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A new 3-hit mouse model of schizophrenia built on genetic, early and late factors

Running title: a new 3-hit mouse model of schizophrenia

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Abstract

Whether the etiology of schizophrenia remains unknown, its multifactorial aspect is conversely now well admitted. However, most preclinical models of the disease still rely on a monofactorial construction and do not allow discover unequivocal treatments, particularly for negative and cognitive symptoms. The main interaction factors that have been implicated in schizophrenia are a genetic predisposition and unfavorable environmental factors. Here we propose a new animal model combining a genetic predisposition (1st hit: partial deletion of *MAP-6 (Microtubule-associated protein)*) with an early postnatal stress (2nd hit: 24h maternal separation at postnatal day 9), and a late cannabinoid exposure during adolescence (3rd hit: tetrahydrocannabinol THC from post-natal day 32 to 52; 8 mg/kg/day). The 2-hit mice displayed spatial memory deficits, decreased cortical thickness and fractional anisotropy of callosal fibers. The 3-hit mice were more severely affected as attested by supplementary deficits such a decrease in spontaneous activity, sociability-related behavior, working memory performances, an increase in anxiety-like behavior, a decrease in *hippocampus* volume together with impaired integrity of *corpus callosum* fibers (less axons, less myelin). Taken together, these results show that the new 3-hit model displays several landmarks mimicking negative and cognitive symptoms of schizophrenia, conferring a high relevance for research of new treatments. Moreover, this 3-hit model possesses a strong construct validity, which fits with gene x environment interactions hypothesis of schizophrenia. The 2-hit model, which associates maternal separation with THC exposure in wild-type mice gives a less severe phenotype, and could be useful for research on other forms of psychiatric diseases.

Keywords

Psychosis, MAP6, multiple-hit, stress, memory, sociability

Introduction

Affecting 1% of worldwide population, schizophrenia is the most frequent chronic psychosis. Patients may present several symptoms (positive and negative symptoms and cognitive deficits), with varying intensities and outcomes (Davidson and McGlashan, 1997). In human, sex ratio is 1.4 for schizophrenia (60% men, 40% women), men being affected earlier and with more severe symptoms (more negative symptoms and cognitive deficits), and more structural and functional brain abnormalities (Leger and Neill, 2016; Leung and Chue, 2000). While etiology is not fully understood, it is now undoubted that schizophrenia results from complex gene x environment interactions (Millan et al., 2016; van Os et al., 2010). Further, among possible causes of the disease, most clinicians agree on the long-standing model of 'stress vulnerability'. It suggests that individuals possess different levels of vulnerability to schizophrenia, determined by a combination of biological, social and psychological factors. A genetic vulnerability could be revealed and would favored schizophrenia appearance in case of an association with early stress (alimentary deficiency during pregnancy, mother stress ...) and late environmental factors (urban birth, heavy cannabis use ...)(Di Forti et al., 2019; Millan et al., 2016).

Efficiency of currently market drugs are only symptomatic. Besides, they mostly target positive symptoms, while negative and cognitive symptoms are often ignored (Murphy et al., 2006). Among the main possible explanations is the relevance of the animal models and, consequently, the lack of confidence of clinicians in the efficiency of drugs that emerge from animal experiments. Overall, there is a consensus for refinement of preclinical research (Burrows and Hannan, 2016; Moore, 2010). Indeed, good construct validity in animal models of schizophrenia is mandatory (Jones et al., 2011; Young et al., 2012) and could be achieved by resorting multiple interventions (Ellenbroek, 2003; Sullivan et al., 2012). Unfortunately, 2- or 3-

hit interventions are rarely investigated in preclinical research(Hida et al., 2013). Herein, we aimed at characterizing a mouse model of schizophrenia based on the combination of three factors: genetic alteration, early stressing event and a late environmental factor. Among candidate genes having been so far associated to schizophrenia, MAP6 revealed several interests(Marchisella et al., 2016; Schloss and Lau, 2012; Shimizu et al., 2006). The gene encoding MAP6 proteins is localized in the human 11q14 chromosomal region containing many susceptibility genes for schizophrenia(Lewis et al., 2003) and polymorphisms in gene MAP6 have been associated with schizophrenia(Shimizu et al., 2006). MAP6 protein expression has been shown to be altered in schizophrenia(Choi et al., 2009) as well as in autism(Wei et al., 2016). Indeed, at the biological level, MAP6 (microtubule-associated protein 6, also known as *stop* for stable-tubule-only-polypeptide) belongs to genes involved in neuron architecture, synaptic plasticity and brain development(Deloulme et al., 2015). Whether full deletion of *map6* gene (MAP6^{-/-}) induces several dysfunctions considered as landmarks of schizophrenia (glutamatergic and dopaminergic neurotransmission alterations), neuronal disconnectivity and severe behavioral defects(Bouvrais-Veret et al., 2008; Brenner et al., 2007; Gimenez et al., 2017) heterozygous mice (MAP6^{+/-}) appear contrastingly only slightly affected with cognitive and social impairments(Merenlender-Wagner et al., 2014, 2010; Volle et al., 2013). MAP6^{+/-} mice were thus proposed as a translational animal model for the genetic ultra-high-risk states of schizophrenia(Volle et al., 2013). The validity of the model was supported by the exacerbation, through severe early stressful events (chronic maternal deprivation from PND 2 to 14) of the phenotype of MAP6^{+/-} mice which become then markedly altered (notably in locomotor reactivity to either acute mild stress or psychostimulant challenge)(Volle et al., 2013). Hence, whether MAP6^{+/-} mice enabled promising results, in line with the stress vulnerability theory, their reactivity to a combination of an early stress event with late environmental factor remains

to be investigated. Early stress event can be achieved through a 24h maternal separation when pups age of 9 days (Ellenbroek et al., 1998). This model induces an altered brain neurotransmission (Marco et al., 2015), associated with exacerbated behavioral responses to pharmacological potentiation of dopaminergic system (Rots et al., 1996; Zimmerberg and Shartrand, 1992). Besides, it induces a social withdrawal in adult mice (Bouet et al., 2011) (one of the negative symptoms of schizophrenia). Finally, considering late environmental factor, growing number of evidences from epidemiological studies argues that consumption of THC (from cannabis) particularly during adolescence is associated with a two-fold increased risk of later psychotic disorder (Arseneault et al., 2002; Di Forti et al., 2019; Rubino and Parolaro, 2014). In preclinical studies, persistent changes in cognitive and emotional processes were described in adult animals after a chronic THC treatment during adolescence (Cha et al., 2006; Rubino et al., 2009). Therefore, three groups of animals were constituted: wild type mice with no environmental manipulation (control mice, n=13), wild type mice submitted to both maternal separation and tetrahydrocannabinol exposure (THC from PND32 to 52) (2-hit group, n=12), and heterozygous MAP6^{+/-} mice submitted to maternal separation and THC exposure (3-hit group, n=7). The possible differences that can be obtained between males and females in such a sophisticated model, as previously reported (Kekesi et al., 2015), would have introduced again another factor. Therefore only males were used in the present study. Several behavioral tests have been selected to assess positive-like (actimeter and open field), negative-like (approach avoidance, anhedonia), and cognitive-like symptoms (spontaneous alternation, Morris water maze). Moreover, high and low resolution (*ex-vivo* and *in-vivo*, respectively) magnetic resonance imaging (MRI) were performed to assess anatomical changes (volume, thickness, and fibers integrity of white matter) in 3 different regions of interest (*hippocampus*, parietal cortex and *corpus callosum*) (Wolf et al., 2008). Finally, given that GABA neurotransmission has been shown

to be affected in schizophrenia(Gonzalez-Burgos et al., 2011; Nakazawa et al., 2012a), and that investigating parvalbumine cells (PV+ cells) is particularly relevant in preclinical, neurodevelopmental, models of schizophrenia(Schmidt and Mirnics, 2015), we performed immunohistochemistry to label and measure PV+ cells density of GABAergic interneurons in *hippocampus* and prefrontal cortex(Du and Grace, 2016; Giovanoli et al., 2014; Nakazawa et al., 2012b; Volk and Lewis, 2014). Our study showed that the partial deletion of *map6* gene, combined to an early stress (maternal separation) and to a chronic exposure to THC during adolescence, induced memory impairments and social behavior disorders that mimic some signs of schizophrenia. This behavioral phenotype came with structural alterations in *corpus callosum* fibers and a decrease in cortical thickness.

Methods

Animals

Heterozygous MAP6^{+/-} male mice and their wild type littermates MAP6^{+/+} were generated from C57Bl6/129SvPas F1 animals (Andrieux et al., 2002). MAP6^{+/-} males were mated with C57Bl6 females and pups were thereafter bred in standard laboratory cages (n=5 per cage). All animals were housed in a normal 12h light-dark room (light on at 8am), with regulated temperature (21±1°C) and humidity (55±10%), and with free access to food and water. At weaning, end tail collection was performed in all mice under anesthesia (isoflurane 5%), for genotyping as previously described (Volle et al., 2013). Experiments were performed in accordance with French and European Economic Community guidelines for the care and use of laboratory animals (2010/63/UE) and authorized under the N°APAFIS#8203-2016120817103795v4.

Animal groups and treatments

At 9 day of age, litters were separated from their dam and placed in a nearby cage during 20s (control group) or 24h (early environmental stress) (Bouet et al., 2011). From P32 to P52, mice received daily intraperitoneal injection of either vehicle (3.5% Tween 80 in serum solution) or tetrahydrocannabinol (THC; 8 mg/kg, subcutaneous, Sigma Aldrich®) and assigned to either control or 2-hit group, and respectively 3-hit group (Fig 1). Such a treatment has been shown to induce behavioral and neurotransmitter systems alterations in genetically modified mice (COMT KO) (Behan et al., 2012; C. M. P. O'Tuathaigh et al., 2010). There was no significant mortality related to any of the three hits or to their combination.

Behavioral assessment

After a one week washout period, a set of behavioral tests was performed (Fig 1) by an experimenter blind to group genotype and/or treatment.

Black and white box (LE810, Leticia[®]) was used to assess anxiety-like behavior (Lelong-Boulouard et al., 2006). The apparatus consisted in a large white illuminated compartment (28cm×27cm×27cm, 900 lux) connected by a small opening to a small dark compartment (17cm×27cm×27cm, 100 lux, red light). Mice were free to explore for 5 min during which the time spent in each boxes and the number of transitions between the two were recorded.

Actimeter. Spontaneous locomotor activity was measured in a restrained environment through an automated actimeter (Imetronic[®]) (Since et al., 2013). Mice were individually placed in standard cages (19cm×11cm×14cm), under two lines of infrared beams. Numbers of beam crossing reflecting locomotor activity were automatically counted during 1h.

Open field. Mice were individually placed for 30min in a white and squared (50cm×50cm×50cm) open arena. Total travelled distance and percentage of time spent in the center part of the arena were automatically collected (Ethovision, Noldus[®]).

Y-maze task was used as previously described (Bazin et al., 2009), to assess spatial working memory. Each mouse was placed for 5 min in the maze, consisting of three equally spaced arms (each sized 22cm×6.5cm×10cm) made of black-painted wood. Number and sequence of arm entries (four-paw criterion), as well as number of rearing were collected. Alternation percentage was calculated by the formula: $\text{number of alternation} / (\text{total number of entries} - 2) \times 100$.

Approach avoidance task allowed to assess social behavior (Bouet et al., 2011). Briefly, the apparatus consisted of a testing compartment (27cm×18cm×21cm) with three interconnected chambers and a compartment for the stimulus animal (6.5cm×18cm×21cm), the two being separated by a transparent plastic perforated wall to ensure visual and olfactory communication. The tested mouse was placed in the center of the testing compartment and the stimulus mouse was placed in the other compartment for 5-min. The stimulus animal was a 45 days old mouse, different for each trial. The time spent by the tested mouse in each of the three

parts (i.e. close, middle, or far from the stimulus mouse) was collected during 5 min. Sociability index was calculated by the formula: (time spent close to the stimulus mouse) – (time spent in the opposite compartment from the stimulus mouse).

Sucrose preference test(Pozzi et al., 2014). Animals were placed one by cage for the duration of the experiment. After measurement of water intake on a 24h period, two bottles (water and sucrose 2%) were simultaneously proposed during a 72h period. Anhedonia was estimated through sucrose preference, calculated with the following formula: (sucrose solution intake/total intake) × 100.

Nesting behavior(Deacon, 2006)

Mice were individually housed for this test, performed just after the previous one. One hour before the dark phase, mice were provided one Nestlet each (without any other enrichment item). The morning after, the nests were assessed and a score between 0 and 5 was attributed(Deacon, 2006).

Morris water maze (MWM) consists of a circular maze (120cm diameter), filled with 21°C opaque water(Bouet et al., 2007). A squared Plexiglas platform (13cm×13cm) was hidden 0.5cm below the surface of water in a fixed position. Swim paths were automatically monitored (Ethovision, Noldus®). Learning phase consisted in 4 trials/day (90s each, 20s of reinforcement and 1h inter-trial interval) during 5 consecutive days. A probe trial (no platform, 60s) was performed 1h after the final trial of learning session.

MRI examinations were performed on a 7T Pharmascan (Bruker®, Biospin, Paravision 6.0), with a mouse brain specific surface coil. To ensure mechanical constraints of the skull for anatomical measures, anatomical T2-weighted images were collected in anesthetized animals (isoflurane: 1-1.5% in N₂O/O₂: 2/1) through a 8min fast spin-echo sequence (RARE8, TE=42ms; TR=5000ms; 4 averages; FOV 2cmx2cm; 10 contiguous slices; matrix: 256x256). Surface of bilateral

hippocampus on 6 consecutive images was manually delineated and multiplied by slice thickness (0.5mm) for volume measurements. Cortical thickness was determined through 8 measures within the medial parietal cortex (4 on each side, equidistant from middle plane), as previously described (Duque et al., 2012). Thereafter, mice ($n=5/group$) were deeply anesthetized with isoflurane (5%) and transcardially perfused with phosphate buffer saline (PBS 0.1M) followed by a 4% paraformaldehyde solution. Brains extracted from the skull were embedded in gelatin (2%). Diffusion-weighted images were obtained from 20 directions, which were collected through 105 min sequence (TE=35ms; TR=3000ms; FOV 14.9mmx14.9mm; 41 contiguous slices; matrix: 128x128; factor $b=1000s/mm^2$). Diffusion maps of anisotropy fractional (FA) as well as radial and mean diffusivities (RD and MD in mm^2/s , respectively) were extracted in the surface of the manually delineated (FSL software[®]) corpus callosum of each mouse. Loss of integrity was characterized by decreased FA (axonal degeneration) as well as increased RD and MD (demyelination) (Beaulieu, n.d.; Leroux et al., 2013).

Immunohistochemistry.

After being used for *ex vivo* MRI, brains were coronally sectioned (40 μ m thickness) on a vibratome apparatus (VT1000S, Leica[®]). Free-floating sections were washed in PBS 0.1M and incubated 1h in 0.3% H₂O₂/50% methanol/0.3% triton in PBS 0.1M. After rinsing, sections were incubated 1h in 5% normal horse serum/0.3% Triton X 100 in PBS 0.1M before adding 1:1000 primary antibody (monoclonal mouse IgG anti-parvalbumin, Swant[®]) for 48h, 4°C incubation. After rinsing, sections were incubated for 90 min at room temperature with 1:200 secondary antibody (biotinylated horse anti-mouse IgG, Vector Laboratories[®]). ABC staining system (Vectastain ABC, Vector Laboratories[®]) was used for staining and peroxidase activity was detected with diaminobenzidine solution (0.05% DAB/0.01% H₂O₂ in PSB 0.1M). Sections were scanned on Aperio high-resolution scanner (ScanScope[®]). Density of PV+ cells was counted in

sub-regions of bilateral prefrontal cortex (Infralimbic IL, prelimbic PrL and cingulate CgL) and of bilateral dorsal *hippocampus* (*Cornus Ammonis* CA1, CA2-3 and *Dentate Gyrus* GD)(Paxinos and Franklin, 2012).

Statistics

Statistical analyses were performed with JMP® to detect differences between the three groups control, 2-hit, and 3-hit (see Fig 1). Except for the MWM for which ANOVA followed by post-hoc test were performed (Fisher PLSD), all data were analyzed with Kruskal-Wallis followed by Dunn's Multiple Comparison Test. Wilcoxon Signed Rank Test was used to compare data to a theoretical value. We conducted intra-group Pearson correlation analyses in the three groups (3-hit, 2-hit and control) to assess possible relationships between brain morphometry data (cortical thickness, volume of the *hippocampus* and values of FA and RD in *corpus callosum*) with behavioral data (total distance and percentage of time spent in the center of open field, time spent in the white box, alternation percentage in Y-maze, sociability index and time spent in target quadrant of MWM), which were significantly different between groups ($p < 0.05$) or with a strong tendency $p < 0.065$).

Results

Behavioral assessment

Anxiety-like behavior

The number of transitions between the two boxes differed between groups (Kruskal-Wallis, $p = 0.043$, Figure 2A). The activity in 2-hit and 3-hit was lower than controls but the large variability observed in the 2-hit group data probably account for an absence of significant difference between pairs of groups with the Dunn's test. Time spent in the white box did not differ between groups (Kruskal-Wallis, $p = 0.303$) attesting for no increased anxiety-like behavior.

Spontaneous activity

In the actimeter box, the number of infrared beam interruptions did not differ between animal groups (Kruskal-Wallis, p value=0.733). On the contrary, when animals were tested in the open field arena, there was a global difference between groups (Kruskal-Wallis, $p=0.021$). Dunn's Test revealed a lower horizontal activity for 3-hit animals compared to control ($p=0.008$) and to 2-hit ($p=0.021$) animals (Fig 2B). Moreover, the percentage of time spent in the center of the open field differed between groups (Kruskal-Wallis, $p=0.028$). Further Dunn's multiple comparison showed a decrease in percentage of time spent in the center for 3-hit animals compared to controls ($p=0.002$) (Fig 2C).

Spontaneous alternation

The number of visited arms in the Y-maze did not differ between groups (Kruskal-Wallis, $p=0.189$). Global analysis did not show a difference in spontaneous alternation between groups (see Fig 2D) ($p=0.464$). The number of rearing in the Y-maze did not differ between groups (Kruskal-Wallis, $p=0.206$). However, Wilcoxon signed rank test revealed that control and 2-hit animals had significant memory performances (*i.e.* alternation significantly above theoretical value of 50%; respectively, medians of 63.2 and 65.0%; $p=0.025$; 0.010), but not 3-hit animals (55.6% and $p=0.218$). The 3-hit therefore displayed deficits in the alternation task.

Approach avoidance

Although no group difference was observed for the sociability index (Fig 2E, Kruskal-Wallis, $p=0.122$), only control animals displayed a value significantly above zero (Wilcoxon signed rank test, $p=0.036$). Although sociability index was very close to the threshold for 2-hit animals ($p=0.054$), 3-hit animals did not prefer the compartment close to the stimulus mouse (negative sociability index, not different from zero $p=0.578$). Hence, while it was normal and close-to-

normal for control and 2-hit animals, respectively, social behavior was severely impaired in 3-hit mice.

Sucrose preference

There was no difference between groups in total water intake (Kruskal-Wallis, $p=0.286$). Besides, when the choice between water and 2% sucrose was given, all groups had a similar behavior (no difference between groups: Kruskal-Wallis, $p=0.106$) and significantly preferred sucrose (Wilcoxon signed rank test, control: $p<0.0001$, 2-hit: $p<0.001$; 3-hit: $p=0.031$).

Nesting behavior

Mice built nests with the cotton Nestlet during the dark phase. All the three groups obtained scores above 4 and there was no global difference between the groups (Kruskal-Wallis, $p=0.128$).

Morris water maze

Spatial learning performances revealed a significant time-dependent decrease in distance travelled to reach platform (ANOVA, time effect $p<0.0001$), but neither groups difference (group effect $p=0.882$), nor interaction between the two ($p=0.998$) (Fig 2F). Even if groups' performances appear similar (with a decrease of the time to reach the platform over trials), further analysis of individual performance showed different proportions of animals within three groups which still displayed a maximum latency to find the platform at the last day of learning (D5): 7.6% in controls, 25% in 2-hit, and 28.5% in 3-hit. Those animals ceased swimming and floated until the limit obtaining a low distance but a high latency, particularly the two last days of learning (D4 and D5). Besides, this floating behavior was increasing along trials. Concerning the time in quadrants during probe test, performed 1h after the last learning trial, there was a global difference between the groups (ANOVA, group effect $p=0.006$), a global difference between quadrants ($p<0.001$), and no significant interaction ($p=0.147$)(Fig 2G). Fisher PLSD

revealed differences between control and 2-hit ($p=0.002$) and between control and 3-hit ($p=0.036$), but not between 2-hit and 3-hit ($p=0.526$). Moreover, the comparison of the time spent in target quadrant with the theoretical value of 15s showed that only control animals stayed more than 15s in the target quadrant (univariate t test, $p=0.011$ for control, $p=0.267$ and $p=0.398$ for 2-hit and 3-hit groups, respectively)(Fig 2H). Therefore, only control animals had significant reference memory performances 1h after the last learning trial even if all groups had similar performances during learning.

Immunohistochemistry

There was no group difference in the density of PV+ cells, neither in the prefrontal cortex, nor in the *hippocampus* ($p=0.961$ and 0.402 , respectively). Furthermore, there was no group difference whatever the sub-region considered in each of these brain structures (IL: $p=0.532$; PrL: $p=0.811$; CgL: $p=0.543$; CA1: $p=0.311$; CA2-3: $p=0.691$; GD: $p=0.618$) (Fig 3A and 3B).

Brain morphometry

A statistical group difference was observed for cortical thickness ($p=0.037$, Fig 3C), with 2-hit and 3-hit groups significantly smaller than control group (Wilcoxon, $p=0.035$ and $p=0.045$ respectively). Besides, the p value obtained when hippocampal volumes were compared between the groups, $p=0.065$ was close to the threshold (Fig 3D), with 3-hit group displaying smaller hippocampal volume than 2-hit group ($p=0.022$). White matter imaging revealed a significant group difference in *corpus callosum* FA and RD values ($p=0.036$ and $p=0.048$, respectively) (Fig 3E and 3F). Both 2-hit and 3-hit groups showed lower FA values compared to control groups ($p=0.037$ and $p=0.037$, respectively), whereas only 3-hit group showed a significant increase in RD compared to control ($p=0.037$) (Fig 3E and 3F).

Correlations

Correlations analyses showed a significant, negative correlation between RD values in corpus callosum and sociability index ($p=0.0037$, $r=-0.98$; r : correlation coefficient) in 2-hit group. All others correlations for each group were not significant.

Discussion

The aim of the study was to characterize the phenotype of mice submitted to three different hits known to be risk factors for the development of schizophrenia. The combination of a partial deletion of MAP6 (1st hit) with an early life stress (caused by maternal separation, 2nd hit) and a chronic exposure to THC during adolescence (3rd hit) induced behavioral deficits that mimic some signs of schizophrenia. Indeed, our results showed an increased anxiety only in few tests (decreased time in the center in open field, decreased activity in black and white box and open field) suggesting the 3-hit mice are probably more sensitive to stressing environments than control mice. Indeed, the fact that a difference between groups was observed in open field and in black and white box but not in actimeter suggests that animals' emotionality, and particularly state anxiety, is different between control and 3-hit animal, more than spontaneous activity *per se*. In open field, and black and white box, the environment is considered to be aversive because of the light and the unknown environment (Lezak et al., 2017; Bourin et al., 2015). In the actimeter, the environment is more familiar for the animal because the box is small, surmounted by a rooftop, and, moreover, because the test duration is long (1h) novelty and related stress decrease with time (Lister et al., 1990). The 3-hit mice also displayed a decreased index of sociability and working memory deficits as well as long-term memory spatial impairments (and, in another cohort of 3-hit animals, deficit in PPI, see supplementary method and data). Imaging study revealed alterations in *corpus callosum* fibers and a decrease in cortical thickness. To our knowledge, even if additional experiment have to be performed, this is the first study presenting such a construct validity in a mouse model, based on the most recent multifactorial theories of schizophrenia (Millan et al., 2016). Combining factors is one of the major issues to build an animal model of schizophrenia with a powerful validity (Ayhan et al., 2016; Robertson et al., 2006; Winship et al., 2019). Our hypothesis was that the genetic deletion

would induce an increased vulnerability to the two other factors. The first hit, considered herein, is a partial deletion of *map6*. Microtubule-associated proteins have a fundamental role in neuronal growth, brain connectivity, synaptic vesicles structure and both short- and long-term plasticity (Andrieux et al., 2002; Deloulme et al., 2015; Gimenez et al., 2017; Peris et al., 2018). Previous study using heterozygous MAP6^{+/-} mice (Merenlender-Wagner et al., 2010; Powell et al., 2007; Volle et al., 2013) reported deficits in social interaction and learning and a slight impairment in sensorimotor gating without drastic alteration in global activity. In another cohort of MAP6^{+/-} mice (Supplementary data 1), we did not observe deficits spontaneous alternation, sociability, anxiety- and depressive-like behavior. Taken together, these observations are consistent with data obtained in our study, and confirm the slight influence of the 1st hit (partial genetic deletion) to social deficits. Interestingly, Volle *et al.*⁵² also showed that MAP6^{+/-} mice are more sensitive to an early stress induced by maternal deprivation (daily for 6h between postnatal days 2 and 14). The second hit, maternal separation, is known to increase dopaminergic system functioning²⁰, increase anxiety level, and decrease social behavior (Bouet et al., 2011; Rentesi et al., 2013), such as observed in 3-hit animals. Concerning the 3rd hit, *i.e.* adolescence chronic THC exposure, animals studies showed that it induced deficits in PPI and in working and spatial memory (Quinn et al., 2008; Renard et al., 2014; Rubino et al., 2009; Schneider and Koch, 2003), in social interaction (Boucher et al., 2007; Zamberletti et al., 2012), and in anxiety level (O'Shea et al., 2004). More precisely, chronic THC (or cannabinoid agonists) exposure during adolescence has been shown to cause cognitive - learning and memory - deficits (working memory, object recognition), in connection with impaired structural and synaptic plasticity in brain regions (Ballinger et al., 2015; Keeley et al., 2015b; O'Tuathaigh et al., 2012). Besides, social behavior and hedonia are also affected (Renard et al., 2016). Interestingly, as reported herein, spatial memory performances in the Morris water maze task seem to be

relatively preserved by cannabinoid exposure (at least the learning phase), and data on anxiety-related behaviour are controversial (Keeley et al., 2015a). Those effects, or part of them, could be involved in the deficits observed in 3-hit animals.

In our conditions, it was not possible to identify all the interactions between factors that take place in the 3-hit mice. However, comparison between 2-hit and 3-hit groups showed a possible genetic vulnerability to the two supplementary environmental factors. The ending phenotype of the 3-hit would be the results of interactions between factors. In regards to literature, it is realistic to consider interactions between genetic and environmental factors, and particularly that the partial deletion of *map6* induces a more pronounced vulnerability to the two additional factors (Volle et al., 2013). This is indeed what is suggested by most behavioral results obtained here (3-hit are impaired but not 2-hit except for the MWM) and by the alterations in volume of the *hippocampus* and radial diffusivity in the *corpus callosum* (decreased in 3-hit but not in 2-hit animals). First, as already shown (Powell et al., 2007), the genetic deletion in itself does not affect the volume of the *hippocampus*. Second, the combination of maternal separation with THC exposure does not affect neither the volume of the *hippocampus* (2-hit). However, in 3-hit animals, the volume of *hippocampus* decreased, accounting for a genetic vulnerability rendering more sensitive to the two subsequent factors. For other parameters, the vulnerability does not change the story, *i.e.* 2-hit and 3-hit displayed similar deficits. This was the case for probe test performances in the MWM, cortical thickness, and fractional anisotropy in *corpus callosum*. Consequently, these three parameters could be impaired because of maternal deletion and/or THC exposure. Interestingly, social index negatively correlated with radial diffusivity index in 2-hit group, but not in control or in 3-hit groups. Thus, in 2-hit group, animals having the lowest sociability index (the most behaviorally impaired) are those having the highest RD value in the *corpus callosum* (*i.e.* the lowest myelination index). This result suggests that altered callosal

fibers are associated with social behavior impairments. A parallel can be done with clinical symptoms; indeed, an increase in radial diffusivity in the *corpus callosum* has been recently related to socio-functional indices in schizophrenic patients (Koshiyama et al., 2018). Surprisingly, no significant correlation was observed in 3-hit animals. But, one should note that - contrary to 2-hit animals - integrity of *corpus callosum* was significantly altered in 3-hit group. Such structural atrophy would have prevented any chances to highlight correlation with behavioral parameters (Deloulme et al., 2015). Even if the respective effects of these two different hits could not be inferred from our study, it is possible that maternal separation and chronic THC, and interactions between the two, have impaired cognitive performances as previously reported with other factors combined with THC (Cha et al., 2006; Nikolaos P. Daskalakis et al., 2013; Rodríguez et al., 2017). Indeed, Zamberletti *et al.* (Zamberletti et al., 2012) showed that maternally deprived rats became more sensitive to a further THC exposure during adolescence. Interactions between genetics and effects of THC have ever been reported, for instance by the observation of strain differences for the effects of THC (Deiana et al., 2007; Keeley et al., 2015b), some strains being more sensitive than others. This has been also observed in animals with deletion of other genes particularly relevant for schizophrenia, *i.e.* Neuregulin 1 (Andrieux et al., 2002; Boucher et al., 2007), COMT (Behan et al., 2012; C. M. O'Tuathaigh et al., 2010; O'Tuathaigh et al., 2012), DISC-1 (Ballinger et al., 2015). These experiments argue for the potential genetic vulnerability for the effects of cannabis. Even if there are several studies on the effects of two hits, investigations on the effects of three different hits are very scarce. To our knowledge, only three publications (two in rats, one in mice) reported combination of three different hits. Chen et al. (Chen et al., 2011) combined repeated maternal separation to avoidance conditioning procedure and PCP administrations in rats, and observed complex interactions, such as alteration PPI by some hits, but prevention by other hits. Kekesi et

al.(Kekesi et al., 2015) combined in rats selected breeding based on pain sensitivity to subchronic ketamine and isolation after weaning, which lead to deleterious effects on cognition, particularly in males. Finally, Sultana and Lee(Sultana and Lee, 2020), combined in mice a genetic mutation (affecting DISC1), to repeated maternal separation and ketamine injections, and reported that the genetic component is predominant to induce schizophrenia-like behavioral phenotypes. These studies are not directly comparable to ours because the hits are quite different, but they highlight the complexity of interactions between factors, some of them being suspected to render more vulnerable to other subsequent hits, while some of them being suspected to promote resilience by preparing to future challenges, as described by Daskalakis et al.(N P Daskalakis et al., 2013).

To conclude, we report here an innovative 3-hit animal model of schizophrenia that could be helpful for schizophrenia research. Given this is a first investigation, further studies are needed to reinforce its knowledge. This model mimics negative-like symptoms (decreased activity in open field, increased stress and anxiety, social withdrawal) and cognitive dysfunctioning (working memory, long term reference memory), in association with a decrease in cortical thickness and impaired integrity of *corpus callosum* fibers. This 3-hit model presents a high construct validity, in relation with epidemiological data. Thereafter, other issues inherent to animal model obtained by combination construct could now be raised, notably the question of respective contribution of each factor to the ending phenotype. However, this innovative 3-hit mouse model based on vulnerability-stress hypothesis(van Os and Kapur, 2009) constitutes to date a powerful tool to mimic a schizophrenia phenotype characterized by predominance of negative and cognitive symptoms. The 2-hit model, which associates environmental stresses, gives a less severe phenotype, and could be useful for research on schizophrenia but also other forms of psychiatric diseases(Pugliese et al., 2019). The new 3-hit model presents satisfying

construct and phenomenological validities, and further studies are needed to confirm these results and to bring a predictive validity to the model by assessing the effects of typical and atypical antipsychotics.

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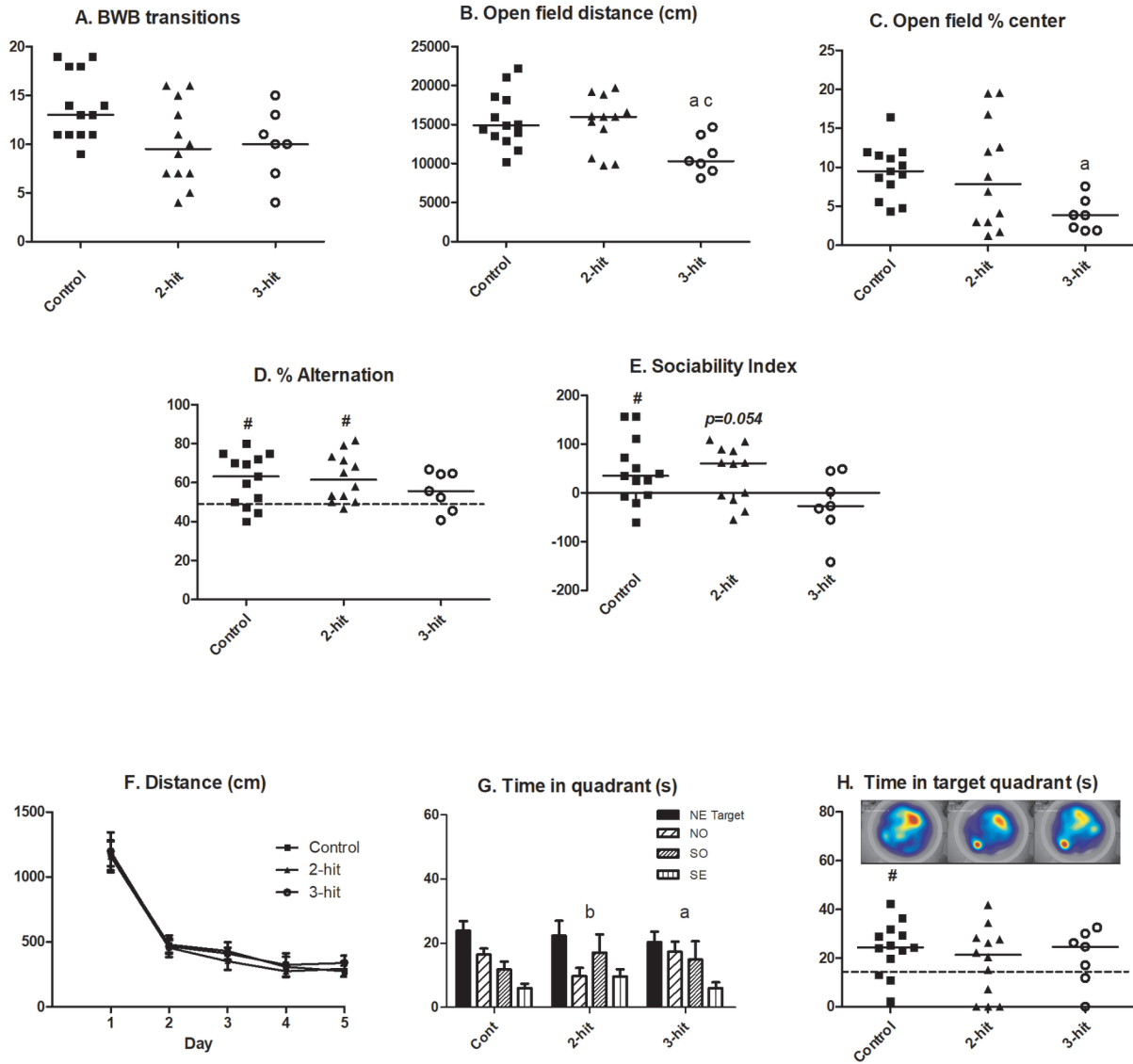
Figure legends

Figure 1. Experimental design and animal groups setting (MAP6: microtubule-associated protein, THC: tetrahydrocannabinol, MRI: Magnetic Resonance Imaging).

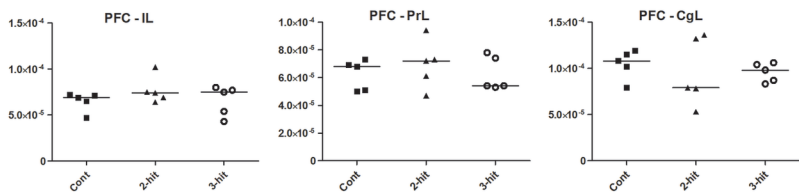
Figure 2. Behavioral effects of 2-hit and 3-hit combinations. A. Number of transitions in the black and white box (BWB). B. Distance (cm) covered in open field during 30 min. C. Percentage of time in the center of the open field. D. Percentage of alternation percentage in Y-maze (horizontal broken: 50%). E. Sociability index. F. Distance (cm) travelled in the MWM across days of learning. G. Time spent in each quadrant (s) during the probe test of MWM. H. Time spent in the target quadrant (s) during the probe test of MWM (horizontal broken: 15s). # indicates a significant difference from 50% (Fig 2D), from 0 (Fig 2E), or from 15s (H). Intergroups differences ($p < 0.05$) were signed as a (control vs 3-hit), b (control vs 2-hit), and c (2-hit vs 3-hit). Data are scatter dot plot and median for Fig 2A, 2B, 2C, 2D, 2E, 2H, and mean \pm SEM for Fig 2F and 2G.

Figure 3. Effects of 2-hit and 3-hit combination on parvalbumin-positive cells (PV+) density and imaging parameters. A and B. Density of parvalbumin-positive cells in the prefrontal cortex and *hippocampus*, respectively. C and D. Parietal cortical thickness and volume of the *hippocampus* from in vivo anatomical images (T2). E and F. Values of fractional anisotropy (FA) and radial diffusivity (RD) in *corpus callosum* from *post-mortem* diffusion-weighted images. Data are scatter dot plot and median. Same conventions as in Fig 2.

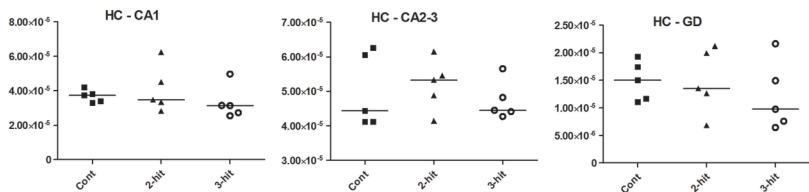
	1 st hit Gene susceptibility		2 nd hit Early stress	3 rd hit Environmental exposure		Behaviour	In vivo	Euthanasia	Post-mortem analyses
Age (days)		0	9-10	32 -52	52-59	60-180	180	182	
		Birth	Maternal separation	THC	Washout				
Control <i>n</i> =13	Wild Type		20s	Vehicle		P60 White and dark box P67 Actimetry P74 Open field P77 Spontaneous alternation P82 Approach-avoidance P110 Sucrose preference P124 Nesting behavior P150-153 Morris water maze	<i>In vivo</i> MRI		<i>Ex vivo</i> MRI Immuno- histochemistry
2-hit <i>n</i> =12	Wild Type		24h	THC treatment 8mg/kg/day					
3-hit <i>n</i> =7	MAP6 +/-		24h	THC treatment 8mg/kg/day					



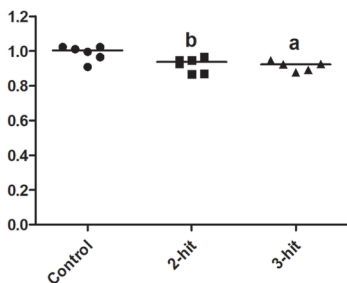
A. PV+ cells in prefrontal cortex



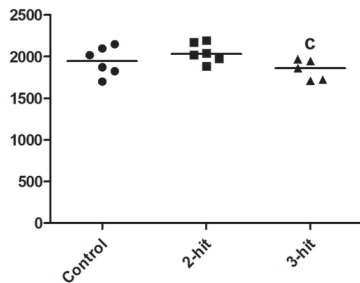
B. PV+ cells in hippocampus



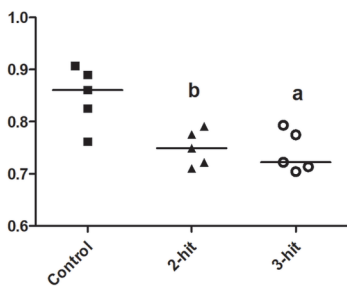
C. Cortical thickness (mm)



D. Hippocampus volume (μm^3)



E. Fractional Anisotropy (FA)



F. Radial Diffusivity (RD)

