process (Fig.12), because the signal disappears in the planes above and below the colocalization.

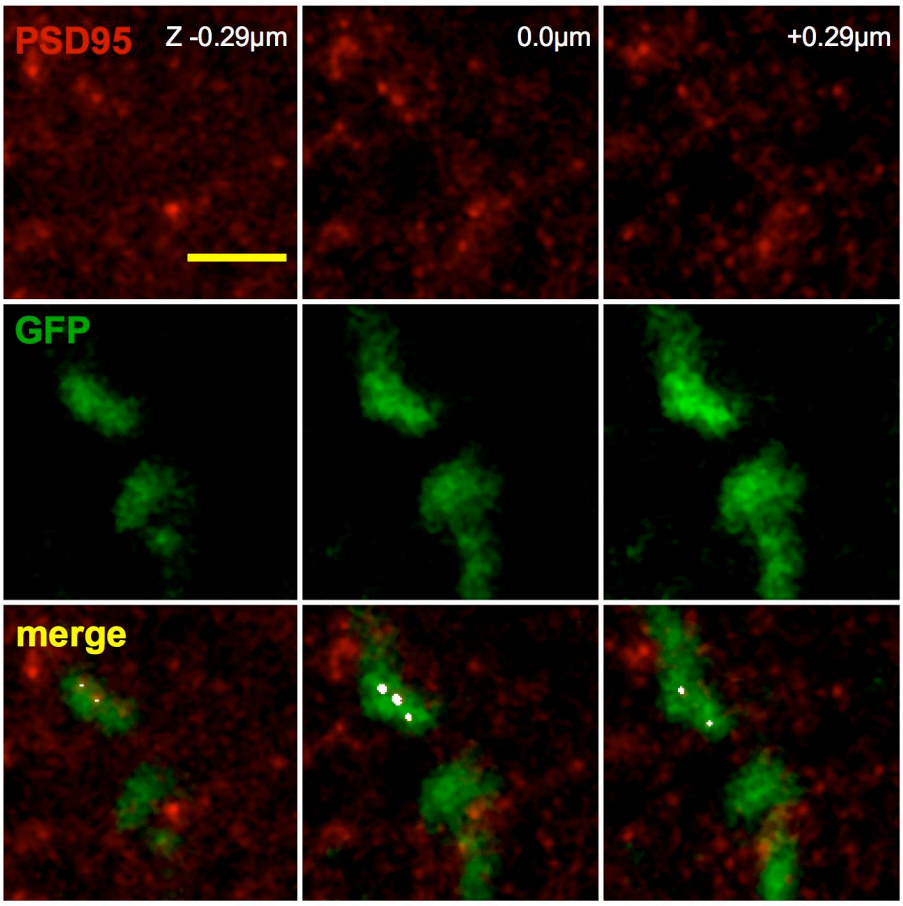


Fig.12 Representative STED image showing colocalization of PSD95 puncta and GFP-positive microglia processes in *Cx3cr1*$^{GFP/+}$ mice at P15 in three consecutive confocal planes (left column: Z = -0.29 μm, center column: Z = 0.0 μm, right column: Z = +0.29 μm). Separate and merged fluorescent channels (PSD95-positive and GFP-positive) are shown (scale bar: 1 μm).

4.3.3 Evidence of engulfment by Electron Microscopy

Electron microscopy was employed for further confirmation of anecdotal examples of engulfment. Cryoimmunolabeling allowed us to stain hippocampal ultrathin sections for PSD95 and for GFP, clearly showing that PSD95 positive gold particles were labelling electron dense structures located at the post synaptic density of dendritic spines (Fig.13A), whereas GFP positive gold particles were labelling microglial cells (nuclear and cytoplasmic localization, Fig.13B).

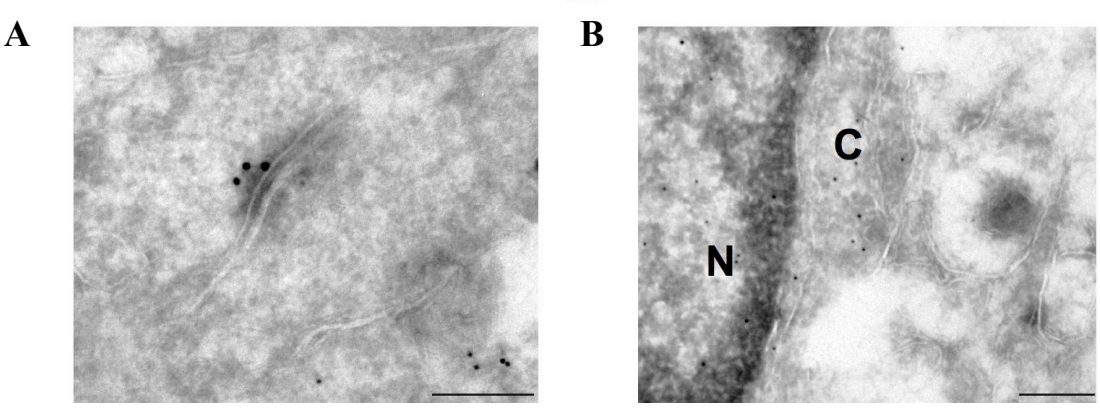


Fig.13 Representative electron micrograph showing (**A**) a PSD95-immunoreactive (15 nm gold particles) electron dense synaptic structure and (**B**) GFP-immunoreactive (10 nm gold particles) nucleus (N) and cytoplasm (C) of a microglia cell.

PSD95 immunoreactive gold particles were occasionally found surrounded by GFP-positive label, confirming our hypothesis that microglia could engulf synaptic material (Fig.14).

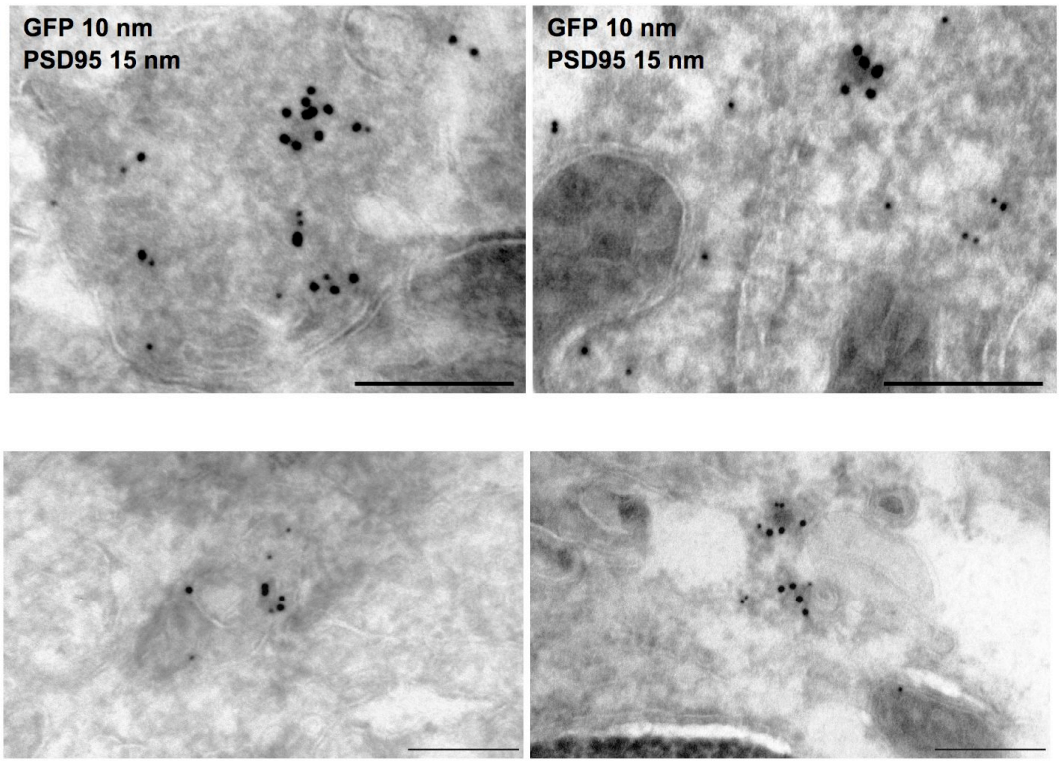


Fig.14 Representative electron micrographs showing PSD95-immunoreactive (15 nm gold particles) electron dense structures surrounded by sparse GFP-immunoreactive (10 nm gold particles) microglia processes (scale bar: 250 nm).

4.4 Quantification of engulfment events and PSD95 density

In order to evaluate the role of microglia in the process of dendritic spine pruning, we decided to compare the frequency of engulfment events (defined as point of colocalization) in *stratum radiatum* CA1 between control mice and littermates with compromised microglial function ($Cx3cr1^{KO/KO}$). We found that microglia with

impaired function showed a reduction in engulfment (Fig.15A). These data correlate with the observation that overall immunoreactive puncta are increased in knockout brains (Fig.15B).

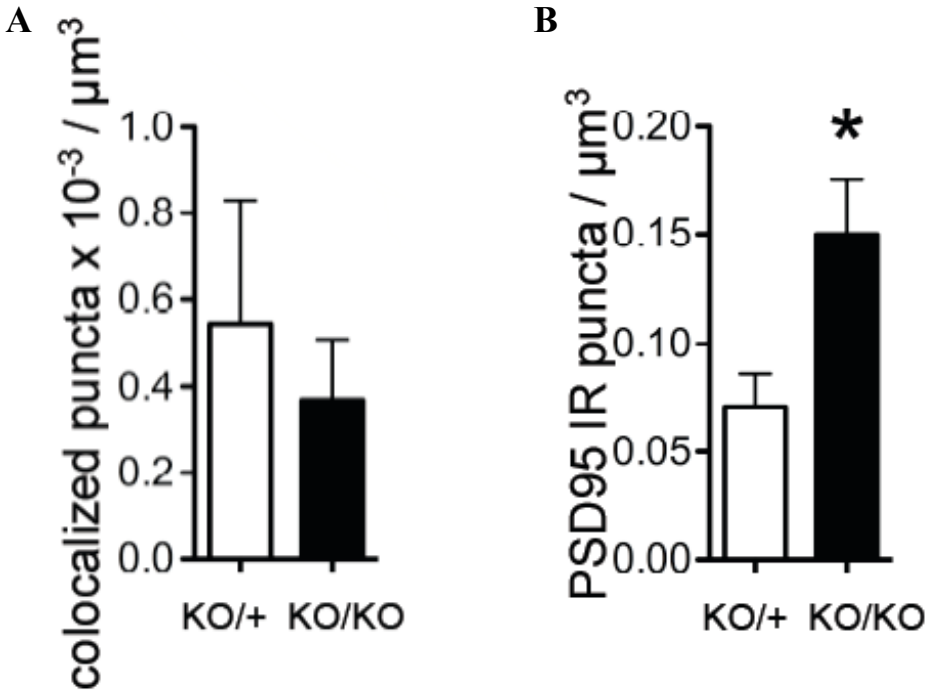


Fig.15 A) Quantification of absolute density of colocalization puncta revealed a trend for a decrease in *Cx3cr1* knockout mice ($0.37 \pm 0.14 \times 10^{-3}$, $n = 3$ mice, sections = 15) compared to littermate controls ($0.64 \pm 0.29 \times 10^{-3}$, $n = 3$ mice, sections = 14). (**B**) Quantification of absolute density of PSD95-immunoreactive puncta revealed a significant increase in *Cx3cr1* knockout mice (0.07 ± 0.02, $n = 3$ mice, sections = 14) compared to littermate controls (0.15 ± 0.02, $n = 3$ mice, sections = 15) ($P = 0.0146$). Data are represented as mean ± SEM (* $P < 0.05$).

4.5 Dendritic spine density analysis

In order to evaluate the real impact of microglia impairment on synaptic pruning we investigated the dendritic spine density of hippocampal pyramidal neurons comparing WT and littermates Cx3cr1$^{KO/KO}$ mice, during synaptogenesis, at P15. We focused our attention on secondary dendritic shafts of apical branches in the CA1 *stratum radiatum* (Fig.16). Thy1-GFP-M mosaic transgene mouse line was used, crossed to the general knock-out for fractalkine receptor. By analysing secondary apical dendrites, we observed a significant increase of dendritic spine density in Cx3cr1 knockout mice compared to wild-type littermates (Fig.17).

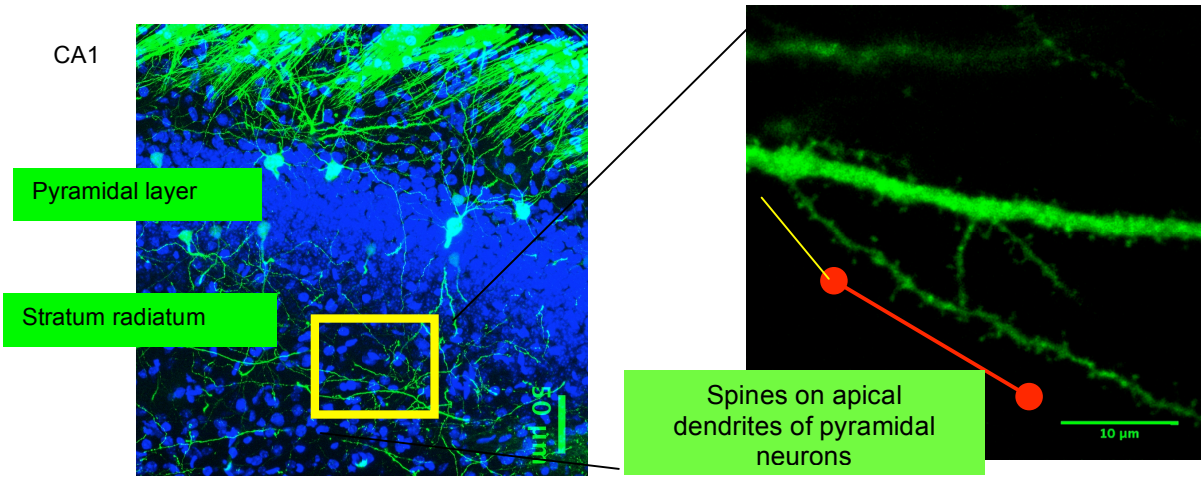


Fig.16 (left) representative example of hippocampal CA1 stratum radiatum of a Thy1::GFP/Thy1::GFP mouse and apical dendritic branches of a pyramidal neuron (yellow box) scale bar 50µm; (right) representative secondary apical dendritic shaft for spine density analysis; scale bar 10µm.

To investigate if the impairment in synaptic pruning was a transient or permanent defect, we analysed dendritic spine density also in adult animals (Fig.18). We observed no differences between adult KO ((15.14 ± 0.27, *n* = 6 mice, segments = 34) and WT (15.13 ± 0.5, *n* = 6 mice, segments = 27) mice in the average number of dendritic spine per 10µm, in CA1 *stratum radiatum.*

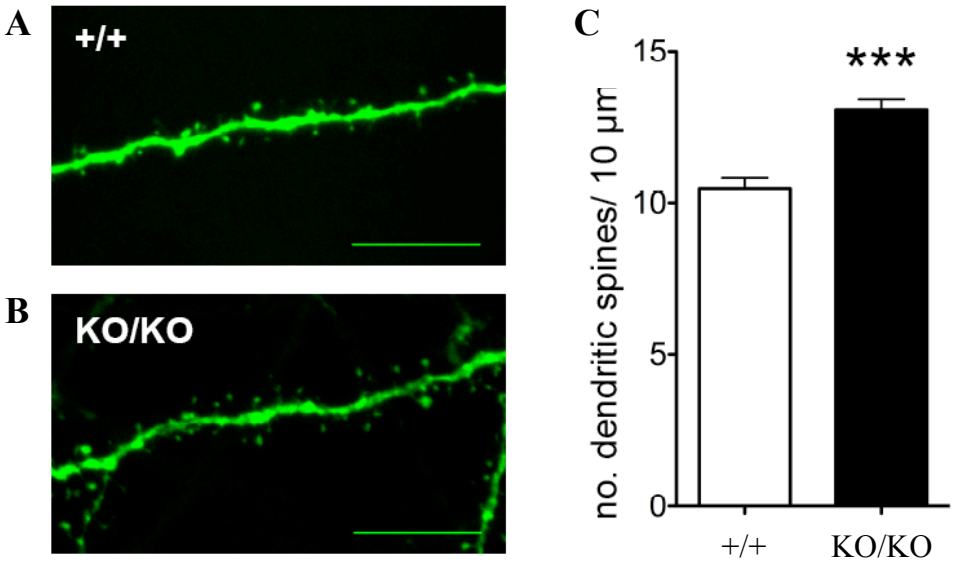


Fig.17. At P15 a significant increase in spine density was observed in segments from Cx3cr1 knockout mice (**B**) (13.05 ± 0.35, *n* = 6 mice, segments = 46) when compared to those from wild-type littermates (**A**) (10.47 ± 0.36, *n* = 6 mice, segments = 38). Number of spines per 10 µm (**C**) is indicated as mean ± SEM; *** P < 0.0001).

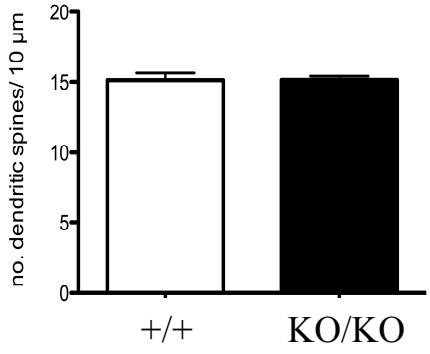


Fig.18 Dendritic spine density in adult was not different in Cx3cr1$^{KO/KO}$ mice when compared to those from wild-type littermates Number of spines per 10 μm is indicated as mean ± SEM.

4.6 Electrophysiological deficit in Cx3cr1$^{KO/KO}$ mice

To determine whether the failure to eliminate synapses during development might lead to altered neural circuit function, we performed (in collaboration with Dr. Davide Ragozzino, University of Rome, La Sapienza) single-cell recordings of spontaneous and miniature synaptic activity in CA1 pyramidal neurons from *Cx3cr1* knockout and wild-type littermates at P13-P16. While in wild-type mice spontaneous Excitatory Post-Synaptic Currents (sEPSC) were significantly greater than miniature EPSC (mEPSC), this was not the case in knockout littermates, suggesting immature connectivity. A small, but significant increase in mEPSC amplitude seen in the knockout was also consistent with immature synapse function. Finally, as expected, the frequency of mEPSC events in knockout mice was significantly increased compared to control littermates confirming an increase in synaptic release sites as a result of deficient pruning.

Next, we examined synaptic plasticity in Schaffer collateral inputs to CA1. Long-term depression (LTD) was significantly enhanced in slices from knockout mice when compared to control littermates at P13, but not at P40, suggestive of a transient deficit, likely a delay, in circuit maturation. All the electrophysiological data are personal communication of Dr. Ragozzino.

4.7 Brain excitability: differences in response to PTZ

In order to investigate the excitability of neural circuits during development, susceptibility to the proconvulsant drug pentylene tetrazole (PTZ) was measured in wild-type and *Cx3cr1*$^{KO/KO}$ littermates at P17-18 (Fig.19).

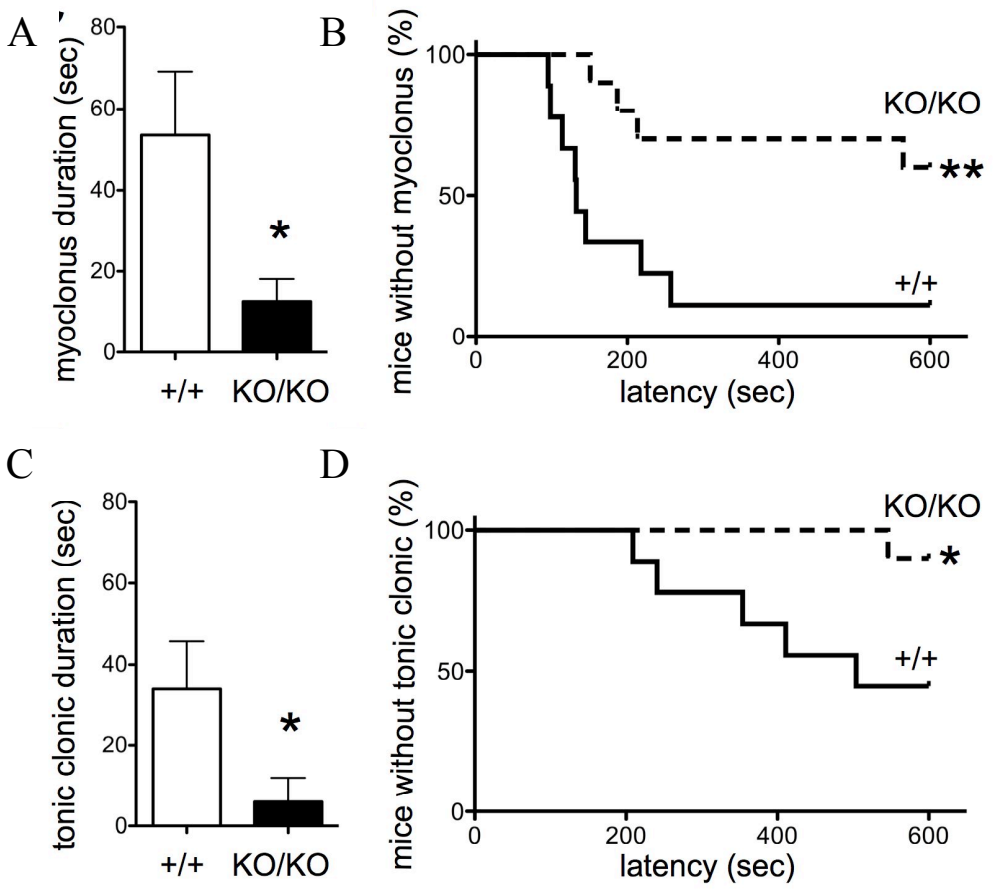


Fig.19 Response to PTZ. A significant reduction in, respectively, the duration of and latency to (**A-B**) myoclonic and (**C-D**) tonic-clonic convulsions was found in knockout mice when compared to wild-type littermates (WT: n = 9; KO: n = 10). Survival curve comparison for myoclonic jerks (**B**) and tonic-clonic seizures (**D**), analyzed by Gehan-Breslow-Wilcoxon test, revealed a significant difference between genotypes (* $P < 0.05$; ** $P < 0.005$).

The analysis of responses to PTZ revealed that Cx3cr1$^{KO/KO}$ mice displayed significant shorter duration of myoclonic and tonic-clonic convulsions and that, moreover, the latency to the first manifestation for both the events was significantly higher.

4.8 Behavioural investigation in juvenile Cx3cr1$^{KO/KO}$ mice

Homing Test analysis, aimed at investigating social behaviour of juvenile mice (P16-P17) revealed that Cx3cr1$^{KO/KO}$ were not able to discriminate between social and inanimate stimulus, compared to their wild-type littermates, which, on the contrary, spent significantly more time exploring the tube containing the dam than the empty tube (Fig.20).

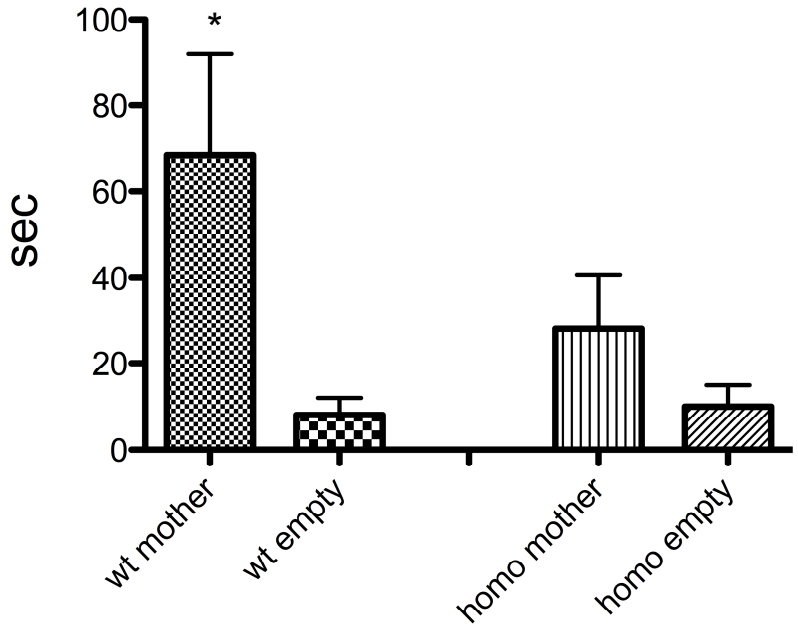


Fig.20 Homing Test; time of exploration (sec) of tubes during the test

Object/Odour Memory Test showed no deficit in novel object and novel odour recognition, with both Cx3cr1$^{KO/KO}$ and wild-type littermates spending significant higher time in exploring the novel object during the test session (Fig.21).

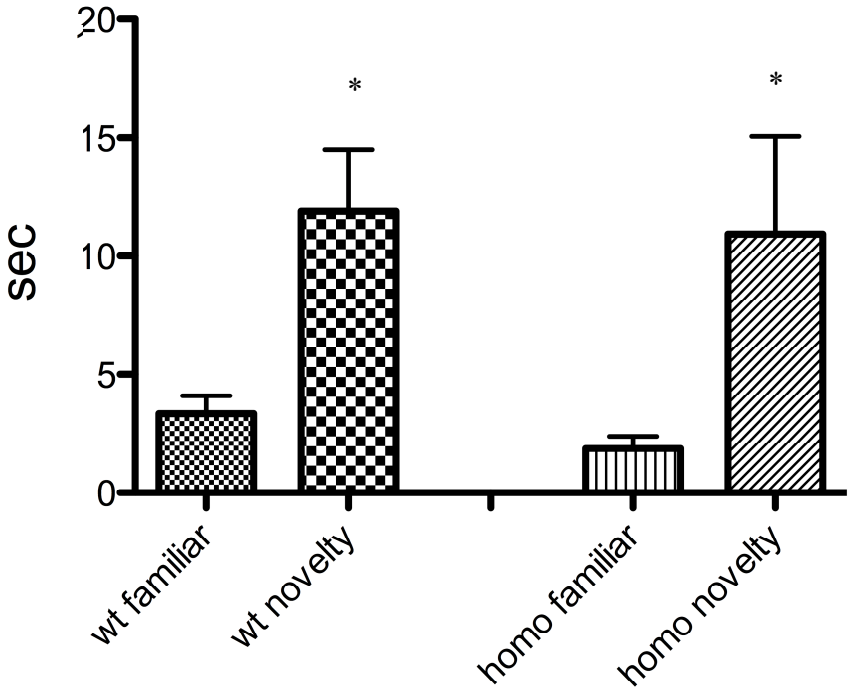


Fig.21 Novel Object/Odour Test: time spent exploring a familiar vs novel object during the test session (sec).

Open Field Test analysis revealed a trend for Cx3cr1$^{KO/KO}$ for reduced locomotion compared to wild-type littermates, as shown by the total distance data. A trend for increased latency to the centre was also observed for knockout mice, but not differences were recorded for time spent into the centre (Fig.22).

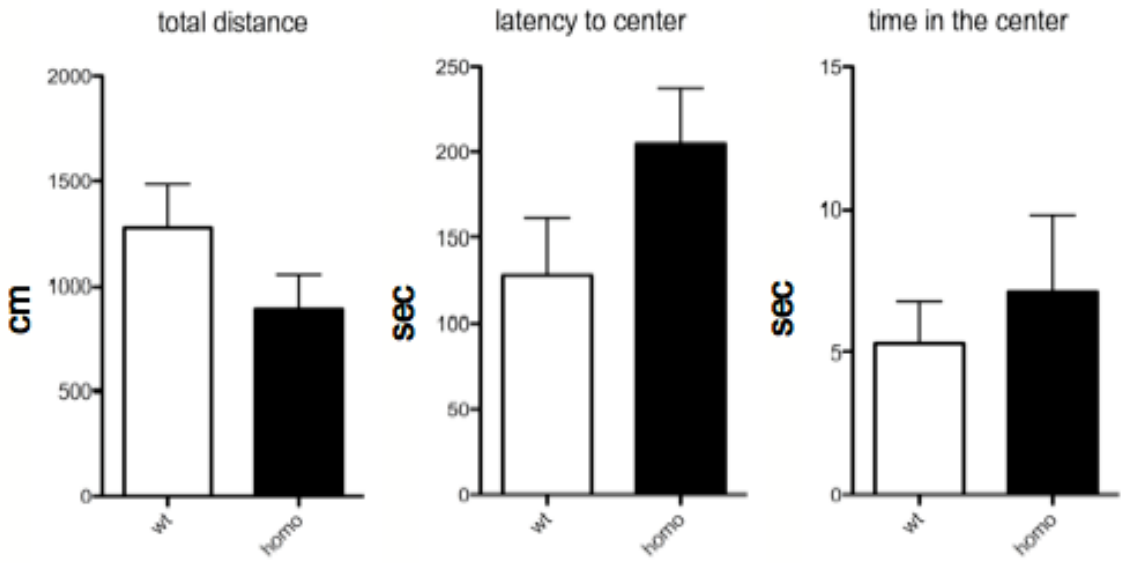


Fig.22 Open Field Test: parameters scored during 5 minutes in an open arena show a trend for reduced total distance and increased latency to the centre; no differences observed for total time spent into the centre.

Other parameters scored related to grooming behaviour revealed a trend for increase time of grooming and decrease latency to groom in Cx3cr1$^{KO/KO}$ compared to wild-type littermates (Fig.23).

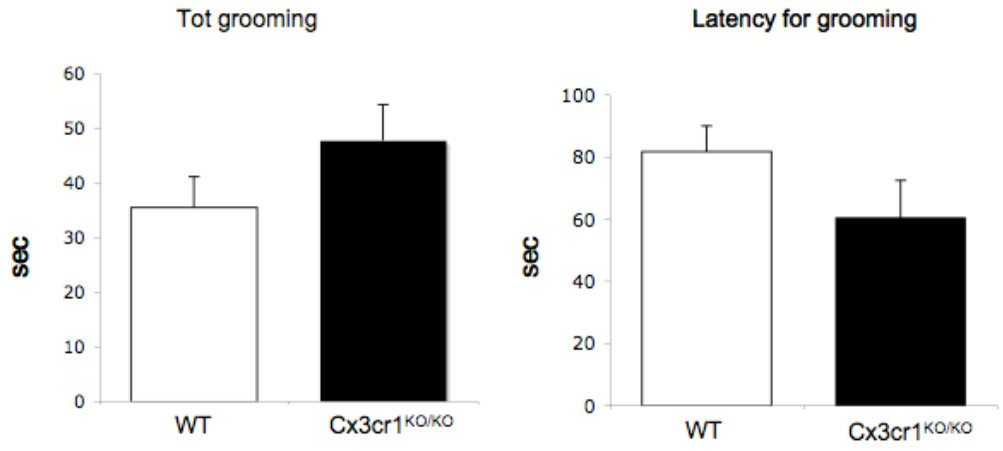


Fig.23 Analysis of grooming behaviour during 5 min Open Field Arena

4.9 C1q apposition to dendritic spines

Investigating a possible role for complement cascade as a mediator of synaptic pruning, we found C1q frequently in apposition to spines on secondary branches of CA1 pyramidal neurons. About 20% of spines analyzed in wild-type mice, in fact, were partially colocalizing with C1q immunoreactive puncta. However, no significant differences were recorded in Cx3cr1$^{KO/KO}$ littermates (Fig.24).

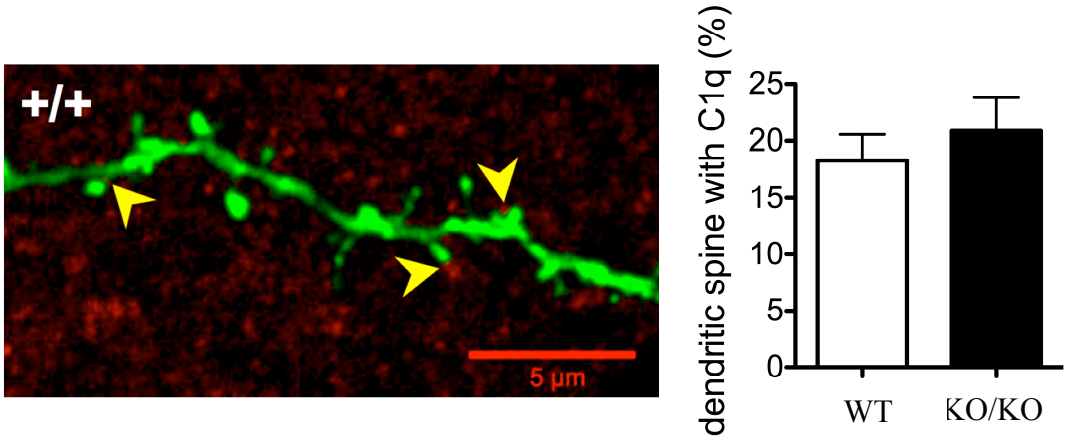


Fig24 (left) Representative confocal single-plane image of a CA1 pyramidal cell dendrite visualized in *Thy1::GFP/Thy1::GFP* mice showing apposition of C1q-immunoreactive puncta with dendritic spines (yellow arrows, scale bar: 5 μm); (right) percentage of dendritic spines found in apposition to C1q immunoreactiva puncta, on secondary apical dendrites from P15 wild-type (n = 13) vs Cx3cr1$^{KO/KO}$ (n = 14) pyramidal neurons.

Investigation of C1q immunoreactive puncta in double GFP mice (Thy1::GFP/+;Cx3cr1GFP/+) allowed us to observe that frequently C1q was found at points of contact between microglia terminal processes and dendritic spines (Fig.25).

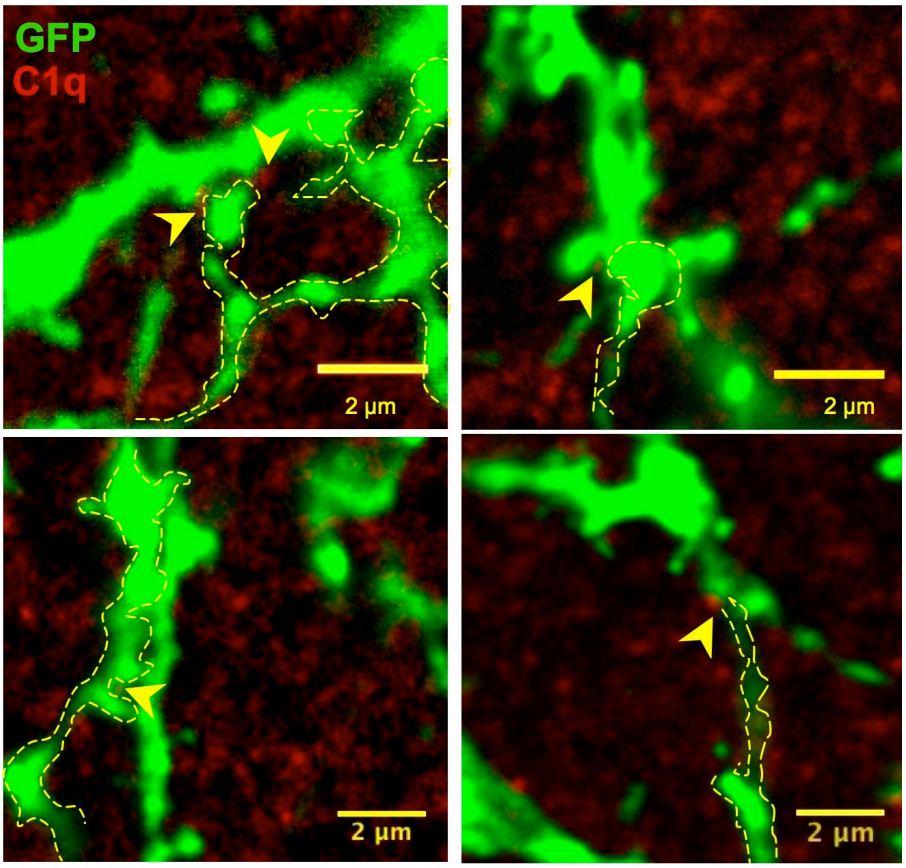


Fig.25 Representative single-plane confocal images of CA1 *stratum radiatum* of *Thy1::GFP/Thy1::GFP*;*Cx3cr1*$^{GFP/+}$ mice at P15 showing C1q-immunoreactive puncta closely apposed to points of contact (yellow arrows) between GFP-positive microglia terminal processes (dotted outline) and GFP-positive dendritic spines (scale bar: 2 μm).

4.10 Blood vessel extension quantification

Blood vessels extension in *stratum radiatum* area was analysed to rule out any possible developmental deficit in absence of fractalkine receptor, since the ligand is

also expressed by endothelial cells. Isolectin B4 staining, performed on coronal slices from WT and KO litermates mice at P15, showed that a compromised microglial function did not have consequences on blood vessels extension (Fig.26). The area positive to IB4 staining was quantified by using ImageJ Software (Fig.27).

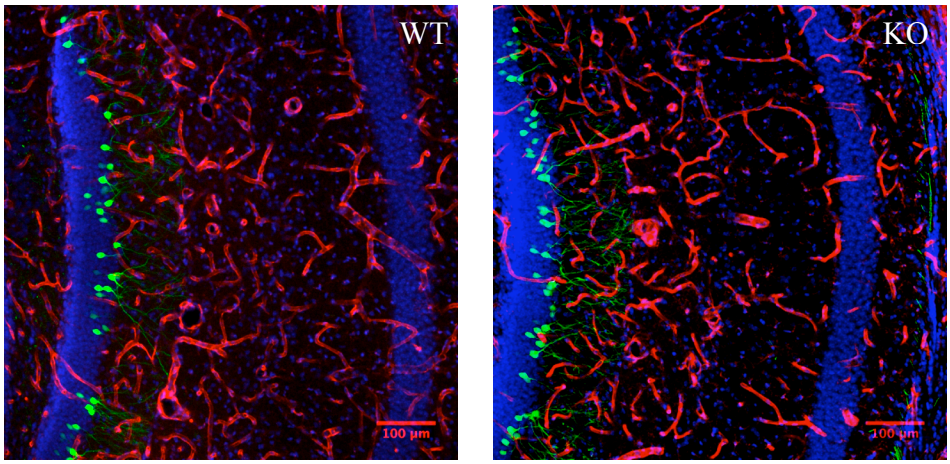


Fig.26 Representative z-stack projection images of CA1 *stratum radiatum* of *Thy1::GFP/Thy1::GFP;Cx3cr1*$^{+/+}$ and *Thy1::GFP/Thy1::GFP;Cx3cr1*$^{KO/KO}$ mice at P15 showing isolectinB4-immunoreactive (IB4) blood vessel-associated endothelial cells.

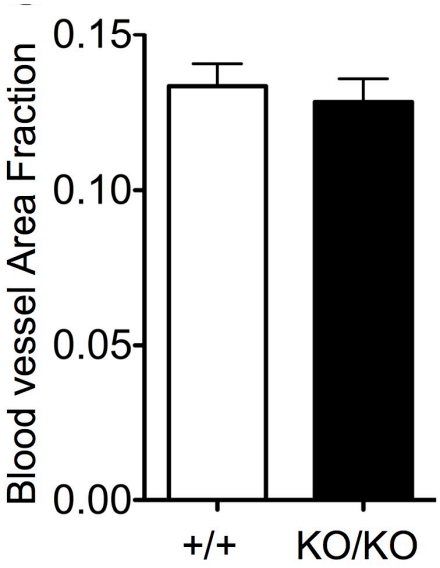


Fig.27 Quantitation of fractional IB4-immunoreactive projection area revealed similar blood vessel ramification in wild-type (+/+, n = 2, sections n = 4) and knockout (KO/KO, n = 2, sections n = 4) littermates.

5. Discussion

Microglia are considered the principal immune cells of the brain and their role in pathological conditions has been extensively studied. However, to date not much is known about their function in the intact brain. We propose here a new role for microglia during development, suggesting that they are necessary for normal synaptic pruning.

Our hypothesis was built on three main observations:

1. microglia cells infiltrate the brain parenchyma and go through a process of maturation limited in time, that strictly matches synaptogenesis (first two-three post-natal weeks) (Dalmau et al., 1998; Monier et al., 2006);
2. microglia cells and neurons display a strong bidirectional communication, as it is shown by particular signalling pathways, where the ligand is expressed by neurons and the specific receptor is present uniquely on microglia, like the chemokine fractalkine and its receptor Cx3cr1;
3. neurons show upregulation of the chemokine fractalkine during synaptogenesis, whose recptor is exclusively expressed by microglia cells.

All together, these hints led us to hypothesize a key role for microglia in synaptogenesis, specifically in synaptic pruning.

In first place, our characterization of mice lacking the fractalkine receptor ($Cx3cr1^{KO/KO}$) revealed that during development this signalling is required for normal morphological and electrophysiological maturation of microglia. Cells lacking the receptor were, in fact, significantly smaller in size compared to controls and exhibited

deficits in electrophysiological properties (reduced Ca^{2+}-activated K^+ outward current; personal communication from Dr. Ragozzino). Moreover, microglia lacking the fractalkine receptor showed a significant reduction in density in CA1 *stratum radiatum* at P8.

If our hypothesis was correct, by employing such a model of microglial impaired function we would then have expected a deficit in synaptic pruning. Indeed, investigation of dendritic spine density in hippocampal *stratum radiatum* of P15 mice revealed a significant increase of spines in $Cx3cr1^{KO/KO}$ mice compared to wild-type littermates, suggestive of a determinant role for microglia in synapses elimination.

Moreover, single-cell recordings of spontaneous and miniature synaptic activity in CA1 pyramidal neurons from mice lacking the fractalkine receptor revealed an altered neural circuit function. While in wild-type mice, in fact, sEPSC currents were significantly greater than mEPSC currents (as a normal feature of development), this was not the case in knockout littermates, suggesting immature connectivity. A significant increase in mEPSC amplitude in the knockout was also consistent with immature synapse function. Finally, the frequency of mEPSC events in knockout mice was significantly increased compared to control littermates, in line with the observed increase in dendritic spines (increased synaptic release sites as a result of deficient pruning).

Supportive results came also from synaptic plasticity analysis in Schaffer collateral inputs to CA1. Long-term depression (LTD) was significantly enhanced in slices from knockout mice when compared to control littermates at P13, but not at P40. Increased LTD is a feature of immature hippocampal circuits *(*Wagner et al.,

1995*)* and these findings corroborate our single-cell data arguing for an increase in excitatory synapses with immature functional characteristics in the absence of fractalkine signalling. Importantly, the disappearance of differences in LTD later in development suggested a transient effect of this chemokine on microglia function.

We also examined susceptibility to seizures following administration of the pro-convulsant drug pentylene-tetrazole (PTZ) in P17-18 mice, as an index of neuronal excitability. Susceptibility to PTZ is significantly lower during the early postnatal period than in adulthood (Klioueva et al., 2001) and administration of PTZ (70 mg/kg) revealed a significant reduction in seizure frequency and duration in knockout mice when compared to wild-type littermates, consistent with immature brain circuit responses at the whole animal level.

Finally, behavioural investigation in juvenile mice lacking the fractalkine receptor revealed normal cognitive performances (as shown by Novel Object/Odour Recognition Test) but deficits in the ability to discriminate social stimuli from inanimate objects (Homing Test). These data supported an association between altered circuit connections, overnumerary dendritic spines and social behaviour impairments, resembling some features of autism spectrum disorders.

Two different mechanisms could explain how deficient fractalkine signalling in microglia leads to reduced synaptic pruning. On the one hand, soluble fractalkine might act to promote microglia migration into the brain or proliferation during development. In this case, the density of microglia would be reduced in the brain of *Cx3cr1* knockout mice and their capacity for synaptic pruning compromised. On the other hand, tethered or locally released fractalkine might be critical for microglia

recognition of synapses prior to or during engulfment in which case the density of microglia might be normal, but the efficiency of engulfment reduced. Consistent with the first hypothesis, we found a significant reduction in microglia density in the brains of *Cx3cr1* knockout mice compared to littermate controls at P8 with a return to normal densities by P40. This finding suggests that reduced synaptic pruning in these mice was likely due to a transient reduction in microglia surveillance and is consistent with normal dendritic spine density in adult mice.

It remains possible that synaptic deficits in *Cx3cr1* knockout mice are due to an effect of this chemokine receptor on general brain maturation rather than a specific deficit in microglia-mediated synaptic pruning. However, several data argue against such a general effect. First, the synaptic deficits we observe are not consistent with a simple delay in brain maturation. *Cx3cr1* knockout mice show increased spine density and functional excitatory synapses (mEPSC frequency) features that are normally seen later in development (Hsia et al., 1998). However, synaptic connectivity (sEPSC/mEPSC ratio), synaptic strength (mEPSC amplitude), plasticity (LTD), and excitability (PTZ-induced seizures) all point to more immature brain circuitries. This phenotype of exuberant immature synapses is best explained by a deficit in synaptic pruning. Second, the transient decrease in microglia density observed in *Cx3cr1* knockout mice matches the transient increase in spine density and LTD in these animals and suggests that pruning does occur in the knockout mice, but at a later developmental stage. Third, although fractalkine is expressed both by neurons and vascular endothelial cells in the brain (Imaizumi et al., 2004) and microglia are known to have a role in the formation of cerebral blood vessels (Checchin et al., 2006), the

absence of its receptor on microglia does not lead to remodeling of brain vasculature ruling out a general effect of fractalkine signalling on cerebral blood supply. Fourth, the relatively frequent occurrence of engulfed PSD95 puncta (~ 0.8%) suggests that microglia-mediated pruning is likely to be a major contributor to synaptic turnover during development.

However, the question about what signals guide microglia-dependent pruning still need to be elucidated. Although our data are consistent with fractalkine signalling being primarily important for controlling the abundance of microglia in the brain, it remains possible that fractalkine also has a role in local neuron-microglia signalling necessary for synaptic pruning. In particular, tethered and released fractalkine may serve different roles in neuron-microglia communication. Other candidates for such signalling include the complement cascade components C1q and C3 who were shown to be necessary for pruning of retino-thalamic axons during development (Stevens et al., 2007). Importantly, C1q knockout mice show reduced pruning of excitatory cortical synapses (Chu et al., 2010), a phenotype reminiscent of that of *Cx3cr1* knockout mice. Consistent with a role of C1q in synaptic pruning-related signalling, we observed C1q-immunoreactive puncta in close apposition to ~ 20% of dendritic spines at P15. In addition, C1q puncta could be seen apposed to points of microglia-spine contact in double transgenic *Thy1::GFP*/*Thy1::GFP*;*Cx3cr1*$^{GFP/+}$ mice. Studies testing the cell-autonomous function of and epistasis among these potential pruning signals will be necessary to address this issue.

6. Conclusion

In conclusion, we have provided the first direct evidence that microglia selectively engulf and eliminate synapses during development. In mice lacking *Cx3cr1*, a chemokine receptor exclusively expressed by microglia in the brain, microglia density was transiently reduced in the developing brain and synaptic pruning was reduced. Deficient synaptic pruning resulted in an excess of dendritic spines and immature synapses and was associated with a persistence of electrophysiological and pharmacological hallmarks of immature brain circuitry. Functional genetic variants in *Cx3cr1* have been reported in humans *(*Kimouli et al., 2009; Zhao et al., 2010*)* but to the best of our knowledge they have not been evaluated for association with neurological or psychiatric disorders. Our data suggest that such mutations along with environmental pathogens that impact microglia function may contribute to susceptibility to developmental disorders associated with altered synapses number, including autism, which is characterized by increased spine density. A detailed mechanistic understanding of microglia-mediated synaptic pruning is likely to lead to a better understanding of synaptic homeostasis and a deeper appreciation of interactions between the brain and immune system.

Project 2

A new juvenile chronic social stress paradigm as a model for early maltreatment in mice

1. Introduction

It is well known that environmental factors, particularly early adverse experiences, represent important risk factors for mental illness. However, not all the individuals experiencing adversity do fall ill, suggesting that genetic or other environmental factors can modulate susceptibility to adversity. Several robust gene-by-environment (GxE) risk factors for mental illness have recently been discovered in human association studies (Caspi et al., 2002; Caspi et al., 2003; Caspi et al., 2005; Rutter et al., 2006). Identifying genetic factors involved in GxE effects is a promising approach to uncover the molecular substrates underlying susceptibility for mental illness and could lead to the identification of new therapeutic targets. Animal models play an essential role as a platform to study the molecular mechanisms and neural circuits underlying such GxE risk factors (Moffitt et al., 2005). In this thesis work the development of a novel early social stress paradigm is proposed, useful for investigating the ability of targeted mutations to moderate the long-term effects of adverse early experiences in mice.

1.1 Early adverse experiences and risk for mental illness

Adverse experiences during the early postnatal period can permanently alter neural and behavioural indices of emotionality (Heim and Nemeroff, 2001; Holmes et

al., 2005; Bradley et al., 2008; Heim et al., 2010). Human studies have shown that psychosocial stress *per se* represents a risk factor for developing psychosis. Two studies from the British National Psychiatric Morbidity Survey have reported that adverse life events experienced during the preceding 6 months were associated with psychotic experiences in a sample of the general population (Johns et al., 2004; Wiles et al., 2006). Other evidences suggest that risk for psychosis increases with the accumulation of life events experienced (Shevlin et a., 2008). Several studies have focused on the long-lasting effects of adversity and on the developmental-programmed effects that these events can exert when occurring early in life (Widom, 1989; Dodge et al., 1990; Loeber & Stouthamer-Loeber, 1998; Barnow, 2001; Barnow et al., 2003; Penza et al., 2003; Barnow and Freyberger, 2003; Barnow et al., 2004). Data from these studies show that individuals with a history of childhood maltreatment (parental loss, child abuse, physical and emotional neglect) often show impulsive aggression, violent and ⁄ or criminal behavior, and antisocial personality symptoms.

Risk of becoming violent offenders and developing antisocial symptoms has been reported in boys experiencing abuse and neglectful parental care during childhood (Rutter et al., 1998; Widom et al., 1989). Moreover, the earlier children experience adversities, the more likely they are to manifest antisocial symptoms and mental disorders (Keiley et al., 2001). Literature on community violence shows associations between youth exposure to violence and aggression, and weaker correlation between exposure to violence and depression, anxiety, and post-traumatic stress disorder (Ng-Mak et al., 2004). Interestingly, from these studies it turns out that male adolescents

are more likely to become violent whereas female adolescents are more likely to show signs of depression (Latzman & Swisher, 2005; Schwab-Stone et al., 1995). In an evolutionary context, the development of such symptoms could be explained as an adaptive mechanism; specifically, the development of aggression in violent contexts has been called *pathological adaptation* (Schwab-Stone et al., 1995). Importantly, we have to consider that individuals exposed to the same adverse environment may develop different adaptive mechanisms and strategies (Cichetti & Rogosch, 2002). All together, this set of unique behavioral trajectories following similar environmental stressors has been defined with the term '*multifinality*' by developmental psychopathologists (Mead et al., 2010).

Although it is generally accepted that early life trauma represents a universal risk factor for pathological aggressive behavior in adult humans, animal models studying developmental stress-induced changes in aggression and their underlying neurobiological mechanisms have not been yet completely exploited. In rodents and primates many studies have been conducted in the attempt to model early trauma long-term effects. Repeated separation from the mother is a well-established model of negligence associated with increased anxiety, depression, and aggression-related behaviour. Similarly, rats and mice exposed to natural variations in maternal care (high and low licking and grooming) exhibit low and high anxiety and depression-related behaviour, respectively, in adulthood (Champagne et al., 2003; Carola et al., 2006). The long-term effects of exposure to an adult male during early life have also been proposed as a model of human exposure to violence during childhood (Hefner et al., 2007); nevertheless, not many examples of such mouse models can be found in

literature. Hefner and colleagues, for example, investigated the long-lasting effects of early postnatal exposure in mice (from postnatal day 2 to postnatal day 14) to an adult cospecific male. This study, however, failed to show any long-term effect on anxiety and depression behaviour, probably because the model employed relied on the use of anesthetized adult males, that thereby were not showing any aggressive behaviour towards the pups (Hefner et al., 2007).

Another manipulation employed as a model for early life neglect is represented by early weaning, but however this model is lacking the important contrubution of abuse and maltreatment during development (George et al., 2010).

This thesis work is aimed at establishing a new behavioural rodent model for chronic social stress that resembles as much as possible childhood maltreatment intended as aggression received during early life. In our model, in fact, juvenile mice are meant to experience physical and psychological stress by being exposed daily to an adult aggressor.

1.2 Gene-by-environment interaction

It is well established that early life stress (ELS) represents an important risk factor for psychiatric disorders. Nevertheless, most individuals exposed to adverse environments do not develop mental illness, suggesting that other environmental or genetic susceptibility elements play a role in modulating environmental risk factors

(reviewed in Caspi and Moffit, 2006). The interplay between environmental and genetic risk factors can derive either from a causal relationship between gene and environment, defined as gene-environment correlation (rG-E, when the genetic component determines the environment), or from an interaction between genes and environment (GxE, when genes moderate susceptibility to environment). In the former case, the individuals may shape their environments because of their genetics (Plomin et al., 1977). In the latter situation, both factors, genetics and environment, can modulate each other to give rise to a particular outcome (Rutter, 2008; Rutter, 2009). The identification of GxE risk factors has been proposed as a powerful method to pinpoint genetic variants that influence risk for mental illness and that could help to dissect the molecular mechanisms underlying these diseases.

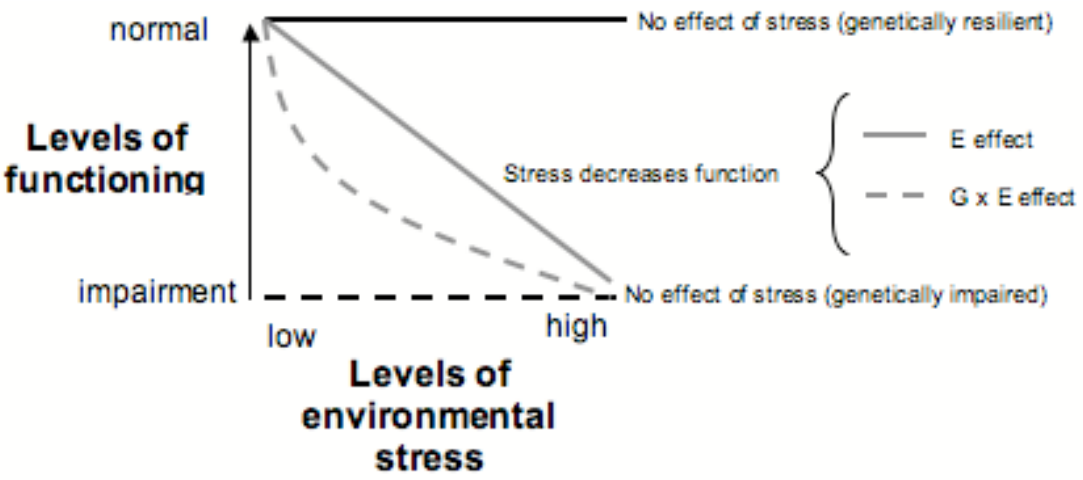


Fig.28 Gene x Environment Interaction Model; adapted by Nugent et al., 2010. Levels of functioning of a certain gene can be influenced by levels of environmental stress.

This interaction is well graphically depicted in the review of Nugent et al. 2010, where gene effects can modulate the outcomes of the environmental stress, conferring resilience or vulnerability to the subjects. Environmental stress can decrease normal levels of functioning alone or by interacting with genetic substrate (Fig.28).

Extensive studies have been conducted trying to model GxE effects and trying to unravel the causes of psychiatric disorders. For example, a common promoter polymorphism in the MAO-A gene has been shown to alter risk for anti-social behaviour in people exposed to childhood maltreatment (Caspi et al., 2002; Kim-Cohen et al., 2006). The same authors also showed that a common polymorphism in the serotonin transporter gene (5-HTT-LPR) moderates the risk for major depression associated with exposure to early maltreatment (Caspi et al., 2003). These studies were the first to identify measured GxE risk factors for mental illness and have been very influential in shifting the focus in psychiatric genetics studies toward the identification of GxE effects.

Sex also represents an important genetic factor, by interact with environmental stressors to moderate risk for human psychopathology. Different strategies for coping with environmental stressors have evolved in males and females, and these differences may underlie the characteristic prevalence of certain types of psychopathology in the two sexes (Schwandt et al., 2010).

The possibility of modelling GxE risk factors in animal models promises to complement human studies in the identification of the neural substrates by which early adverse experiences moderate risk for mental illness (Moffitt et al. 2005, Lynam et al., 2007). The chance to manipulate the genome essentially at will in the

laboratory mouse makes these species a particularly powerful tool for investigating the specific contribution of genetic component to such GxE risk factors. In this context, in fact, environment can be strictly controlled, allowing the experimenter to assess the exclusive effects of genetic variants tested under identical environmental conditions.

Clinical and experimental studies indicate that the prefrontal cortex (PFC) and hippocampus play a key role in the cognitive deficits and aberrant emotional behaviors originating from early-life adversity. Mediators of neural plasticity in the PFC and hippocampus, such as brain-derived neurotrophic factor (BDNF) are strongly affected by early adverse experiences (Roth et al., 2009). Moreover, many clinical studies point out a clear correlation between BDNF levels and mood disorders, such as anxiety and depression (Duman and Monteggia, 2006). Supported by literature, we propose here that BDNF can represent a good candidate as a modulator of long-term effects of early maltreatment.

1.3 BDNF and its physiological functions

Brain-derived neurotrophic factor (BDNF) is an abundant neurotrophin, present in many regions of the nervous system, including in the CNS the hippocampus, cerebral cortex, hypothalamus, substantia nigra, amygdala and spinal cord (Zhou et al., 2004). It plays an important role in promoting survival, growth and differentiation of

peripheral and central neurons, both during development and adulthood (Hashimoto, 2007; Cohen-Cory et al., 2010). Expression of the *Bdnf* gene is highly controlled by neuronal activity, through mechanisms dependent on intracellular Ca^{2+} levels (Carvalho et al., 2008). In mammals, BDNF is synthesized as a pre-pro-precursor that undergoes post-translational modification and is proteolytically cleaved to generate mature BDNF. The proBDNF (32kDa protein) is produced in the endoplasmic reticulum (ER), then transits to the Golgi apparatus and finally accumulates in the trans-Golgi network. The most relevant extracellular protease in the cleavage of neurotrophins is plasmin (Pang et al., 2004). Plasmin is a serin protease expressed as an inactive zymogen (plasminogen) which becomes active upon cleavage by tissue plasminogen activator tPA (Plow et al., 1995). Once cleaved, mature BDNF (mBDNF) homodimerizes and binds to its main target, the tropomyosin-related kinase B (TrkB), a tyrosine kinase receptor expressed widely in the brain. Binding of BDNF to trkB has been shown to activate several signalling cascades, including the ERK/MAPK and PI3K cascades. However, mBDNF can also bind with minor affinity to the neurotrophin pan-receptor, $p75^{NTR}$.

BDNF has been extensively studied as a modulator of experience-dependent synaptic maturation and plasticity. BDNF powerfully modulates long-term potentiation (LTP) in hippocampal slices cultures and in vivo (Patterson et al., 2001; Gruart et al., 2007). In both the hippocampus and visual cortex, BDNF moderates the maturation of inhibitory synapses (Marty et al, 2000; Huang et al., 1999) playing an instructive role in regulating the critical period for visual plasticity. Huang and colleagues investigated the maturation and plasticity of visual cortex in transgenic

mice that overexpress BDNF, showing that the maturation of GABAergic innervation was accelerated. In this study it was proposed that BDNF promotes the maturation of cortical inhibition during early post-natal life, thereby regulating the critical period for visual plasticity. BDNF has also been shown to be necessary for maturation of pre-synaptic plasticity, acting as a retrograde signalling, in neocortical neurons (Walz et al., 2006). In the developing mouse barrel cortex, BDNF was shown to act synergistically with neural activity to promote unmasking of silent synapses, by activating AMPA receptors trafficking to the synaptic site (Itami et al., 2003). Silent synapses are synapses that express NMDA receptors but lack AMPA receptors and thus do not generate postsynaptic currents at resting potentials (Busetto et al., 2008). In a recent study Cabezas and Buño (2011) showed that in hippocampus BDNF is required for the coordinated changes on both sides of the synaptic cleft that lead to the functional conversion of silent synapses. They are prominent in early postnatal development and are thought to play a key role in the activity- and sensory-dependent refinement of neural circuits.

The juvenile period in rodents (P14-P35) in which BDNF has been shown to control experience-dependent maturation of the visual system is also a period of significant synaptic maturation in cognitive structures such as cortex, hippocampus, and amygdala. However, the functional consequences of BDNF-driven synaptic maturation in these circuits have not been studied yet. The present proposal is based on the assumption that BDNF is likely to play a role in the experience-dependent synaptic maturation of forebrain circuits that are sensitive to early environmental conditions

1.4 Role of BDNF in modulating mood disorders

Increasing evidence suggests that BDNF plays a role in the pathophysiology of psychiatric diseases, particularly mood disorders such as anxiety and depression (Duman and Monteggia, 2006). In humans, a common genetic variant in the BDNF gene (Val66Met) that compromises protein processing, activity-dependent secretion, and neural activity (Egan et al., 2003; Hariri et al., 2003) is associated with increased anxiety traits in some studies (Sen et al., 2003; Hashimoto, 2007). However, other studies failed to replicate similar results (Jiang et al., 2005; Willis-Owen et al., 2005). Similarly, mice heterozygous for a null allele of BDNF show altered anxiety behaviour in some studies (Lyons et al., 1999; Chourbaji et al., 2004), but not in others. MacQueen and colleagues, for example, failed to show such a correlation between BDNF levels and mood disorders (MacQueen et al., 2001). Recently, a mouse has been engineered to carry the human Val-Met mutation, and these mice show increased anxiety and fear behaviours (Chen et al., 2006) and resilience to social defeat (Krishnan et al., 2007). One possible interpretation of these contradictory findings between BDNF genotype and anxiety is that environmental influences can act to moderate the BDNF phenotype. Support for this interpretation comes from mouse studies in which chronic stress in adulthood increased anxiety behaviour in mice overexpressing BDNF in forebrain, but not in non-transgenic littermates (Govindarajan et al, 2006). Other evidences come from studies in our laboratory showing GxE interactions between maternal care and heterozygous mutations in BDNF on anxiety behaviour in adulthood. Here, the mouse model

employed for early stress was represented by cross-fostering to 'good' and 'bad' mothers, respectively C57Bl6/J and BALB/cByJ females providing litters with high and low maternal care. In this study BDNF was shown to confer significant susceptibility to maternal care, modulating its long-term effects on anxiety-like behaviours (Carola et al., 2010).

Finally, conditional genetic manipulations of BDNF have begun to shed light on the neural circuits involved. While deletion of BDNF in forebrain structures in adult mice had no effect on anxiety (Camk2a-Cre/BDNF, Chan et al., 2006), deletion of BDNF in cortex and hippocampus during early development was associated with increased anxiety in the open field test (Emx1-Cre/BDNF). Deletion of BDNF in dopaminergic neurons, on the other hand, decreased susceptibility to social defeat in adulthood (Berton et al., 2006). These findings point to a role for forebrain BDNF in the developmental programming of anxiety.

1.5 Early critical period for long-term circuit alterations

An important issue when modelling human adverse early experiences in animals is how best to match human and animal developmental periods. For example, the first postnatal week, that is so important for rodent maternal environment, corresponds roughly to the last trimester of gestation in primates (Khazipov et al., 2001). Thus, the neural substrates of altered maternal care in rodents may not overlap with the

substrates of altered rearing environment in humans. The juvenile period in rodents, corresponding to the time when pups venture away from their mothers and become independent (earliest possible weaning date, usually P14) and their attainment of sexual maturity (P35-P42), is thought to correspond to the early postnatal period in humans. This is an especially sensitive period, as stressors experienced by rodents during this time may have far reaching effects on neurotransmission (Jacobson-Pick et al., 2008) and on behavioural changes measured in adulthood (Avital et al., 2006; Maslova et al., 2002; Jacobson-Pick et al., 2010). The particular sensitivity of this developmental phase could be related to extensive reorganization of neurotransmitter systems ad pruning of synapses that occur at this time (Jacobson-Pick et al., 2011).

However, although much effort has been made to investigate the long-term consequences of early adverse environment in rodents on anxiety- and depression-like behaviours, the majority of these studies remain focused on early post-natal days manipulation, such as maternal care models, and not so many studies to date have been focused on manipulating juvenile environment, especially in the early weaning period (P21-P28). In our study we establish a chronic social stress paradigm covering two weeks, focusing on juvenile period, from P14 to P27, which is a developmental stage crucial for synaptogenesis and refinement of neural circuits. This processes occurring in the early post-natal days in mice can be considered corresponding to late-gestational and early postnatal development in the human foetus (Noel et al., 2010).

2.Aim of the project

Early adverse experiences represent an important risk factor for developing mood disorders. We propose that juvenile mice subjected to chronic social stress can develop later in life behavioural deficits. In this thesis a new paradigm for juvenile chronic social stress has been established and its immediate and long-term effects have been investigated on mice, by employing a broad battery of behavioural tests.

Moreover, the ability of BDNF heterozygous null mutation to moderate such effects has been studied, in the light of possible gene x environment interaction mechanisms.

3. Material and Methods

3.1 Animals

Bdnf$^{KO/+}$ and wild-type littermates C57BL/6J mice were used as experimental subjects, obtained by crossing WT females to *Bdnf*$^{KO/+}$ male breeders. CD1 adult males (3-4 months old) were used as unfamiliar aggressors during the Juvenile Chronic Social Stress Paradigm. All animals were kept on a 12 h light/dark cycle (lights on at 7:00) with constant ambient temperature (21.5 ± 1°C) and humidity (55 ± 8%) with food and water available *ad libitum*. All mice subjected to the Juvenile Chronic Social Stress Paradigm were weaned at postnatal day 28, divided by sex and grouply caged (four/five per cage). CD1 aggressor males were singly caged, whereas CD1 intruder males, employed for priming of aggressors, were grouply caged (four per cage).

In total 277 mice were used in this project, coming from three different cohorts. All mice were handled according to protocols approved by the Italian Ministry of Health and commensurate with NIH guidelines for the ethical treatment of animals.

3.2 Establishment of a new juvenile chronic social stress paradigm

A new juvenile chronic social stress paradigm has been established (Fig.1), based on daily exposure to an unfamiliar aggressive male. Every day young mice from the Stress Group were exposed for 10 minutes to an aggressive conspecific adult male (CD1 strain). Each male-exposure session was composed of a short period of physical contact (3 minutes) followed by a period of sensory contact (7 minutes, where the aggressor was constrained under a transparent plexiglass tube with small holes in it). However, when the attacks received could physically hurt the pups, the CD1 aggressor was constrained under the plastic tube even earlier than 3 min. Each day of stress, every juvenile mouse was exposed to a different CD1 aggressor, so that never had to encounter the same CD1. A the same time of the day, a littermate control group was exposed for an equal amount of time (10 min of total exposure) to a novel cage containing only fresh bedding; we refer to this group as Novelty. Another control group, defined as Control, was represented by unhandled mice, kept with their mother until weaning (P28). Just before each session, all resident CD1 males were exposed to a sex and age-matched CD1 intruder for 15 minutes, in order to elicit aggression. CD1 Resident-Intruder sessions were videorecorded and aggressive behaviour was scored in real time, in order to ensure that all CD1 resident males displayed a similar amount of aggression.

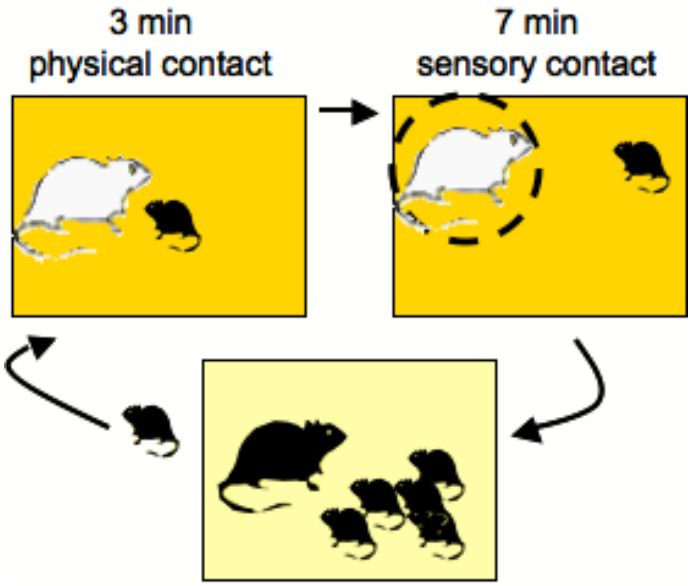


Fig.29 Juvenile Chronic Social Stress Paradigm. Every day, for 14 consecutive days, pups were individually placed into the homecage of an adult CD1 aggressor. After 3 minutes of physical contact, during which pups received attacks, the aggressor was constrained under a transparent plexiglass tube. At the end of 10 minutes, each pup was returned back to the nest.

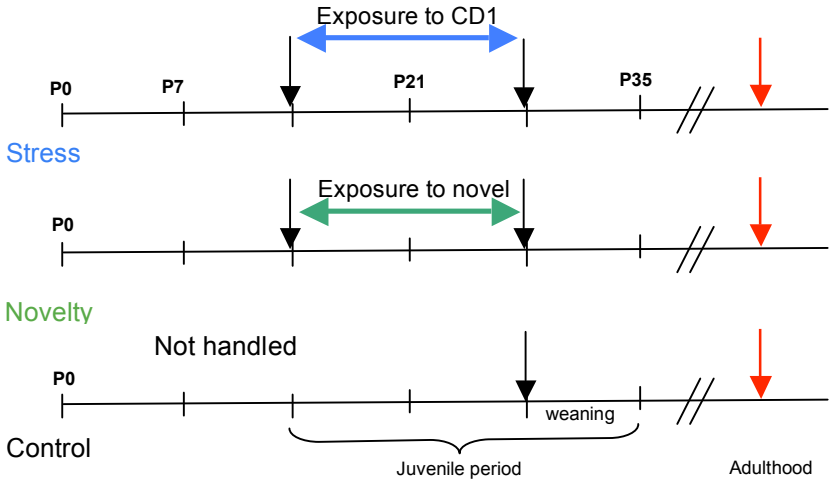


Fig.30 Outline of the Juvenile Chronic Social Stress Paradigm. From P14 to P27, mice were exposed to the chronic paradigm. Littermates were assigned to Stress or Novelty Group. A third group, in which mice were completely unhandled until weaning (P27), was defined as Control. Red arrows indicate the starting of behavioural tests.

The Juvenile Chronic Stress Paradigm lasted 14 days in total, consisting of single daily exposures to an adult aggressive male or to a novel cage, according to the experimental group, at random time of the day, ranging from 9 am to 7 pm (Fig.30).

3.3 Immediate effects of the paradigm

3.3.1 Analysis of behavioural effects

Every daily session of the paradigm was videorecorded. Behavioural immediate effects of stress were scored in real-time by sampling analysis: every 15 seconds, behaviour performed by mice was annotated. During the first 3 minutes the following behaviours (influenced by the CD1 leading behaviour) were scored: Attacks received, Attacks escaped, Social grooming, Self-grooming, Ano-genital sniffing received, Body sniffing received. During the sensory contact (7 minutes of observation) the following parameters were scored: Movement, Jumping, Rearing, Immobility, Grooming, Tube exploration, Interaction through the tube, Sniffing environment. All data per each experimental subject across the entire paradigm duration were then collected and statistically analyzed.

3.3.2 Analysis of physiological effects

Immediate physiological effects of stress were also investigated, by analysing the daily weight gain of each mouse across the period of stress (P14-P27): mice from Stress and Novelty group were weighted every day after exposure to the stressors, whereas mice from Control group were only weighted on day P14 and P27. Levels of corticosterone (CORT) either after one single exposure (acute stress) or after 14 days of exposure (chronic stress) were measured. For CORT analysis, blood was collected 30 min after exposure to Stress or Novelty and from unhandled mice (Control) on the first and last day of the paradigm (n = 4 per each experimental group).

Blood samples were always collected at 1 pm and transferred into tubes containing 10µl EDTA. Blood plasma was separated by centrifugation at 3000 rpm at 4 °C for 15 min before being stored at −80 °C until assayed. Corticosterone levels were determined using the radioimmunoassay (RIA) method. The analysis was performed by the Bios International Company (Bios S.r.l., Roma).

3.4 Outline of the experiment

After attaining adulthood, starting from about P80, animals from Stress, Novelty and Control groups underwent a battery of behavioural tests, including general anxiety test (Open field, Ligth-Dark Exploration Test), cognitive tests (Object Memory Test),

social investigation tests (Social preference, Social interaction) and aggressive behaviour tests (Resident-Intruder assay). The behavioural experiments, spanning across three months, were performed following the chronological order that is depicted in Fig.31.

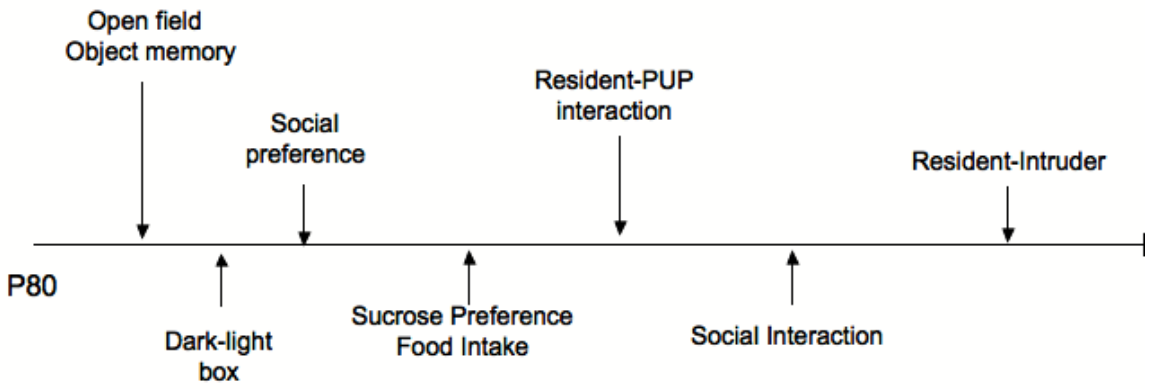


Fig. 31 Outline of behavioural tests performed, following the chronological order as shown. Test started when experimental subjects were about P80.

3.5 Behavioural Tests

3.5.1 Open Field Test

Open Field Test is a well-established behavioural test employed for assessing general anxiety and exploratory behaviour in mice. Mice were habituated to the testing

room for at least 30 minutes before the test started. They were individually placed into a grey square plastic arena (50 cm x 50 cm), surrounded by 25 cm high plastic walls. Animals were initially placed along one side of the arena and their behaviour was videorecorded for 20 minutes. The arena was carefully cleaned with alcohol and rinsed with water between animals. The center region was defined as an imaginary 25 cm x 25 cm central area. Each session was scored offline using the computer-based video-tracking VideoMot Software (TSE-Systems, Chesterfield, MO) that provides data such as time spent into the center, latency to reach the center, distance traveled into the center, total distance traveled. All data are derived from an analysis of the first 5 min of Novelty wild-type N = 38; Novelty $Bdnf^{KO/+}$ N = 20; Stress wild-type N = 53; Stress $Bdnf^{KO/+}$ N = 28.

3.5.2 Light-Dark Exploration Test

The Light-Dark Exploration Test exploits the natural tendency of a mouse to search for dark environments, where it feels more confident and safe (Crawley, 1981). This test was conducted in an apparatus (square arena made by plastic, 50 cm x 50 cm) divided in two compartments: a light chamber and a dark chamber completely enclosed by black plexiglass walls. The dark chamber, a rectangular box (50 cm x 25 cm), was placed into the square arena, generating a light compartment of equal size. The two chambers were accessible one to each other through a small opened door

centrally located on the wall of the dark box. Initially, the mouse was placed into the light compartment with the head facing the dark box door. Each session, lasting 5 min, was videorecorded and scored offline using VideoMot Software. The latency for the first transition from the dark to the light chamber, the distance travelled and the time spent into the light were measured and statistically analysed. The data presented derive from the analysis of one cohort of animals; Novelty wild-type N = 16; Novelty $Bdnf^{KO/+}$ N = 4; Stress wild-type N = 12; Stress $Bdnf^{KO/+}$ N = 5.

3.5.3 Social Preference Test

Social Preference Test was adapted from Jacqueline Crawley work on sociability and social approach (Chadman et al., 2008). Sociability, in this regard, is defined as the attitude of the mouse to spend more time exploring a social novel mouse than a non-social novel object. We used a three-compartment social approach task, which scores time spent in a side chamber with a social stimulus (novel mouse within a plexiglass tube) versus time spent in a side chamber with an inanimate object (plexiglass tube only). In this case, the plexiglass tube with holes on its wall serves as the novel inanimate object on one hand and as a container for the novel social target on the other side (Silverman et al., 2010). As a social stimulus we employed sex-matched juvenile conspecific mice P21-P24 old. Mice were habituated to the testing room for at least 30 min before the test started. The three-chamber apparatus was

made by grey plastic. It was filled every time with fresh bedding, carefully cleaned with alcohol and rinsed with water between animals. The test started with 5 min habituation. In this step mice were individually placed into the center and left free to explore the entire apparatus. At the end of 5 min, mice were constrained under a plexiglass black tube in the central area for few seconds, just the time to introduce into the side chambers the two targets: on one side an empty tube, on the other side a tube containing a social stimulus. The mice were then freed and the social preference assessment lasted 10 minutes. After that, the plexiglass tubes were removed and the experimental subjects were free to physically interact with the social target for 3 minutes (direct interaction). Each session was videorecorded and scored offline using Biobserver Software. Parameters such as time spent in the side chambers during habituation and during social preference test were measured and statistically analyzed. Number of attacks to the social stimulus during the direct interaction was scored in real time. The data presented derive from the analysis of Novelty wild-type N = 39; Novelty $Bdnf^{KO/+}$ N = 19; Stress wild-type N = 47; Stress $Bdnf^{KO/+}$ N = 28.

3.5.4 Food-Intake Test

Daily food-intake was measured across 5 days of test, during which mice were singly caged. Per each animal, pellet food was weighted at the beginning and at the end of the test (on day 1 and on day 5). Then the difference registered was assumed as

food consumed and averaged in order to have an index of daily food-intake. The resulting data were compared among groups and genotypes and statistically analysed. The test was performed on animals belonging to one cohort, N = 6 per group.

3.5.5 Resident-Pup Intruder Test

Aggressive behaviour toward pups was assessed by employing a classical resident-intruder test, having as intruder sex-matched pups (P21-24). The test was composed of one daily session, on three consecutive days. During the test an unfamiliar innocuous pup was introduced into the resident homecage of the experimental subject. Each exposure, lasting 5 minutes, was videorecorded. Latency to the first attack and total number of attacks given were scored in real time. Only male mice were tested. The data presented derive from the analysis of Novelty wild-type N = 14; Novelty $Bdnf^{KO/+}$ N = 3; Stress wild-type N = 12; Stress $Bdnf^{KO/+}$ N = 7.

3.5.6 Social Interaction Test

Social Interaction Test was performed across three consecutive days, aimed at investigating the social approaches between conspecific sex-matched mice before and

after a single social defeat exposure to an aggressive CD1 male. Every day, mice were habituated to the testing room for at least 30 minutes before the test started. In detail: on day 1 experimental subjects were individually placed in a new cage, with fresh bedding, together with a novel social stimulus (age- and sex-matched conspecific C57Bl/6J mouse) for 10 minutes. Stimulus mice were shaved on the back in order to recognize them from the top.

On day 2 experimental subjects were placed for 10 minutes into the homecage of an adult aggressive CD1, previously isolated for 1 month. This was considered a single exposure of social defeat, because isolated CD1 males showed clear aggressive behaviour against the C57 mice, which were acting in this case as subjugated intruders. After the initial 10 minutes of free interaction, CD1 aggressors were constrained under a plexiglass transparent tube, and the behaviour of the experimental subjects was scored. Exposure on Day 2 was similar to the daily exposure of the Juvenile Chronic Social Stress Paradigm that the experimental subject underwent early in life.

On day 3 the experimental subjects were placed into a new cage with fresh bedding, together with a novel social stimulus (age- and sex-matched conspecific C57Bl/6 mouse) for 10 minutes, as on day 1. Each session was videorecorded and scored offline using The Observer XT Software (Noldus Information Technology, Wageningen, Netherlands), in order to measure the amount of time each mouse spent for different behaviours. The following parameters were scored: climbing (number of times that the mouse was standing on the hind paws lining against the cage wall), rearing (number of times that the mouse was standing on the hind paws in the center

of the cage), digging, allosniffing (defined as the total time spent to sniff the social stimulus body), anogenital-sniffing (defined as the total time spent to sniff the social stimulus genitalia), self-grooming, social grooming (defined as the time spent grooming the social stimulus body and mouth). Social Interaction Test was performed only on WT male mice, belonging to the third cohort: Novelty N = 6, Stress N = 14.

3.5.7 Resident-Intruder Test

In order to assess aggressive-like behaviour on male subjects, Resident-Intruder Test was performed. Before the test, mice were individually caged, without changing their bedding, for at least one month. Intruder animals were sex- and age-matched CD1 grouply caged mice. The test was composed of three daily sessions, performed on consecutive days. In each session, one CD1 intruder mouse was introduced into the homecage of a resident experimental subject for 15 minutes. In every session, the experimental subject encountered a different intruder CD1 mouse. Each session was videorecorded. Latency to the first attack given and received was scored. Total number of attacks given was counted in real time. The data presented derive from the analysis of Novelty wild-type N = 21; Novelty $Bdnf^{KO/+}$ N = 8; Stress wild-type N = 28; Stress $Bdnf^{KO/+}$ N = 9.

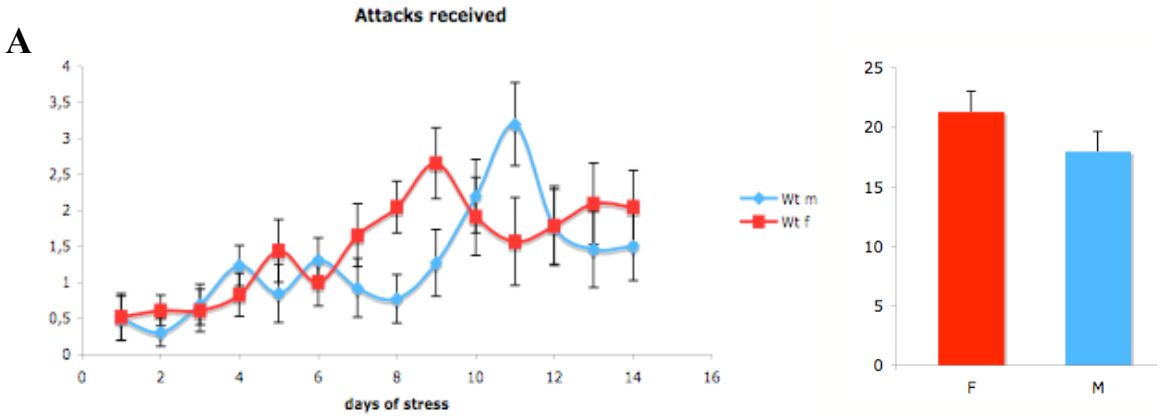
3.6 Statistical Analysis

Data were analyzed by ANalysis Of VAriance (ANOVA) statistical test: one-way, factorial or repeated measurements, according to the experiment. In case of non-normal distribution, effect of genotype was analyzed by non-parametric test (Kruskal-Wallis Test). All data are shown as mean ± SEM.

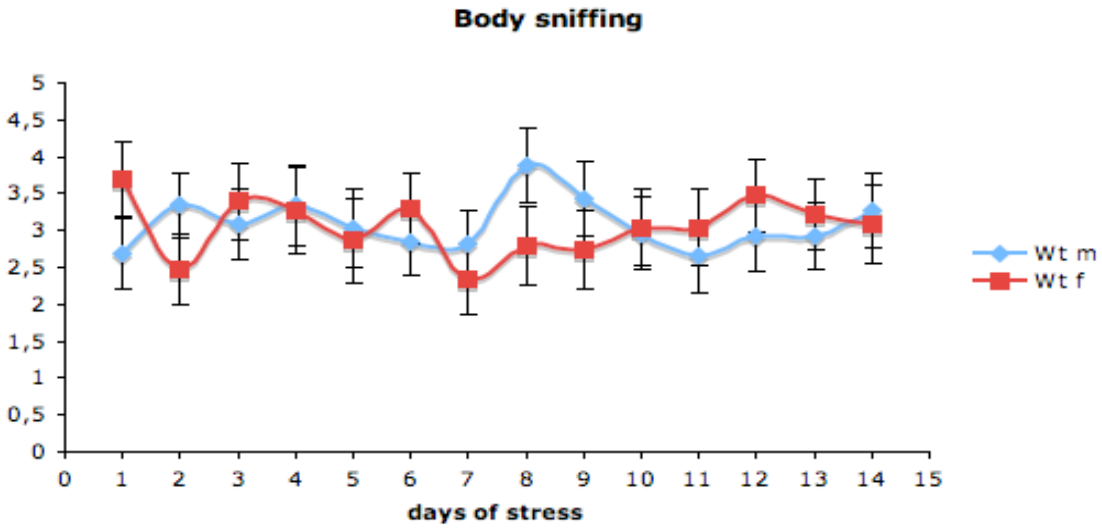
4 Results

4.1 Immediate behavioural effects of juvenile chronic social stress

Immediate effects of juvenile chronic social stress paradigm have been investigated by analysing pup behaviours during each session of daily exposure to an aggressor. The following data are expressed as a frequency of events during 3 minutes of direct contact between the experimental subjects and the CD1 aggressor, and during 7 minutes of sensory contact. We analysed the acute effects of stress on wild-type animals, comparing behaviours between males and females. We do not observe any significant difference in none of the parameters measured: numbers of attacks received, frequency of free movement, immobility and rearing in the aggressor homecage, amount of anal-sniffing or body-sniffing received by the resident CD1 mouse, amount of grooming during the sensory phase, exploration of the plexiglass tube containing the aggressor (Fig.32 A-G)



B


Body sniffing
5
4,5
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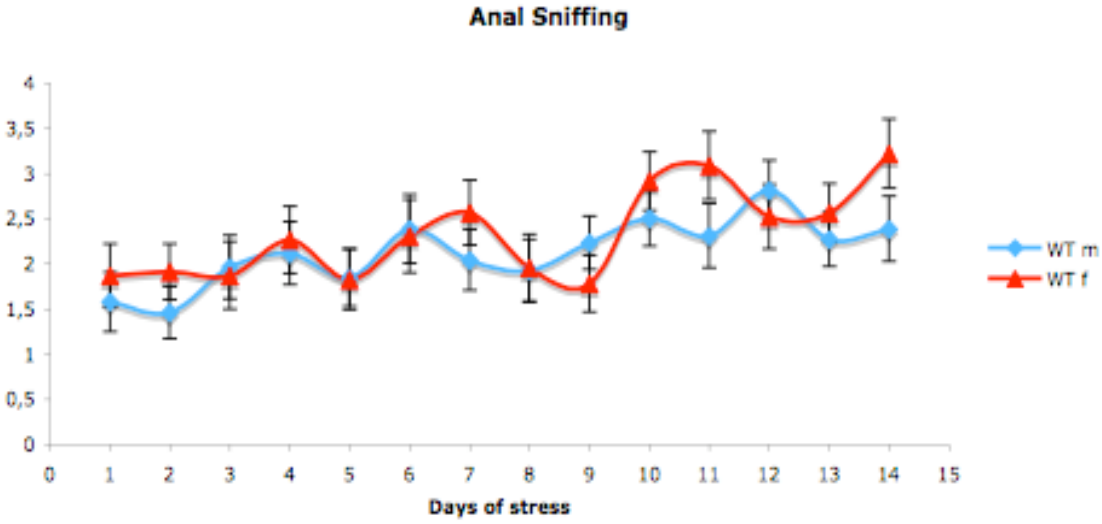
days of stress
Wt m
Wt f

C


Anal Sniffing
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Days of stress
WT m
WT f

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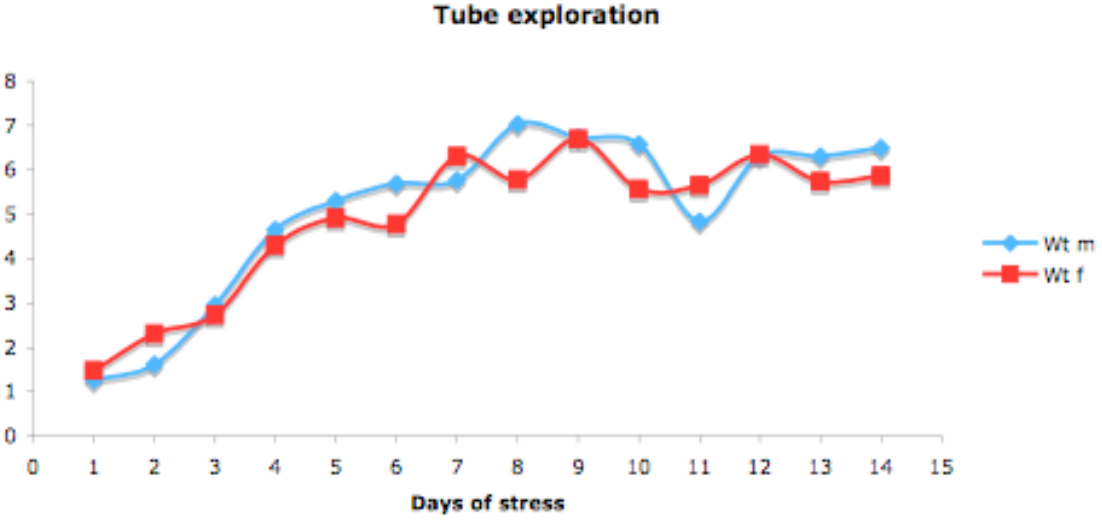
Tube exploration
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Days of stress
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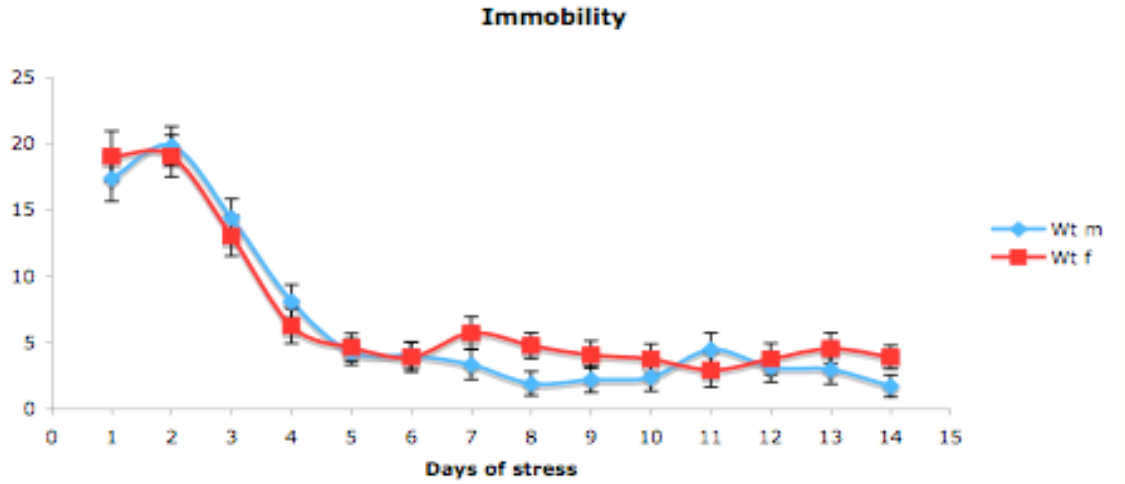
Immobility
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Days of stress
Wt m
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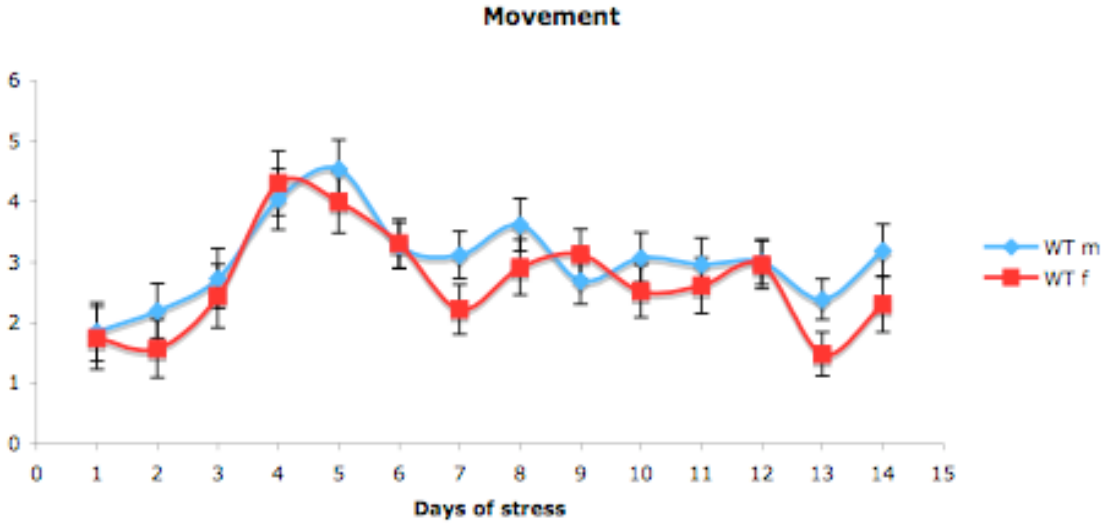
Movement
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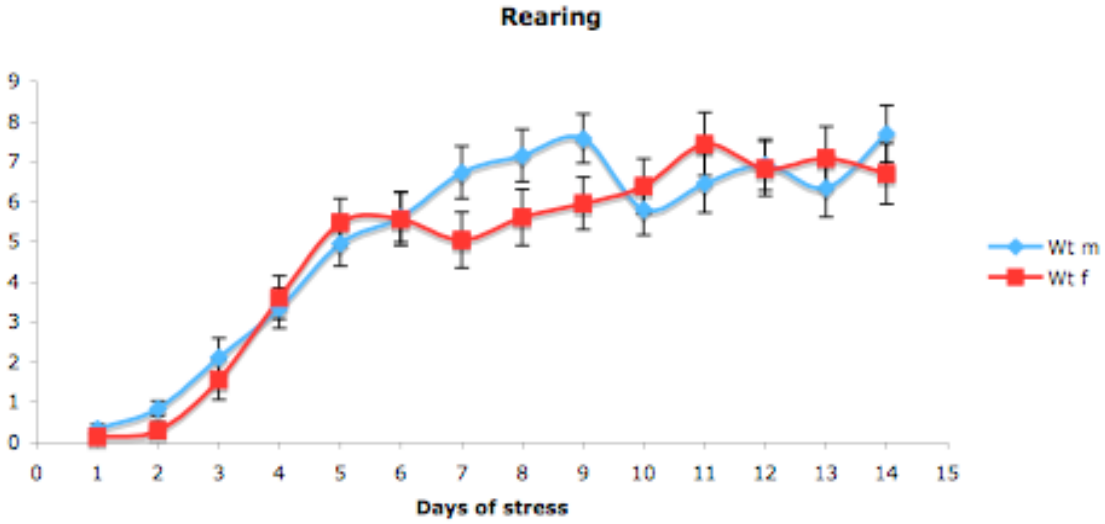
Days of stress
WT m
WT f

G


Rearing
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0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days of stress
Wt m
Wt f

Fig.32A-G Analysis of immediate behavioural effects of chronic social stress did not reveal any difference comparing wild-type females and males littermates. Graphs show behaviours across the 14 days of stress received: **A**) number of attacks received; **B**) frequence of body sniffing received; **C**) frequence of ano-genital sniffing received; **D**) frequence of tube exploration during the 7 min of sensory contact; **E**) frequence of immobility defined as absence of movement; **F**) frequence of locomotion; **G**) frequence of rearing.

4.2 Immediate physiological effects of juvenile chronic social stress

Body weight of pups belonging to Novelty and Stress group was measured every day from P14 to P27, soon after each exposure to the paradigm. Mice from Control group, unhandled by definition, were only weighted before weaning at P27. The body weight gain was daily compared between Novelty and Stress mice, and repeated measure ANOVA analysis revealed no effect of treatment (Fig33). No effects of treatment-sex interaction on body weight gain were observed between the two groups (Fig.34). Effect of genotype was also analysed, and no differences between Wt and Bdnf $^{KO/+}$ littermates were recorded (Fig. 35). However, handling of mice at this stage, either by exposing to Novelty or to Stress environment, induced a significant decrease in body weight, as it is shown by comparison to Control animals at P27 (Fig.36).

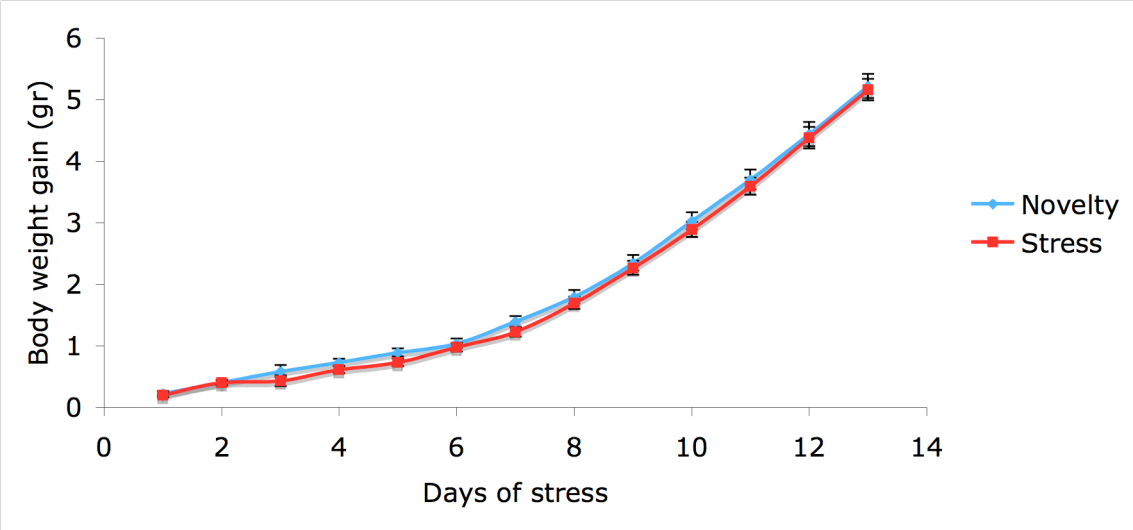


Fig.33 Body weight gain across the whole duration of the Juvenile Chronic Social Stress Paradigm (14 days in total); statistical analysis did not reveal any effect of treatment (Novelty n = 60; Stress n = 76. P = 0.99)

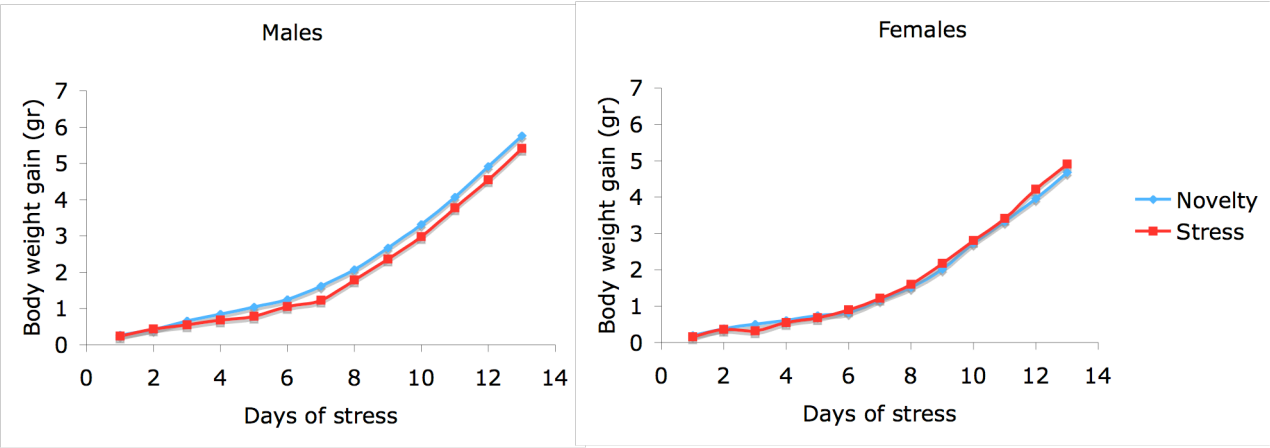


Fig. 34 Body weight gain across the whole duration of the Juvenile Chronic Social Stress Paradigm; no effect of treatment-sex interaction (Males: Novelty n = 26; Stress n = 33. Females: Novelty n = 34; Stress n = 42. P = 0.57)

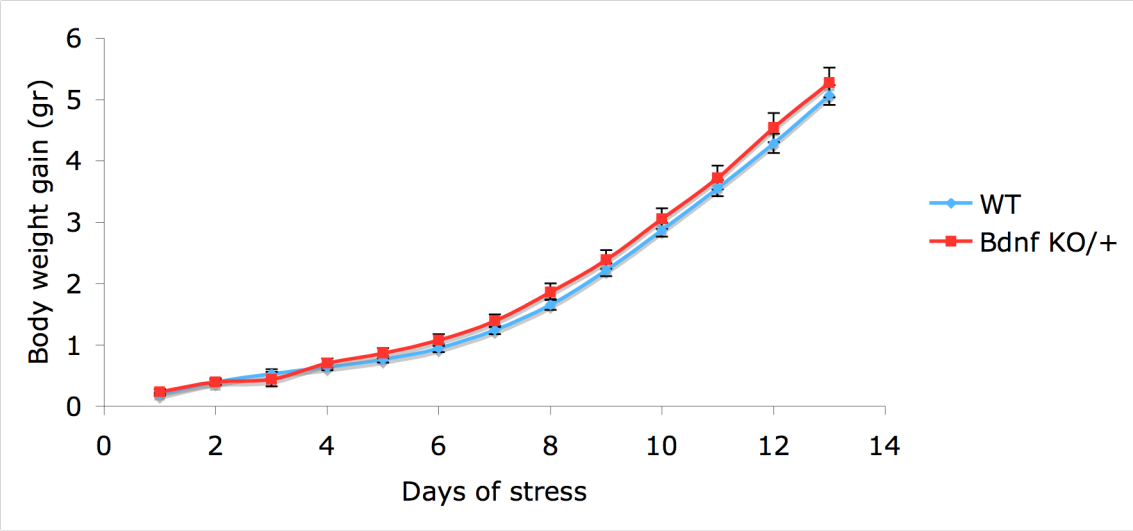


Fig.35 Body weight gain across the whole duration of the Juvenile Chronic Social Stress Paradigm; statistical analysis did not reveal any effect of genotype (WT n = 93; $Bdnf^{KO/+}$ n = 43. P = 0.9)

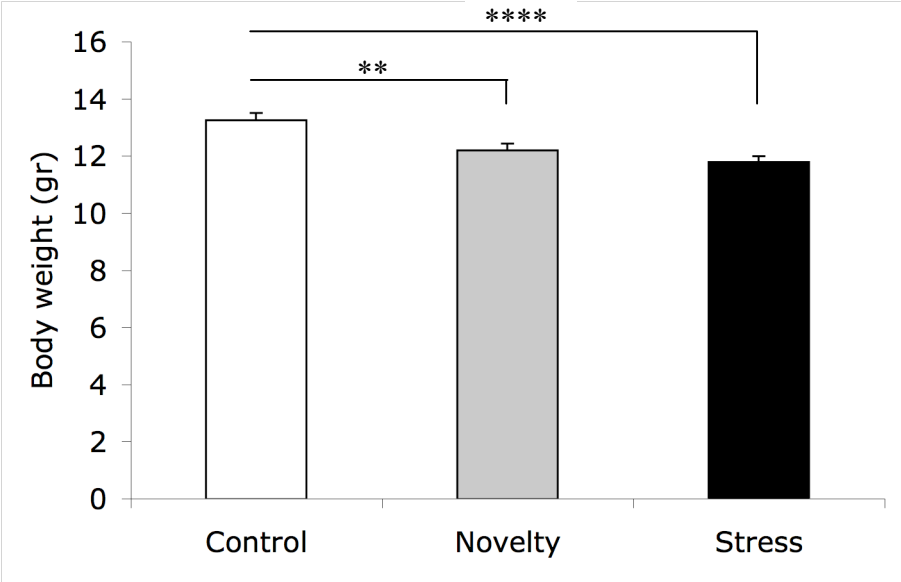


Fig.36 Absolute body weight is significantly decreased at P27 in Novelty and Stress mice compared to Control (Control n = 53, Novelty n = 54, Stress n = 72; Novelty vs Control, P = 0.0093; Stress vs Control, P = 0.00005)

Corticosterone levels. Plasma corticosterone levels were measured in Control pups, as basal reference, and in Novelty and Stress pups, in order to evaluate if the exposure to our Juvenile Chronic Social Stress paradigm was effectively stressful to the animals, either after one single (acute) or repeated (chronic) exposures. These data reveal that both Novelty and Stress exposures are stressful experiences for P14 pups, since corticosterone levels are significantly increased compared to Control group (Fig.37A). However, after chronic exposure we observed a significant increase in cort levels only for Novelty group and a trend for increased levels in Stress mice (Fig.37B).

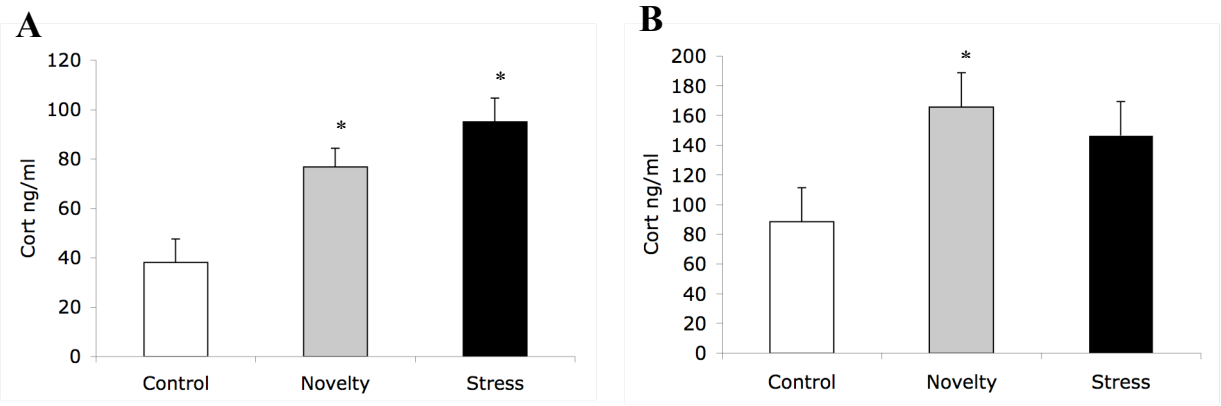


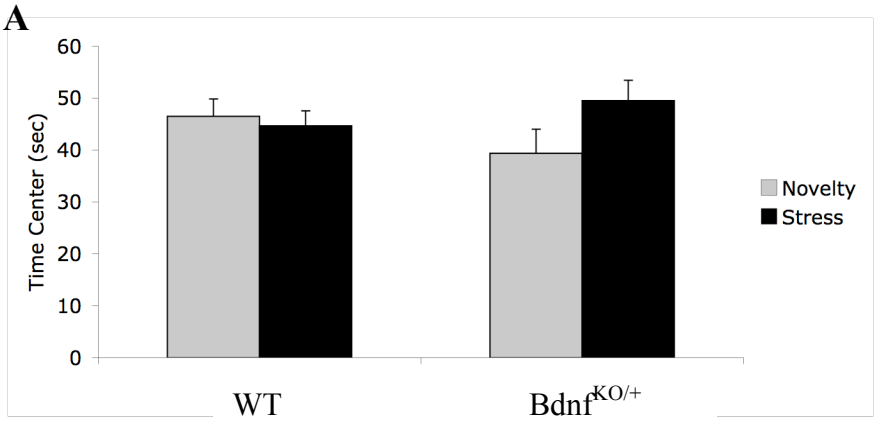
Fig.37 A) Analysis of corticosterone levels at P14, after acute exposure to the paradigm, revealed a significant differences in Novelty and Stress mice compared to Control group (Control n = 4, Novelty n = 4, Stress n = 4; Novelty vs Control, P = 0.04; Stress vs Control, P = 0.09). **B**) Analysis of corticosterone levels at P27, after chronic exposure to the paradigm, revealed significant differences in Novelty mice compare to Control (Control n = 4, Novelty n = 4, Stress n = 4; Novelty vs Control, P = 0.04; Stress vs Control, P = 0.09)

4.3 Long-term behavioural effects of chronic social stress

After attaining adulthood, all mice underwent a battery of behavioural tests, listed here in chronological order: Open Field Test, Light-Dark Box, Social Preference Test, Food Intake Test, Resident-Pup Intruder Test, Social Interaction Test, Resident-Intruder Test. Since our pure control for the Stress group is actually represented by the Novelty group, we will show only the behavioural results for these two groups, leaving behind the Control animals.

Open Field Test

Parameters normally scored in Open Field Test, such as time spent in the centre, latency to reach the centre, distance in the centre, and ratio between distance in the centre and total distance did not reveal any significant difference between Novelty and Stress group (Fig.38A-D), suggesting that exposure Juvenile Chronic Social Stress does not have long-lasting effects on general exploratory and anxiety behaviour.



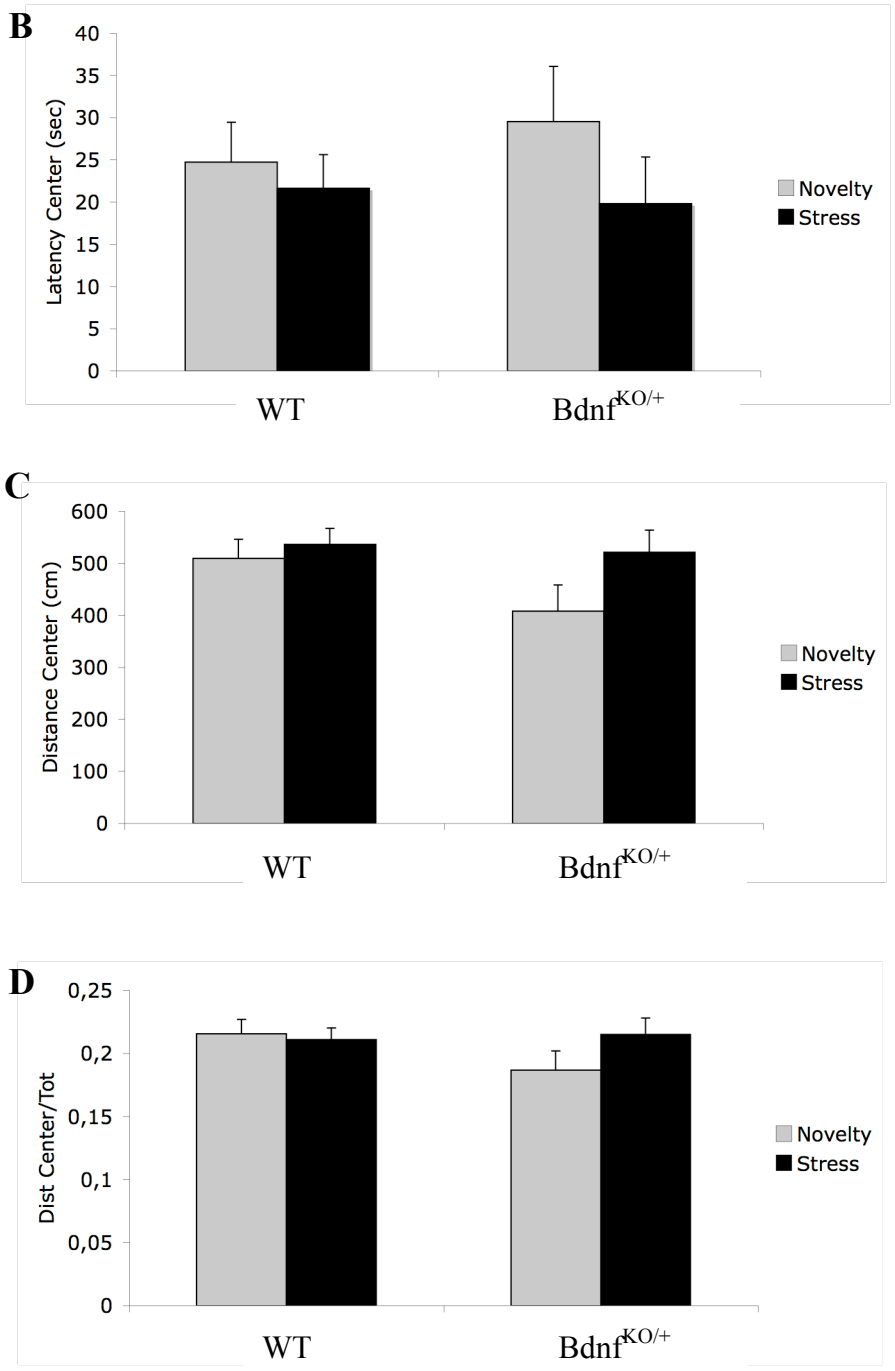


Fig.38 Open Field Test revealed no differences in general anxiety-like behaviours between Novelty and Stress groups, as shown by the analysis of different parameters scored: **A**) Time in the center; **B**) Latency to the center; **C**) Distance in the center; **D**) Distance in the center/ Total distance travelled.

Light-Dark Box Test

Generalized anxiety-like behaviours were also investigated in the Light-Dark Exploratory Test. No significant differences were observed in time spent in the light compartment comparing Novelty (N) and Stress (S) mice for both genotypes (Fig.39A). Similarly, no significant differences were observed in latency to visit the light compartment (Fig.39B)

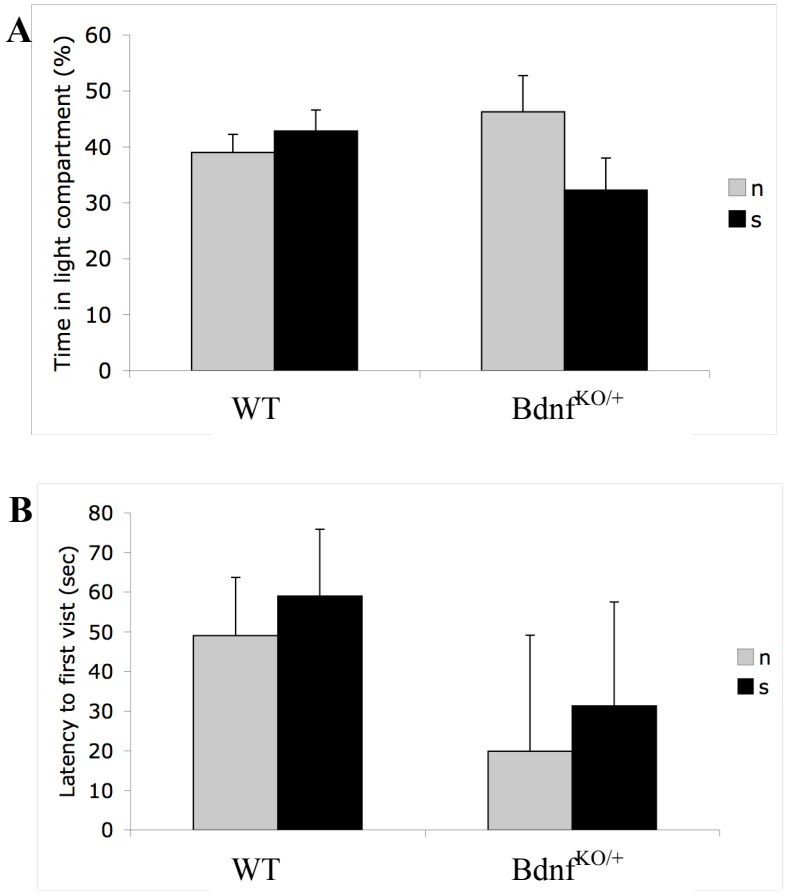
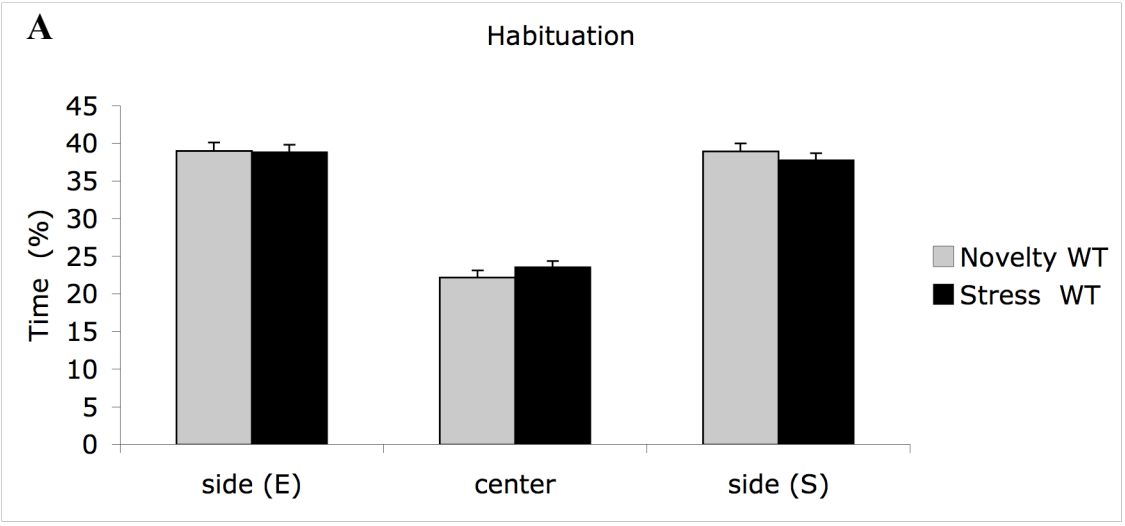


Fig.39 Light-Dark Box Test did not reveal significant differences in generalized anxiety behaviour. **A**) No differences observed for time spent in light compartment; effect of group: P = 0.31; effect of genotype: P = 0.74; effect of group*genotype: P = 0.08. **B**) No differences observed for latency to visit the light compartment; effect of group: P = 0.64; effect of geneotype: P = 0.22; effect of group*genotype: P = 0.97.

Social Preference Test

The sociability of our mice was assessed in the 'Social Preference Test', comparing the time spent in a compartment with a social stimulus versus the time spent in an empty compartment of the experimental apparatus. During the habituation phase, when the social stimulus was absent, mice did not show any preference to the two side compartments. 'Side (S)' represents the side compartment that, later on during the test phase, will contain the Social Stimulus. 'Side (E)' is indicating the side compartment that will contain the empty tube. 'Centre' represents the central compartment, between the two side chambers. T-Test analysis revealed that time spent in both side compartments was not significantly different for both Novelty and Stress mice (Fig.40A-B). However, in the test phase, once the social stimulus was introduced in the experimental box, all mice showed a clear preference to the social compartment (Fig.41A-B).



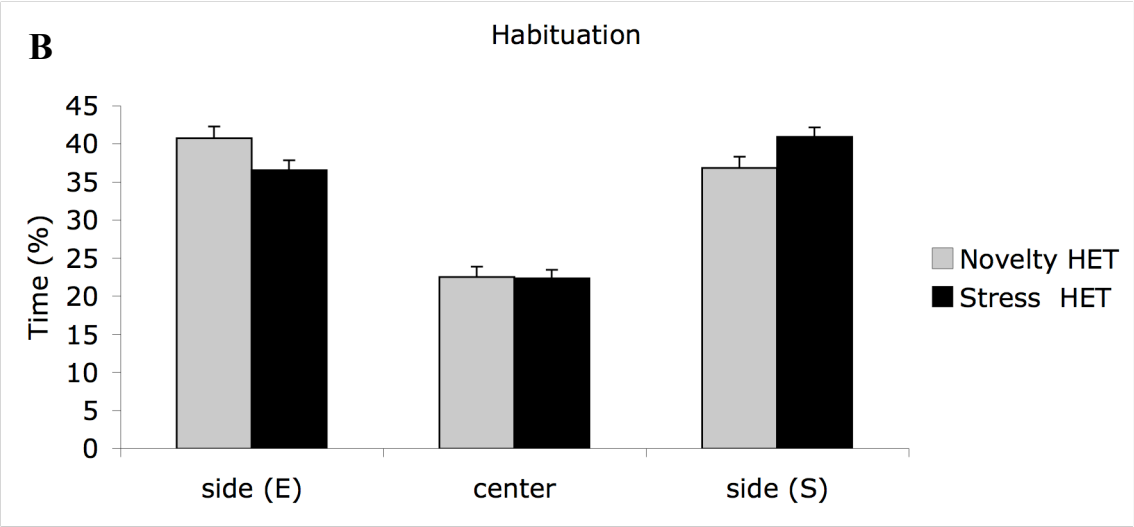
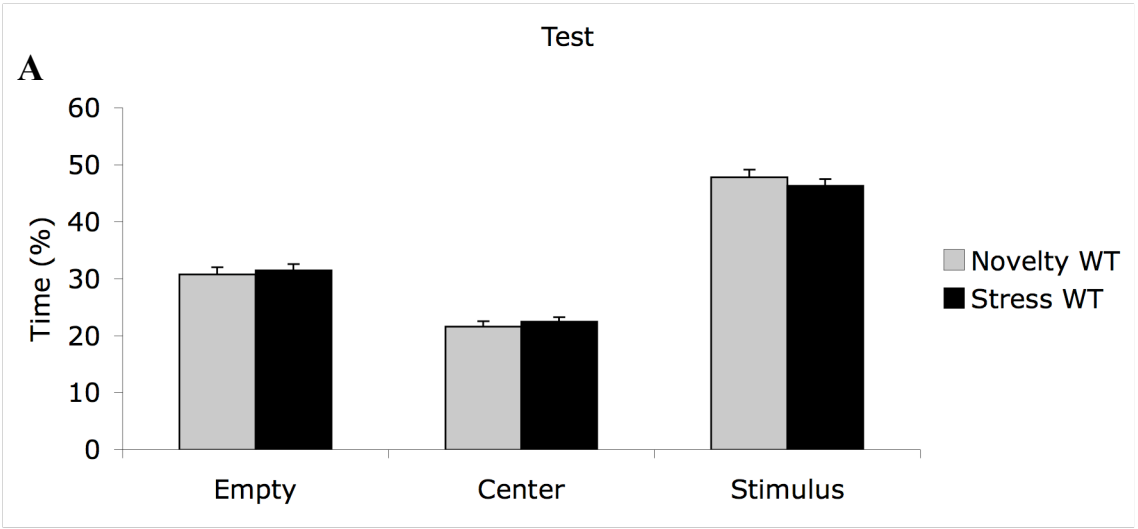


Fig.40 Social Preference Test. During the habituation, in absence of any stimulus, mice did not show preference toward the side compartments. **A**) Time spent in each compartment during the habituation of WT mice; **B**) Time spent in each compartment during the habituation of $Bdnf^{KO/+}$ mice.

ANOVA statistical analysis did not reveal any effect of environment (P = 0.6) nor of gene*environment interaction (P = 0.73). However, a significant effect of genotype (P<0.05) was observed in the time spent exploring the social compartment, with Bdnf heterozygous null mutant mice showing higher preference than WT.



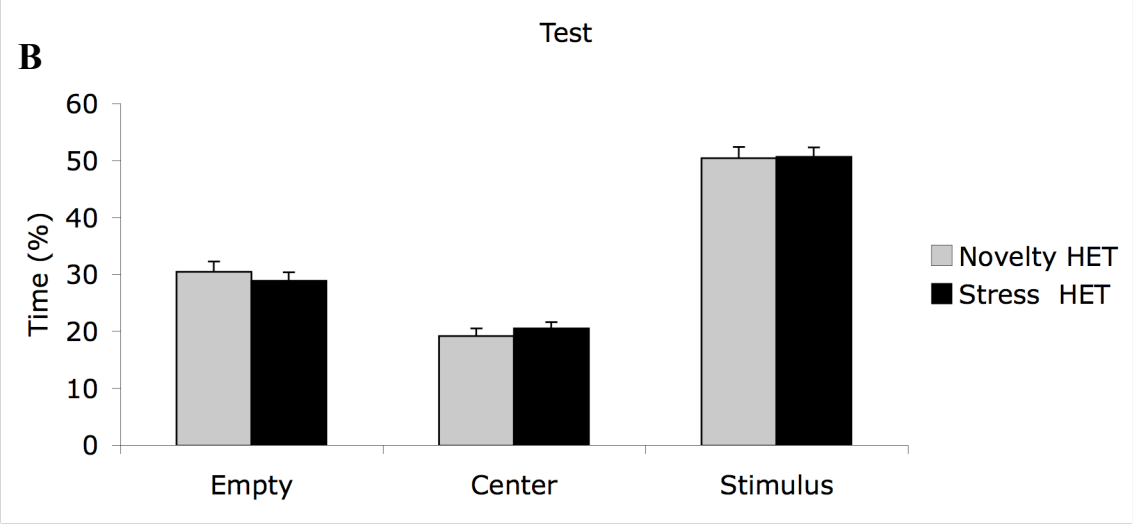


Fig.41 Social Preference Test. When the social stimulus was introduced, all mice showed a clear preference, spending significantly more time in the social compartment. **A**) Social preference displayed by wild-type mice. T-Test for Social vs Empty Compartment: Novelty WT: P <0.0001; Stress WT: P <0.0001. **B**) Social preference displayed by BdnfKO/+ mice. T-Test for Social vs Empty Compartment: Novelty HET: P<0.0001; Stress HET: P<0.0001.

Food Intake Test

Food Intake Test, performed across five consecutive days, did not show any significant difference in daily food intake between groups (Fig.42).

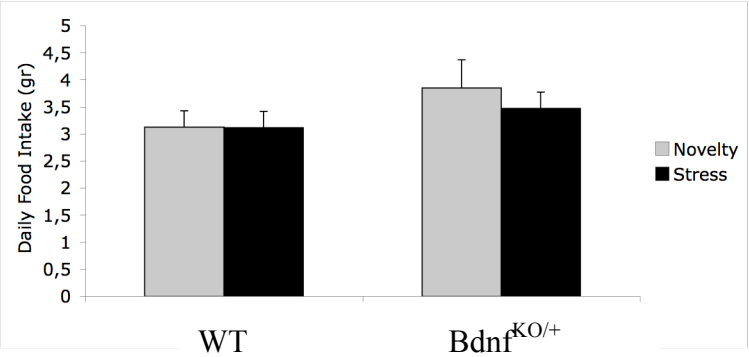


Fig.42 Food Intake Test: no effects of environment, effects of genotype nor gene*environment interaction were observed

Resident-Pup Intruder Test

Aggressive-like behaviour toward pups revealed no differences between Novelty and Stress mice. Number of attacks (Fig43A), in fact, was not significantly different, nor latency to the first attack (43B). However, a significant effect of genotype was observed, with Bdnf heterozygous null mutant mice displaying significantly shorter latency to attack the pups.

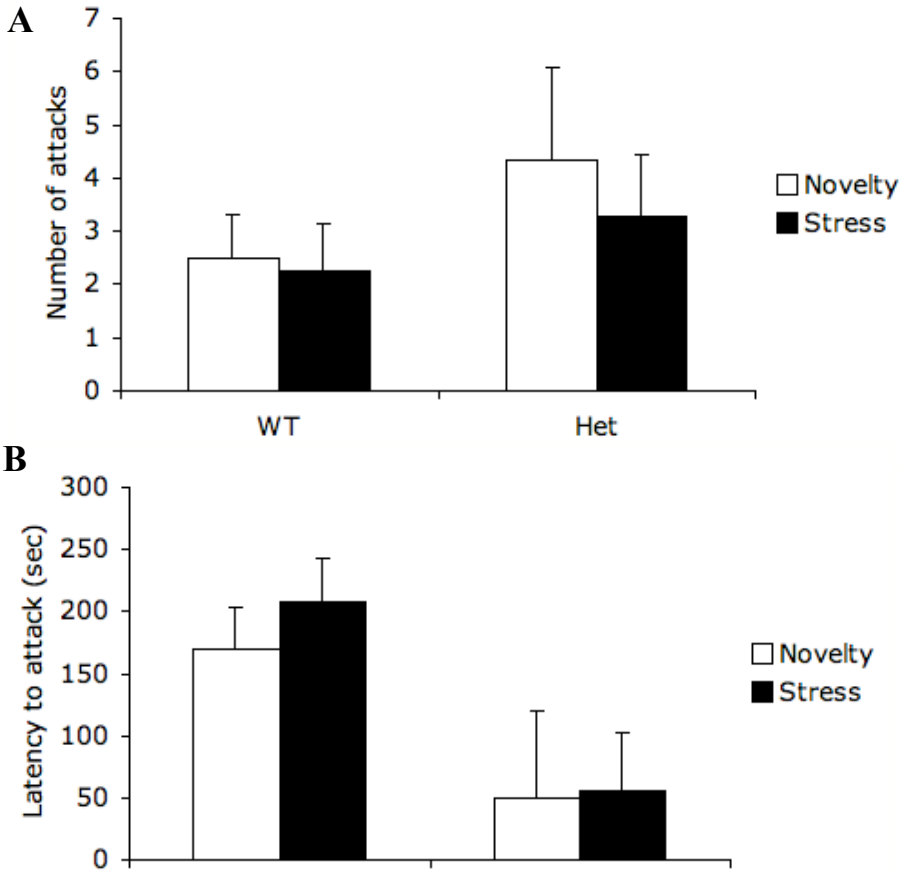
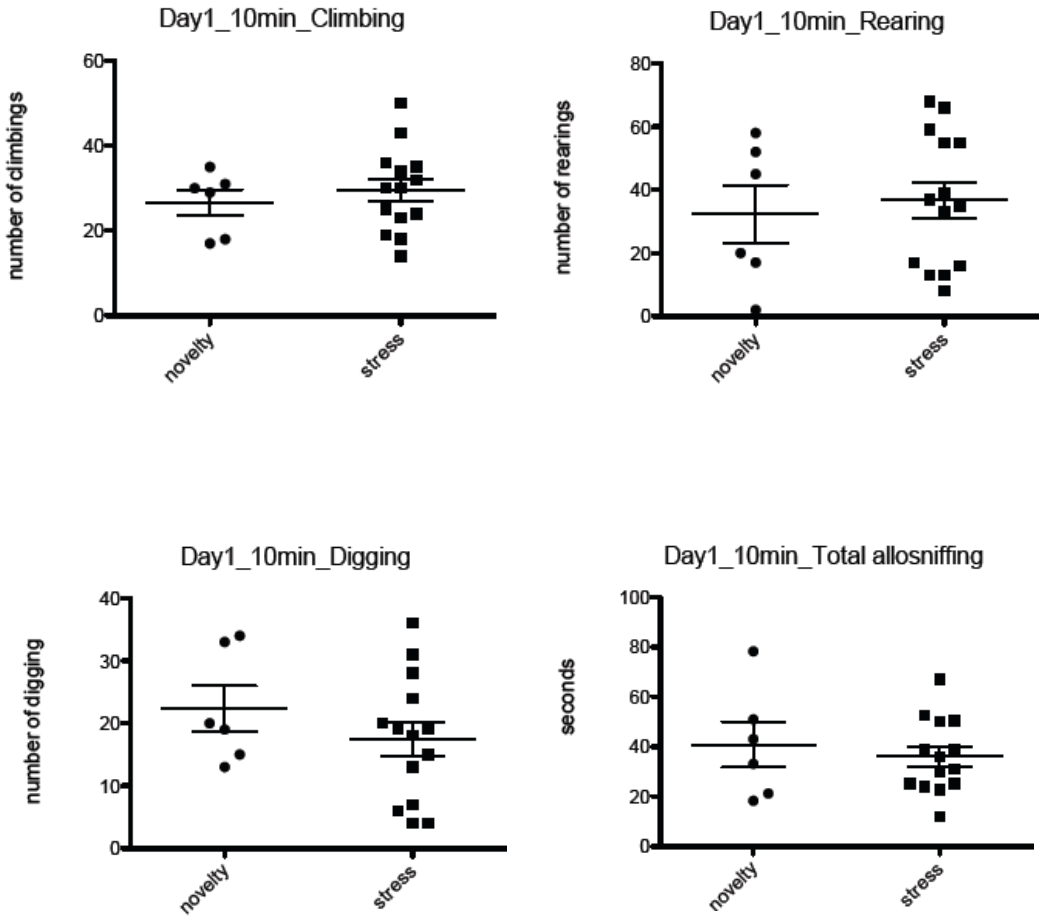


Fig.43 Resident-Pup Intruder Test revealed no effect of juvenile treatment on aggressive-like behaviour towards pups. **A**) Total number of attacks. **B**) Latency to the first attack.

Social Interaction Test

This test was performed only on WT animals, aimed at studying in depth the long-term effects of the Juvenile Chronic Social Stress Paradigm on social interaction. On Day1, social approach to an unfamiliar conspecific C57Bl/6J mouse was investigated. No significant differences were observed comparing Novelty and Stress mice behaviour, during the 10 minutes of interaction (Fig.44).



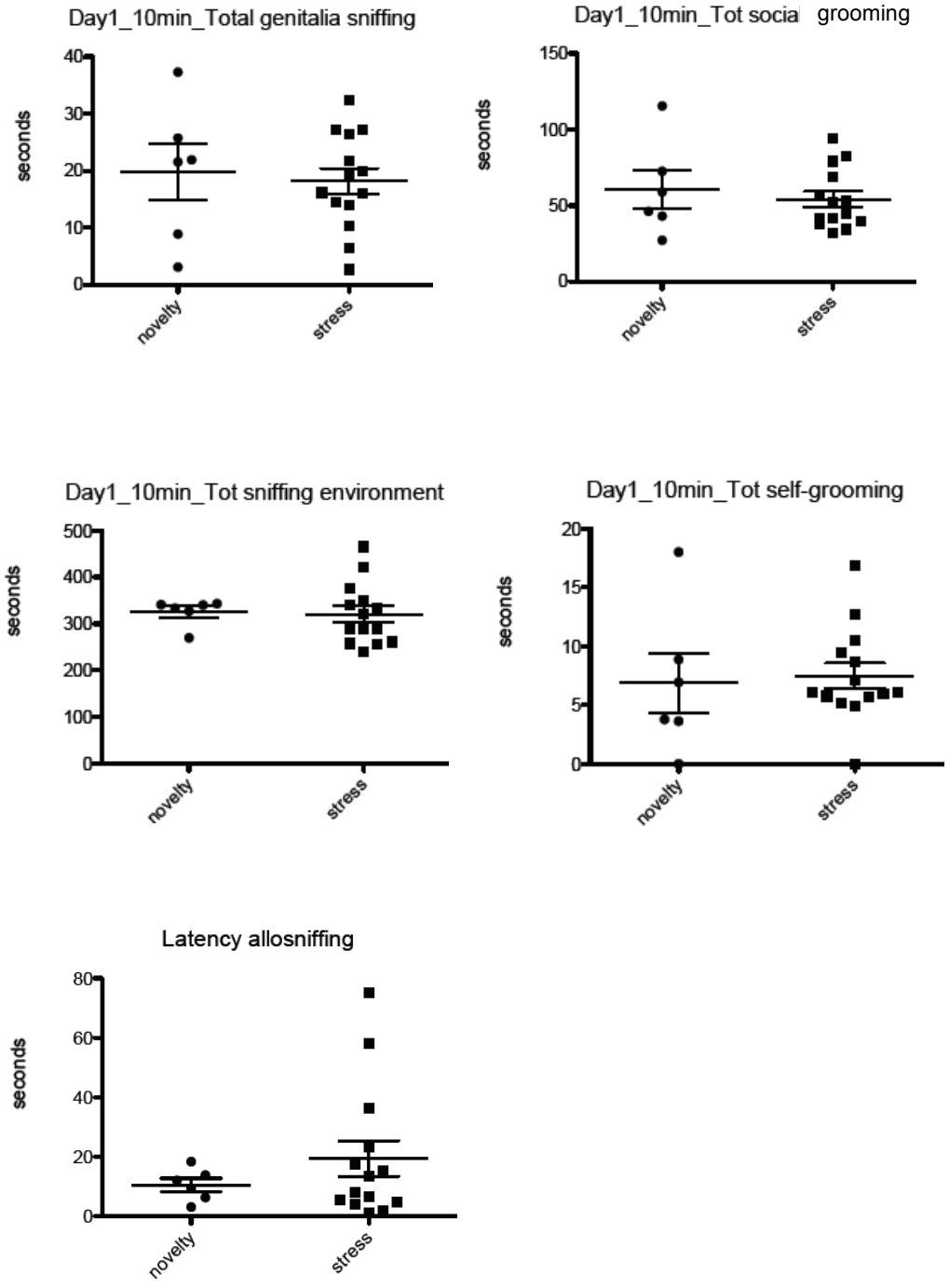
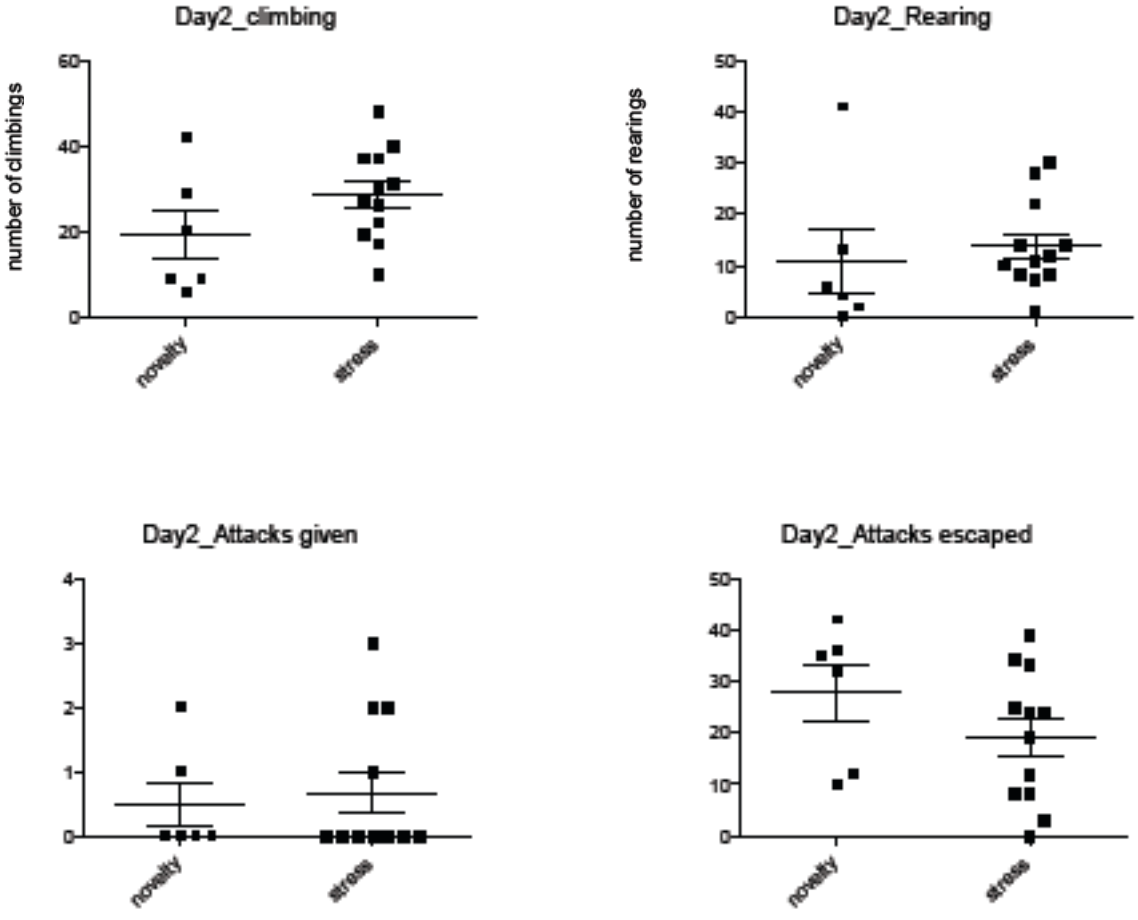
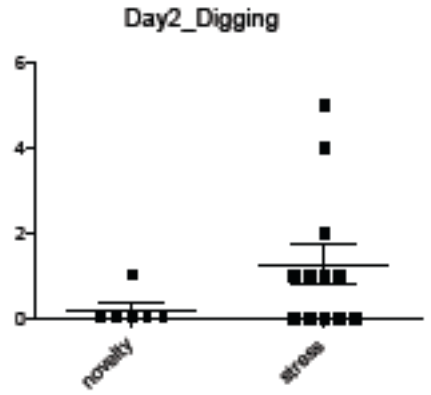
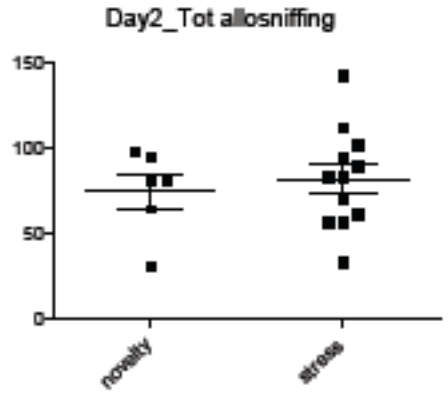


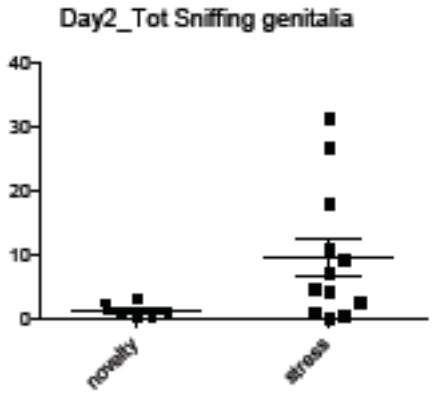
Fig.44 Social Interaction Test, Day 1. Novelty and Stress mice, exposed to a sex-matched social stimulus in a neutral environment, did not show differences in social interaction.

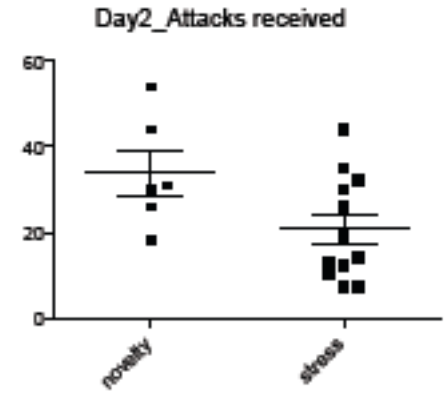
On Day2, our experimental subjects were exposed to the homecage of a resident CD1 and their 'intruder' behaviour was analysed. Stress mice showed a trend to increased climbing, sniffing of environment, digging behaviour and self-grooming, interpretable as an index of motor agitation. Number of attacks received and time spent in defence posture were also decreased in Stress compared to Novelty mice, and sniffing of genitalia and number of attacks escaped were increased. Taken together, all these results can be considered as an index of increased aggressive-like behaviour (Fig.45).

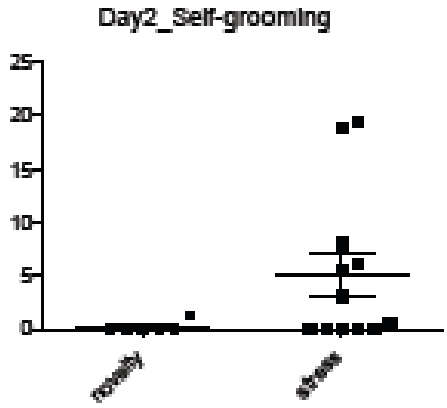


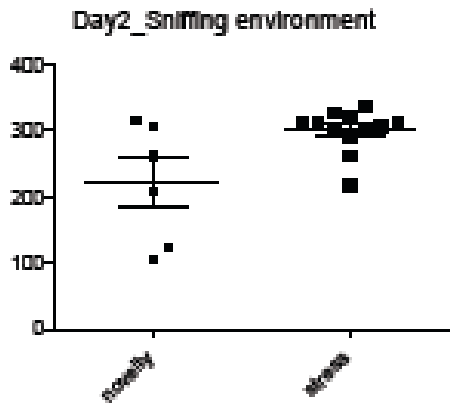

Day2_Digging
6
4
2
0
novelty
stress


Day2_Tot allosniffing
150
100
50
0
novelty
stress


Day2_Tot Sniffing genitalia
40
30
20
10
0
novelty
stress


Day2_Attacks received
60
40
20
0
novelty
stress


Day2_Self-grooming
25
20
15
10
5
0
novelty
stress


Day2_Sniffing environment
400
300
200
100
0
novelty
stress

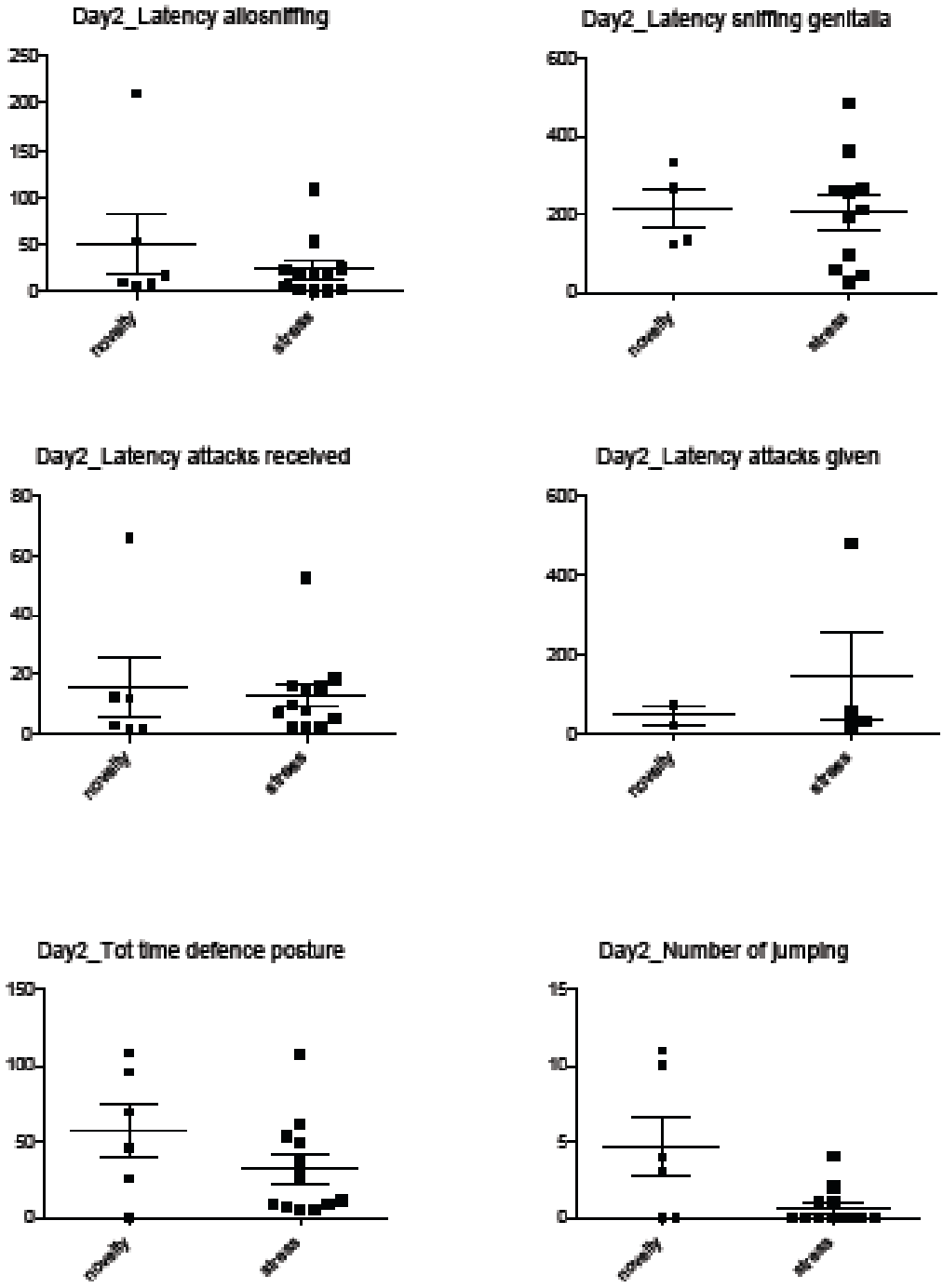
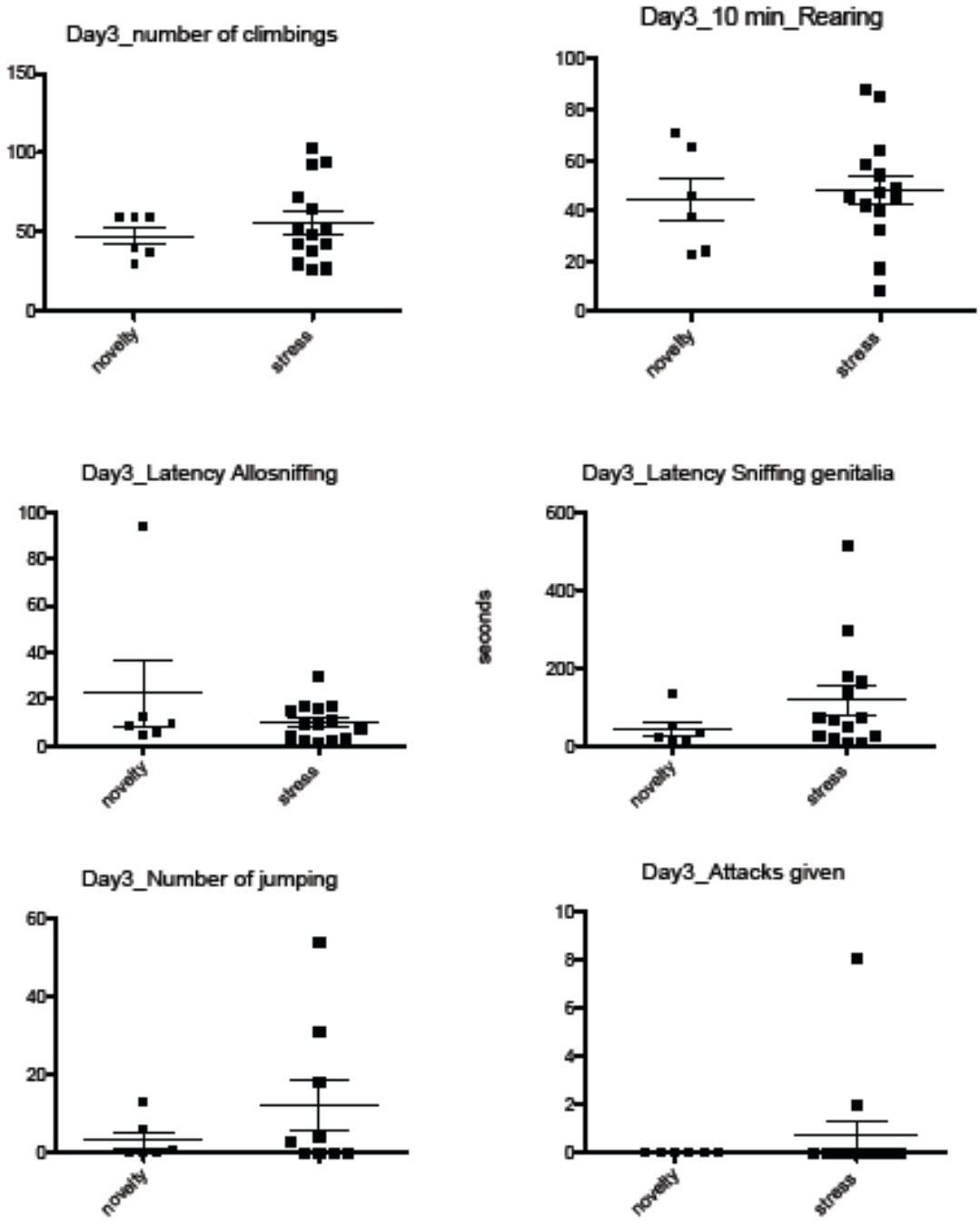


Fig.45 Social Interaction Test, Day 2. A single exposure to an aggressive CD1 mouse revealed an increased aggressive-like behaviour in Stress mice compared to Novelty.

On Day3, mice were exposed again (like on Day1) to an unfamiliar C57Bl/6J mouse, in a new cage with fresh bedding (neutral context). No differences in behaviour between Stress and Novelty mice were observed (Fig.46).



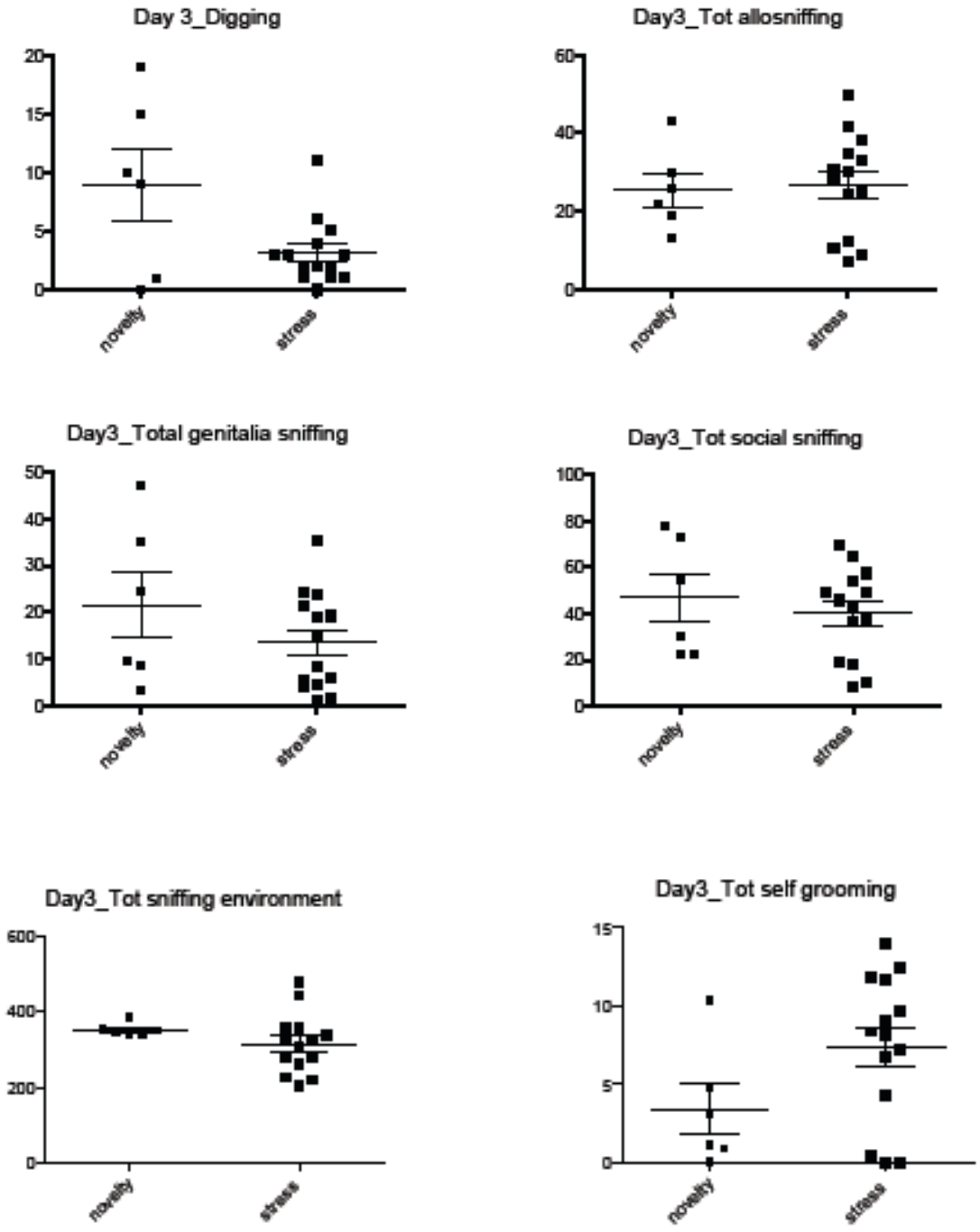


Fig.46 Social Interaction Test, Day3. No differences observed between Stress and Novelty mice in social interaction with a sex-matched stimulus, in a neutral environment.

Resident-Intruder Test

Aggressive-like behaviour was investigated in the Resident-Intruder Test, scoring the latency to the first attack and the total number of attacks given during the three consecutive days of test. A significant decrease in latency (Fig.47A) and an increase in number of attacks (Fig.47B) were observed on the last day in Stress WT mice compared to Novelty WT (Kruskal-Wallis Test: P = 0.003 and P = 0.008, respectively)

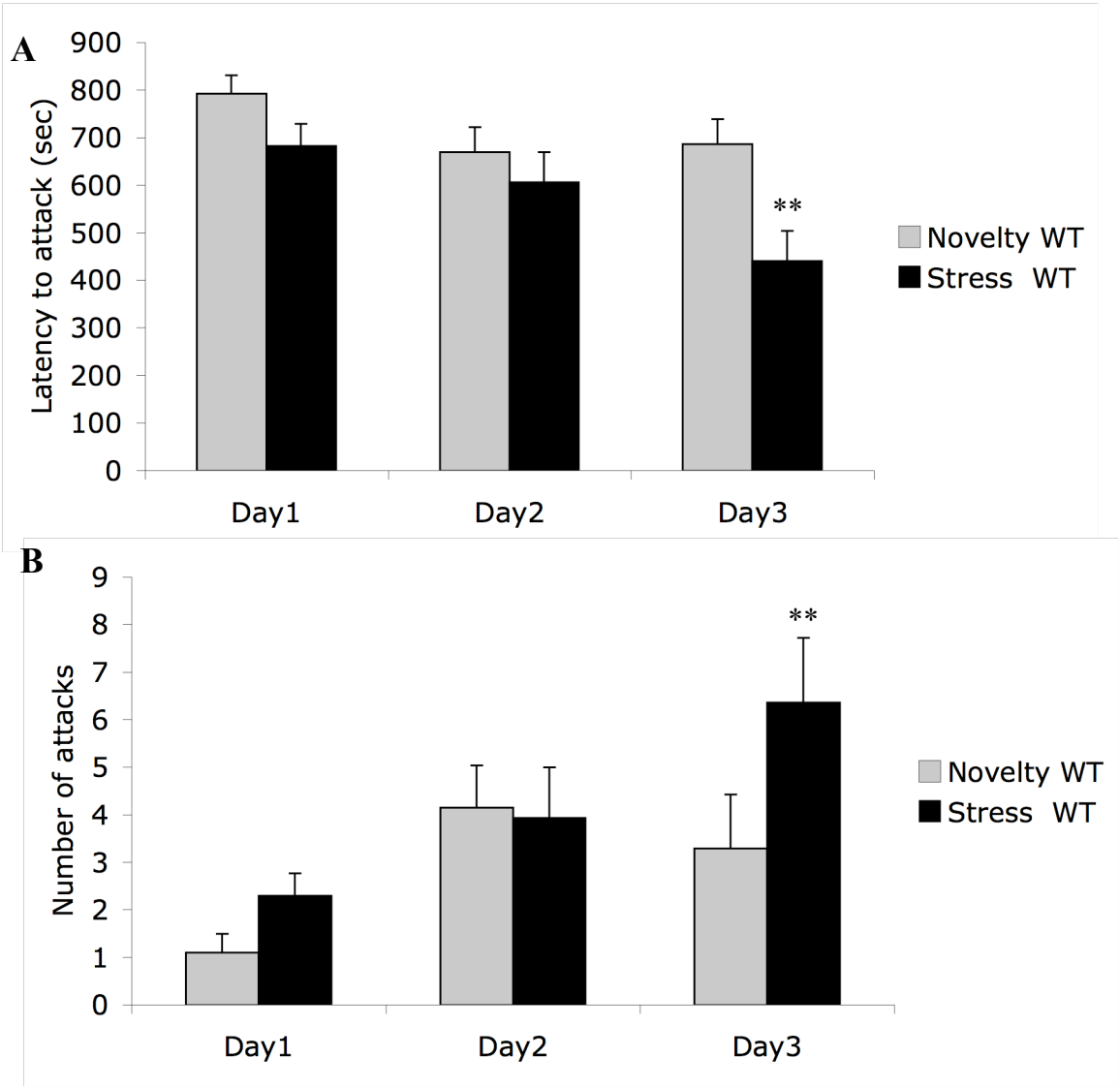


Fig.47 Resident-Intruder Test. On Day 3 **A**) wild-type stress mice showed significant shorter latency to the first attack and **B**) significant increase in number of attacks to the intruder.

However, environment effect was not significant in heterozygous mice, with Stress and Novelty groups showing no differences in latency (Fig.48A) and in total number of attacks (Fig.48B).

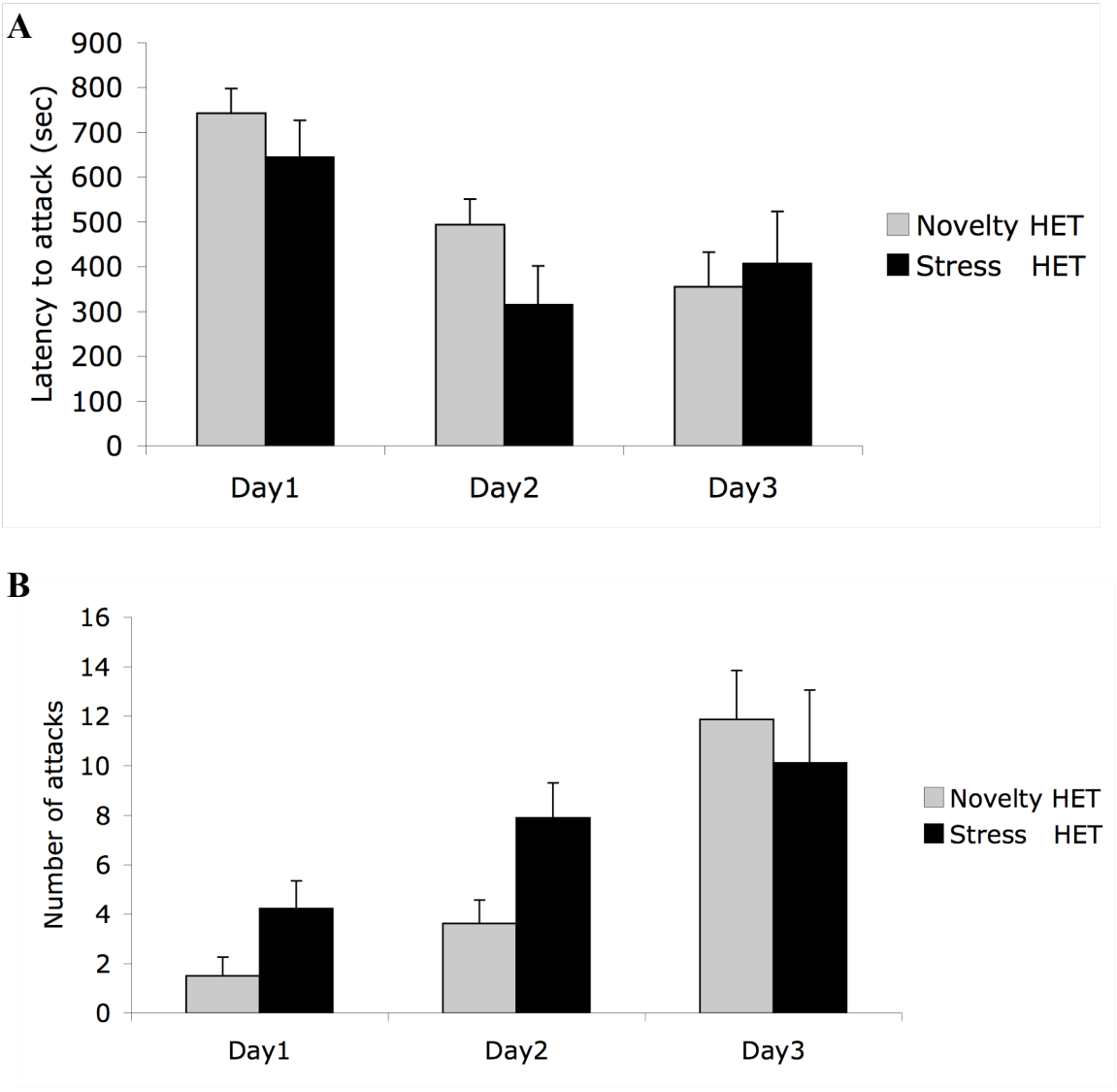


Fig.48 Resident-Intruder Test did not show significant differences when performed on *Bdnf* heterozygous null mutant mice. No differences were observed **A**) in latency to the firsts attack, nor **B**) in total number of attacks given to the intruder.

Factorial ANOVA analysis on Day 3, revealed a significant effect of genotype on both parameters scored (latency to first attack: $P < 0.05$; number of attacks: $P < 0.01$) showing that *Bdnf* heterozygous mice display a more aggressive phenotype, regardless the treatment received (Fig49).

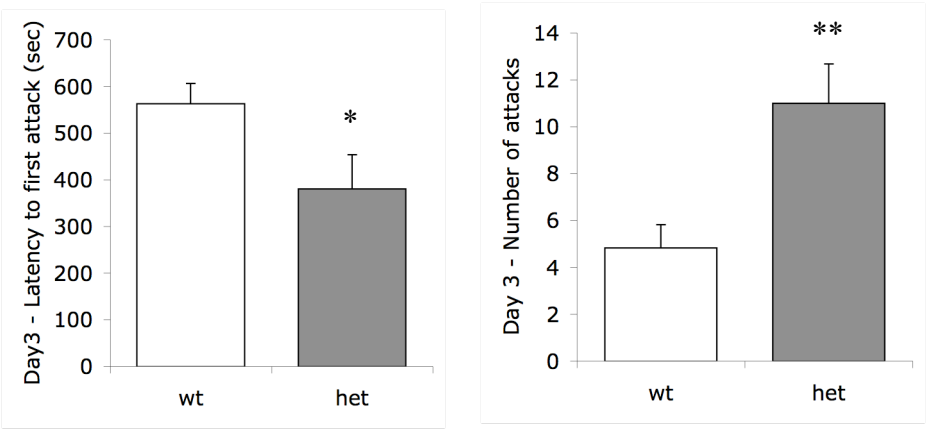


Fig.49 Resident-Intruder Test, Day 3. Genotype effect on latency to the first attack and total number of attacks, with *Bdnf* heterozygous null mutant mice displaying significantly increased aggressive-behaviour than wild-type littermates.

4.4 Long-term physiological effects of juvenile chronic social stress

Long-term effects of stress were investigated on body weight gain, comparing absolute weight of wild-type and *Bdnf* heterozygous null mutant male mice, at three different time point (about P80, P90 and P100). The analysis revealed an increase in body weight of wild-type Novelty and Stress mice, compared to wild-type Control. An

interaction between genotype and environment was observed, with *Bdnf* heterozygous null mutant Stress mice anticipating the obese phenotype compared to Novelty littermates (Fig.50).

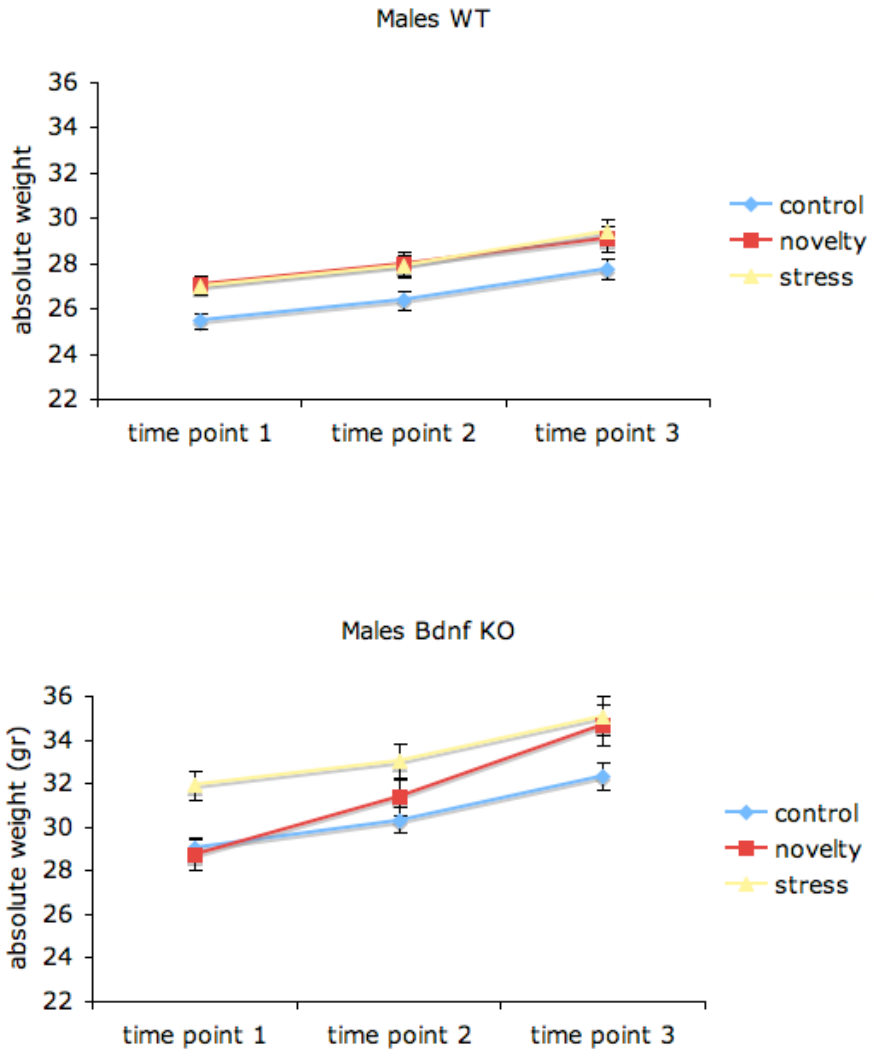


Fig.50 Absolute body weight analysis across time on male mice revealed an effect of genotype on wild-type mice (with Novelty and Stress heavier than Control group) and a gene*environment interaction effect on *Bdnf* null mutant mice (with a clear early onset of obesity in Stress mice).

5. Discussion

5.1 Immediate effects of Juvenile Chronic Social Stress Paradigm

Early adverse experiences have been shown to affect neural circuits development and alter behavioural outcomes in adulthood (Heim and Nemeroff, 2001; Barnow et al., 2003; Penza et al., 2003; Barnow and Freyberger, 2003). Social Stress is considered an important risk factor, responsible for a determinant contribution to mental illness aetiology. In the attempt to establish a mouse Juvenile Chronic Social Stress Paradigm to model and study the effects of environment in human mental disorders, we first needed to validate our paradigm, assessing its immediate effects on experimental subjects. Mice behaviour during daily exposure to an adult unfamiliar aggressor was videorecorded and scored to ensure that all the subjects were equally exposed to stressful experiences across the whole duration of the paradigm. In this regard, number of attacks received, time of body sniffing and ano-genital sniffing were not significantly different among mice, regardless sex or genotype. Considering that adult mice usually do not display aggressive behaviour toward pups, because they do not perceive them as a threat, we decided to prime our CD1 aggressors by subjecting them to a single resident-intruder session (encountering others CD1 mice) just before each pup exposure. Priming appeared to be necessary for triggering aggressive behaviour, because CD1 mice that were not exposed to this procedure did not attack the intruder pups (data not shown). Analysis of latency to the first attack

and total number of attacks for CD1 priming revealed an overall significant effect of time in exacerbating aggression in these mice, as supported by literature about pathological aggression (Caramaschi et al., 2008). Moreover, no differences were observed in relation to other general parameters scored, like locomotion, immobility, jumping, rearing. Taken together, these data suggest that all the experimental subjects were exposed to a similar amount of social stress during the juvenile period and that there were no significant immediate effects of sex or genotype on behaviour. We also needed to show that the exposure to the Juvenile Chronic Social Stress Paradigm was, in fact, effectively stressful to the pups. Analysis of physiological immediate effects on mice subjected to the paradigm versus non-handled mice (Control group) revealed indeed a significant effect on body weight and plasma corticosterone levels, which can both be considered as an index of stress. Although no significant differences were observed between Novelty and Stress mice, suggesting that this kind of manipulation during juvenile period is stressful *per se,* we could observe a trend showing higher plasma corticosterone levels (at P14) and lower body weight (P27) in Stress group.

5.2 Long-term effects of Juvenile Chronic Social Stress Paradigm

The establishment of a new Juvenile Chronic Social Stress Paradigm was aimed at studying early social adverse experiences as a risk factor for developing mood disorders later in life. Long-term behavioural effects, in fact, were the main focus of

our investigations. A large piece of evidence from literature has shown that rearing in a negative social environment can contribute to the manifestation of mental illness in adulthood. Thus, we analysed a broad spectrum of behaviours, spanning from generalized anxiety to social behaviour and aggressive-like behaviour. In order to assess the direct contribution of social stress, we compared mice that during their juvenile period have been chronically exposed to an adult aggressor (presence of social component, Stress group) versus mice that have been chronically exposed to a novel empty cage (absence of social component, Novelty group). The Novelty group was designed as a control group, to reduce at maximum all the possible variables: littermate mice from both groups were daily separated from their mother for the same amount of time, were subjected to the paradigm at the same time of the day, were also exposed to a cage of similar size, containing an identical plastic tube; the only difference resided in the fact that Stress mice were placed in an adult aggressor homecage and received psychological threat and physical attacks.

Interestingly, no differences were observed in general anxiety behaviours, as shown by Open Field Test and Light-Dark Exploratory Test, suggesting that our paradigm did not specifically affect neural circuits related to anxiety-like behaviours. No significant differences were also found in Food Intake Test, assuming that no anhedonia-like behaviour was present in our mice. Social Preference Test revealed similar attitude to explore a social stimulus in both Novelty and Stress mice, with both groups spending significantly higher time in the social chamber than in the empty one. This is suggestive of a normal social behaviour in approaching new individuals, in the light of the innate interest that mice display for innocuous stimuli (Crawley 2007).

Resident-Pup Intruder Test, as well, revealed a normal social behaviour toward pups in both Novelty and Stress mice; here the pup intruder was not perceived as a threat, because young mice represent innocuous social stimuli. Similarly, Social Interaction Test on Day1 and Day3 (testing the social approach toward an innocuous sex- and age-matched conspecific mouse) did not show any behavioural differences between Novelty and Stress groups. On the contrary, interestingly enough, social behaviour appeared to be different on Day2 of the same test, when our experimental subjects had to encounter an aggressive CD1 male in its homecage. In this situation, in fact, Stress mice showed a different strategy to cope with the aggressor compared to Novelty group, as was suggested by the significant increase in jumping and digging, increase in sniffing genitalia and sniffing environment, increase in attacks escaped and decrease in attacks received, along with a decrease in defence posture. All together, this pattern of behaviours recorded for the Stress group depicts an 'assertive' strategy to cope with an aggressive social stimulus. This interpretation found confirmation in the Resident-Intruder Test, which revealed a significant effect of early environment on aggressive-like behaviour. Stress mice, when compared to the Novelty group, displayed shorter latency to the first attack and higher number of attacks toward their intruder, leading us to conclude that early adverse experiences (modelling early maltreatment in humans) can represent a risk factor for developing enhanced aggressive behaviour later in adulthood. Specifically, Stress mice showed increased impulsive aggression, defined as 'a form of aggression characterized by high levels of autonomic arousal and precipitation by provocation associated with negative emotions such as anger or fear' (Siever, 2008). Fear could represent, in our case, the precipitation factor for triggering

aggressive behaviour. Impulsive aggression is different from premeditated aggression; the latter is also defined as instrumental or predatory aggression and responds to clearly planned goals. Stress mice do not show predatory aggression since they behave normally when exposed to 'innocuous' social stimulus, as was shown during Social Preference Test, during Resident-Pup Intruder Test or during Social Interaction Day1 and Day3. On the contrary, when encountering a CD1 aggressive mouse, either as intruders (Social Interaction Test Day2) or as residents (Resident-Intruder Test), Stress mice display increased aggressive behaviour, compared to their littermate Novelty mice.

The outcomes of our study contribute to corroborate human data, according to which individuals with history of early maltreatment are likely to develop pathological violence and abnormal aggressive behaviour (Veenema et al., 2006; Siever, 2008; Mead et al., 2010). Recent studies have explored the role of maltreatment in exacerbating vulnerability for child antisociality. Interestingly, similar environmental risk exposures to violence do not affect all children equally: some individuals perpetuate violence, some others develop depression, and others appear to be not affected at all, displaying normal behaviour (Cicchetti & Toth, 1995; 2005). These observations clearly support a key role for a complex interplay of genetic vulnerability and maltreatment risk exposure on antisocial outcomes among children (Mead et al., 2010), suggesting that Gene X Environment interaction may be responsible for effects that are even bigger than the main effect of gene or environment alone. A supportive example, in this regard, comes from the pioneer studies of Moffitt on serotonin transporter and monoamine oxidase type A (MAO-A) polymorphisms, showing how

variants in such genes can confer susceptibility and interact with childhood maltreatment to predispose to violence (Caspi et al., 2002; Kim-Cohen et al., 2006).

5.3 Role of BDNF in modulating stress effects

Genetic factors play a determinant role in the modulation of environmental effects, by conferring susceptibility for developing mood disorders and antisocial behaviours. The Juvenile Chronic Social Stress Paradigm we established has been used as a tool to screen *Bdnf* heterozygous null mutation as a possible moderator factor of long-term behavioural effects. Unfortunately, we were not able to identify any Gene-by-Environment interaction in our model. This could be explained by the main effect that the genotype itself had on aggression, the behaviour mainly influenced by our Juvenile Chronic Social Stress Paradigm. Heterozygous *Bdnf* knockout mice, in fact, are known to have increased aggression (Lyons et al., 1999). A recent study showed that aggressive behaviour is significantly increased also when *Bdnf* is selectively knocked out exclusively from the hippocampal region CA3, identifying a new specific neural correlate for aggression (Ito et al., 2011).

In human studies, as well, BDNF Val66Met polymorphism was associated with increased aggression in a population of schizophrenic patient (Spalletta et al., 2010), confirming a role for this neurotrophic factor in influencing aggressive behaviour.

Thus, we hypothesize that the *Bdnf* heterozygous null mutation may have been associated with a ceiling effect in our aggression tests, possibly masking an interaction with early adverse environment. Nevertheless, the paradigm may still be useful to screen additional genetic mutations as candidate risk factors for developing mood disorders and behavioural deficits, such as MAO-A or serotonin transporter, already known for their modulatory effect on early environmental risk factors in rodents and primates (Kim-Cohen et al., 2006; Reif et al, 2007).

6. Conclusion

In conclusion, we established in this study a new Juvenile Chronic Social Stress Paradigm as a model for early maltreatment in humans. Our paradigm, based on daily exposure to psychological and physical aggression, resulted to have long-lasting effects on neural circuits affecting aggressive-like behaviour. Mice subjected to the Stress Paradigm during juvenile period, in fact, showed later in life increased aggression in Resident-Intruder Test, when compared to control littermates. These results allowed us to consider the Paradigm a good tool for screening genetic variants as possible modulator of environmental effects, in a Gene-by-Environment interaction model.

The gene of choice for our study was brain-derived neurotrophic factor because of its robust association with mood disorders and its key role in neural circuit

maturation. However, we failed to identify a role for Bdnf in modulating the effect of early environment, probably because of a ceiling effect due to the aggressive phenotype that Bdnf heterozygous null mutant mice display. This abnormal aggression intrinsic to Bdnf mutant mice, may have masked the effects of a possible Gene-by-Environment interaction.

Nevertheless, this new Juvenile Chronic Social Stress Paradigm potentially remains an useful tool to better understand themolecular mechanisms by which early maltreatment programs adult behavioural strategies and in order to investigate genetic variants as possible candidate modulators of these long-term environmental effects; other genetic mutations, however, need to be screened in order to validate our paradigm as a powerful Gene-by-Environment interaction model.

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