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Exploratory, anxiety and spatial memory impairments are dissociated in mice lacking the LPA_1 receptor

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ABSTRACT

Lysophosphatidic acid (LPA) is a new, intercellular signalling molecule in the brain that has an important role in adult hippocampal plasticity. Mice lacking the LPA_1 receptor exhibit motor, emotional and cognitive alterations. However, the potential relationship among these concomitant impairments was unclear. Wild-type and $maLPA_1$-null mice were tested on the hole-board for habituation and spatial learning. $MaLPA_1$-null mice exhibited reduced exploration in a novel context and a defective intersession habituation that also revealed increased anxiety-like behaviour throughout the hole-board testing. In regard to spatial memory, $maLPA_1$ nulls failed to reach the controls' performance at the end of the reference memory task. Moreover, their defective working memory on the first training day suggested a delayed acquisition of the task's working memory rule, which is also a long term memory component. The temporal interval between trials and the task's difficulty may explain some of the deficits found in these mice. Principal components analysis revealed that alterations found in each behavioural dimension were independent. Therefore, exploratory and emotional impairments did not account for the cognitive deficits that may be attributed to $maLPA_1$ nulls' hippocampal malfunction.

1. Introduction

Lysophosphatidic acid (LPA, 1-acyl-2-*sn*-glycerol-3-phosphate), acting through 6 G protein-coupled receptors (LPA_{1-6}), has gained increasing attention over the last few years as an intercellular messenger with several effects on different target tissues (Anliker & Chun, 2004; Birgbauer & Chun, 2006; Choi et al., 2010; Chun 2005, 2007; Fukushima, Ishii, Contos, Weiner, & Chun, 2001; Ishii, Fukushima, Ye, & Chun, 2004; Moolenaar, van Meeteren, & Giepmans, 2004; Noguchi, Herr, Mutoh, & Chun, 2009; Rivera & Chun, 2008). A growing body of evidence indicates that the LPA pathway is involved in normal and abnormal brain development and function (Anliker & Chun 2004; Choi, Lee, & Chun, 2008; Chun, 2005; Estivill-Torrus et al., 2008). The most extensively studied of these receptors is LPA_1 (Chun, 2005; Contos, Fukushima, Weiner, Kaushal, & Chun, 2000; Estivill-Torrus et al., 2008; Fukushima et al., 2002; Herr & Chun, 2007; Kingsbury, Rehen, Contos, Higgins, & Chun, 2003; Matas-Rico et al., 2008).

Recently, Santin et al. (2009) described the behavioural phenotype of the $maLPA_1$-null mouse, a stable variant of the LPA_1-null mutant strain formerly characterised by Contos et al. (2000) and described in Estivill-Torrús et al. (2008). Impaired spatial memory retention, abnormal use of searching strategies, altered exploration in the open field and increased anxiety-like responses in the elevated plus maze have been reported in the absence of retinal and auditory malfunctions. However, concomitant neurological deficits were observed in olfaction and somesthesis, limb reflexes, co-ordinated limb use and neuromuscular strength (Santin et al., 2009). Interestingly, these behavioural alterations are accompanied by impairments in both hippocampus and cerebral cortex that may be partially responsible for the phenotype (Estivill-Torrus et al., 2008; Matas-Rico et al., 2008).

The complexity of the behavioural phenotype exhibited by the $maLPA_1$-null mice with impairments in several behavioural domains is frequently observed when transgenic mice are used in research (e.g. Acevedo, Pfankuch, Ohtsu, & Raber, 2006; Kalueff, Fox, Gallagher, & Murphy, 2007; Santin et al., 2009). However, the

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potential relationship among sensorimotor, emotional and cognitive variables is not generally well-addressed and may lead to inaccurate interpretations. To date, it is known that anxiety-related behaviours, exploration and cognition may reflect dissociated or common processes in animal testing (Matzel, Grossman, Light, Townsend, & Kolata, 2008; Miyagawa et al., 1998; Ohl, Roedel, Binder, & Holsboer, 2003; Ohl, Roedel, Storch, Holsboer, & Landgraf, 2002). In this regard, it has been reported that memory could be influenced by the rodent's inborn anxiety or by its reactivity to a stressor (Herrero, Sandi, & Venero, 2006; Ribeiro et al., 1999; Wright, Lightner, Harman, Meijer, & Conrad, 2006). The relevance of this point is emphasized in reports that suggest that the performance of some mouse strains in certain tasks may reflect the strain's anxiety-related behaviour, rather than cognitive functions (Dockstader & van der Kooy, 2001; Ohl et al., 2002). It is important to note that stressors, such as a novel environment or forced swimming, are usually an unavoidable part of the experimental setting even when studying non-emotional cognitive processes. Furthermore, the degree of aversion varies from one task to another, and that may explain disparate memory results between procedures (Hodges, 1996). On the other hand, anxiety levels could be related to increased or reduced locomotion (Kameda et al., 2007; Ramos & Mormède, 1998), and motor activity could influence anxiety and memory when their assessment involves spatial–temporal parameters (Brody & Holtzman, 2006; Kalueff et al., 2007; Strekalova, Spanagel, Dolgov, & Bartsch, 2005).

The main purpose of this work is to study exploration, anxiety and spatial memory in maLPA$_1$-null mice, with a focus on the interrelationship among these characteristics, to determine whether motor activity or anxiety impairments might account for cognitive performance. To address this issue, we used the hole-board test and the principal components analysis (PCA) multivariate approach. The hole-board is a frequently used hippocampal-dependent task for measuring spatial learning that is similar to the water maze in that extra-maze cues are used to solve the task (Oades, 1981). Moreover, the hole-board, as well as its modified version, allows the simultaneous evaluation of various potentially interrelated emotional, exploratory and spatial memory measures (Ohl et al., 2002, 2003; Takeda, Tsuji, & Matsumiya, 1998). PCA is useful to resolve variables into the independent dimensions (factors) that underlie behaviour (Ohl et al., 2002, 2003; Ramos & Mormede, 1998). Although PCA has successfully been applied to assess behavioural paradigms and inbred strains, it has been less frequently used in studies using transgenic animals (Carola, D'Olimpio, Brunamonti, Mangia, & Renzi, 2002; Fernandes, Gonzalez, Wilson, & File, 1999; Gross, Santarelli, Brunner, Zhuang, & Hen, 2000; Ohl et al., 2003). In this study, we further show the utility of PCA in analysing behavioural research using mutant mice.

2. Materials and methods

2.1. Animals

The generation and characterization of maLPA$_1$-null mice have been previously described (Estivill-Torrus et al., 2008; Matas-Rico et al., 2008). The original-null mice were obtained by targeted gene disruption using homologous recombination and Cre-mediated deletion in a 129X1/SvJ background. These animals were then backcrossed with C57BL/6J mice. Intercrosses of these mice, as well as with mice generated from one additional backcross (Contos et al., 2000), were begun immediately. An LPA$_1$-null mouse colony, termed maLPA$_1$ from the *Málaga* variant of LPA$_1$ knockout, was spontaneously derived during the original colony expansion by crossing heterozygous foundation parents (maintained in the original hybrid C57BL/6J × 129X1/SvJ background). Intercrosses were performed with these mice and subsequently backcrossed for 20 generations with mice generated within this mixed background. MaLPA$_1$-null mice carrying the *lpa*$_1$ deletion were born at the expected Mendelian ratio, and they survived to adulthood. Targeted disruption of the *lpa*$_1$ gene was confirmed by genotyping (according to Contos et al., 2000), and immunochemistry confirmed the absence of LPA$_1$ protein expression.

Fourteen maLPA$_1$-null male mice and 23 analogous wild-type littermates were used in this study. All mice were approximately 4 months old at the onset of the behavioural testing and were housed singly in standard cages with a 12 h light/dark cycle (lights on at 7:00 a.m.). For 4 days before the experiment, mice were handled daily by the experimenter in the testing room, to get adapted to the experimental conditions, and they were fed a restricted diet so their body weights were reduced to 80–85% of their free-feeding weight. Food restriction processes remained throughout the experiment. Experiments were conducted between 9:00 a.m. and 3:00 p.m. in a testing room illuminated at 300 lux. All procedures were in accordance with the European animal research laws (European Communities Council Directive 86/609/EEC, 98/81/CEE and 2003/65/CE and Commission Recommendation 2007/526/EC) and Spanish National Guidelines for Animal Experimentation and use of genetically modified organisms (Real Decreto 1205/2005 and 178/2004 and Ley 32/2007 and 9/2003).

2.2. Habituation in the hole-board

The hole-board (40 × 40 cm) contained 16 equidistant holes (5.5 cm apart, 2.5 cm diameter, 3 cm depth) placed in the central zone of the apparatus, which was surrounded by an arena 6.5 cm in from the clear Plexiglas walls (20 cm high) of the maze. Several spatial cues (black cards in different geometric shapes) were located on the walls of the testing room to allow mice to orient in space. For habituation, mice spent 1 session of 3 min on 2 consecutive days in the hole-board. All 16 holes were baited with a small food pellet (0.03 gr) in order to habituate mice to visit holes to eat food. After each session, the number of faecal boli laid in the arena was counted, and the apparatus was cleaned with a solution containing neutral soap.

Sessions were videotaped, and locomotion (mm travelled) and thigmotaxis (percent of time spent by the animal in the periphery, defined as the 6.5 cm of arena in from the walls) were registered using a video tracking system (Ethovision XT, Noldus Information Technology, Wageningen, The Netherlands). Frequency of head dipping (the mouse introduced its nose in a hole), rearing (the mouse stood on its hind paws, with forelegs supported or unsupported on the walls), risk assessment (the mouse stretched its head and shoulders, before return to its initial posture) and grooming (the mouse licked/scratched its fur, washed its head and/or licked its tail or genitals) were assessed with an observational software (Smart, 2.5, Panlab, Barcelona. Spain). To control for the fact that animals may not eat the same amount of rewards during the habituation phase, all measures with the exception of thigmotaxis and defecation were expressed as a rate (per minute or per second), and the time each mouse spent eating was observationally recorded and subtracted from the total time employed to calculate the rates. Importantly, the locomotion rate in our study, although expressed in units per second, is not the same as velocity. When velocity is assessed, all the time the animal is resting (not just when eating) should be excluded from analysis (Bothe, Bolivar, Vedder, & Geistfeld 2004).

2.3. Spatial learning in the hole-board

The day after habituation, only a fixed set of 4 holes was baited (0.03 gr of food pellet), in a pattern that remained constant

throughout the rest of the experiment. Spatial learning was conducted on 4 consecutive days with 2 sessions of trials each day with an intersession interval of 2 h. Each session consisted of 2 consecutive trials with an intertrial interval of 45 s. Mice were introduced manually onto the hole-board from 1 of 4 random starting locations corresponding to the 4 arena corners. Each trial lasted until the mouse obtained all 4 rewards from the baited holes. Then, the animal was manually removed from the maze and returned to its home cage, and the box was cleaned with a solution containing neutral soap. All holes contained food pellets at the bottom, on which a perforated false bottom was placed, so the mice were unable to discriminate between baited and non-baited holes by orientating based on olfactory cues. All tests were videotaped and the latency for each mouse to finish each trial was recorded. Once again, locomotion, rearing and head dipping were expressed as rates per unit of time to control for the influence of the distinct trial durations (i.e., latency to find the 4 rewards) between genotypes in this phase. Moreover, locomotion and head dipping expressed per time were intended as measures of exploration in this study. In contrast, a total measure of distance or head dipping needed to find all rewards would have probably been related to the accuracy in performing the task. Regarding the cognitive measures, the reference memory ratio was defined as the number of visits and revisits to the baited holes divided by the total number of hole visits (visits and revisits to baited and non-baited holes). The working memory ratio was expressed as the number of food-rewarded visits divided by the number of visits and revisits to the baited holes (Douma et al., 1998). A hole visit (or revisit) was scored when the mouse introduced its nose into a hole.

2.4. Statistical analysis

On habituation phase, data from the 2 habituation sessions and from the first and last 30 s from the first session were analysed for intersession and intrasession habituation, respectively. Analyses were carried out by two-way ANOVAs with repeated measures followed by post hoc Fisher's LSD for intra-group and inter-group comparisons. In the spatial learning phase, all registered data were averaged to yield 1 daily score per subject for the assessment of the measures across days. The study of the influence of the interval between trials on working and reference memory was carried over the memory ratios of the 24 h, 2 h and 45 s delay trials from each day (the 2 daily 45 s interval trials were averaged in 1 measure). To analyse different trial stages that involved different difficulties (Olton & Papas, 1979), a reference and working memory ratio were calculated for each of the 4 rewards in each trial. The 4 trial stages were defined as follows: '1°': from trial start to the finding of the first reward; '2°': from the finding of the first reward to the finding of the second; '3°': from the finding of the second reward to the finding of the third; '4°': from the finding of the third reward to the finding of the last one. Data from each trial stage were collapsed per animal in each day, and measures from training days 1 and 2 and from training days 3 and 4 were averaged. All data from spatial learning were analysed by ANOVAs with repeated measures and post hoc Fisher's LSD. Only probabilities less than or equal 0.05 were considered significant.

The investigation of the correlation of the assessed parameters by PCA was conducted according to previous studies (Ohl et al., 2003). Datasets from the habituation days and spatial learning days (averaged for a daily measure per animal) were analysed separately for wild-type and-null genotypes using a principal components solution with varimax orthogonal rotation. Varimax rotation ensures that factors are independent of one another and therefore reflect separated and not correlated processes (Ramos & Mormede, 1998). Factors were selected until they accounted for at least 85% of the total variance. The contribution of each behavioural variable to each factor is referred to as "factor loading". Factors were named after the measures best reflecting each factor, defined as the ones that loaded high in that factor but low in the others. Loadings less than 0.6 were considered a weak representation of the variable in the factor and, therefore, were not taken into account.

3. Results

3.1. Response to novelty and habituation

To study the response to a novel context and habituation learning, 14 maLPA$_1$-null and 23 analogous wild-type male mouse littermates were tested by hole-board test in 2 sessions of 3 min each carried out on 2 successive days, with all holes baited. Locomotion, percent of time in the maze's periphery (time in periphery), rearing, head dipping, risk assessment, grooming and defecation were assessed.

Repeated measure ANOVAs carried out over the 2 habituation days revealed a significant 'genotype' effect in locomotion ($F_{(1,35)} = 7.388$, $P < 0.001$), time in periphery ($F_{(1,35)} = 32.689$, $P < 0.001$), head dipping ($F_{(1,35)} = 42{,}279$, $P < 0.001$), grooming ($F_{(1,35)} = 6.290$, $P < 0.05$) and defecation ($F_{(1,35)} = 11.033$, $P < 0.05$). On the novel environment (i.e., first hole-board exposure), Fisher's Least Significant Difference (LSD) post hoc comparisons showed that maLPA$_1$-null mice exhibited a pattern of reduced activity and exploration in comparison with controls that consisted of hypolocomotion ($P < 0.05$, Fig. 1A) and reduced rearing ($P < 0.001$, Fig. 1C), head dipping ($P < 0.001$, Fig. 1D) and grooming ($P < 0.05$). MaLPA$_1$ knockouts also exhibited increased defecation, but the time in periphery was similar to wild-type mice's and did not support a greater anxiogenic reaction to novelty (Fig. 1B).

On the next day of habituation testing, mice were placed in a familiar environment (i.e., second hole-board exposure) to study intersession habituation, and they were assessed for changes in behaviour from the first to the second habituation day. Familiarity with the context was proven to change the behavioural response, as reflected by a significant 'day' effect on time in periphery ($F_{(1,35)} = 49.697$, $P < 0.001$), rearing ($F_{(1,35)} = 17.057$, $P < 0.001$), head dipping ($F_{(1,35)} = 13.658$, $P < 0.001$) and risk assessment ($F_{(1,35)} = 45.598$, $P < 0.001$). However, familiarity differentially affected the mice according to genotype, and the effect for the 'genotype x day' interaction was significant for most of the measures (locomotion: $F_{(1,35)} = 4.523$, $P < 0.05$; time in periphery: $F_{(1,35)} = 18.469$, $P < 0.001$; rearing: $F_{(1,35)} = 6.607$, $P < 0.05$; head dipping: $F_{(1,35)} = 3.957$, $P < 0.05$, grooming: $F_{(1,35)} = 4.126$, $P = 0.05$). Post hoc comparisons revealed that the intersession habituation process was evident in wild-type mice by the change in behavioural scores between sessions but was defective in knockouts. In this way, wild-types reduced locomotion ($P < 0.05$), time in periphery ($P < 0.001$), rearing ($P < 0.001$) and risk assessment ($P < 0.001$), increased head dipping ($P < 0.001$) and showed no defecation in the familiar context. In contrast, maLPA$_1$-null mice only reduced the risk assessment behaviour ($P < 0.001$) in response to familiarity, thus maintaining their baseline levels of locomotion, time in periphery, rearing and head dipping ($P > 0.05$, Fig. 1A–D). Although head dipping did not increase in the LPA$_1$-null group, it is relevant to note that they ate normally when food was placed in their home cage, suggesting that their defective head dipping likely reflected a lack of exploration instead of a low motivation to eat. On the other hand, the intrasession habituation was preserved in the null genotype. In this way, both groups increased their locomotion ($P < 0.001$) and head dipping, reduced time in periphery ($P < 0.001$), and maintained rearing and grooming frequency within the first habituation session ('genotype' effect for locomotion:

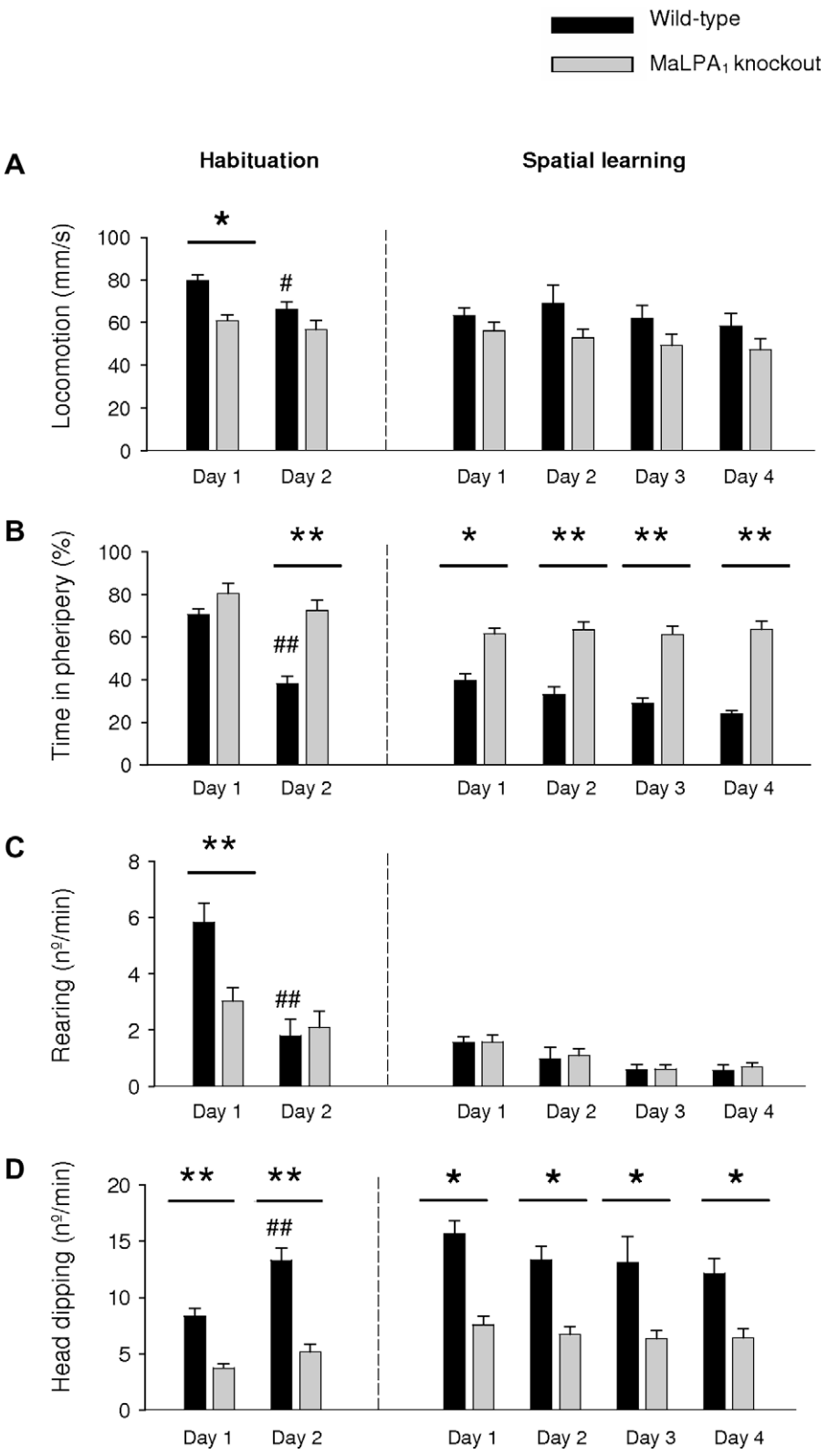

Fig. 1. Impaired exploration and increased anxiety-like behaviour in mice lacking the LPA_1 receptor. Locomotion, percent of time in the maze periphery, rearing and head dipping were tested on wild-type and $maLPA_1$-null mice across the hole-board training. The task comprised 2 days of habituation and 4 days of spatial learning. $MaLPA_1$-null mice exhibited lower locomotion and rearing on the first exposure to the hole-board (A and C) along with decreased head dipping on all days (D) The null genotype was unable to reduce its preference for the periphery of the maze (B) Fisher's LSD post hoc comparisons: differences between groups ($^{*}P < 0.05$, $^{**}P < 0.001$); change from the first day of habituation within the group ($^{\#}P < 0.05$, $^{\#\#}P < 0.001$).

$F_{(1,35)} = 8.980$, $P < 0.05$; time in periphery: $F_{(1,35)} = 4.885$, $P < 0.05$; rearing: $F_{(1,35)} = 3.811$, $P = 0.05$, head dipping: $F_{(1,35)} = 9.892$, $P < 0.05$; 'intrasession change' effect for locomotion: $F_{(1,35)} = 29.682$, $P < 0.001$; thigmotaxis: $F_{(1,35)} = 19.516$, $P < 0.001$; head dipping:

$F_{(1,35)} = 2.979$, $P < 0.001$; 'genotype x intrasession change' was never significant).

3.2. Spatial learning

The day after habituation, a fixed pattern of 4 holes was baited. Spatial learning took place over 4 consecutive training days (days 1–4 from now on) that comprised 2 sessions of 2 consecutive trials per day, with an intersession delay of 2 h. Reference and working memory ratios, locomotion, time in periphery, rearing, head dipping and latency to find all rewards (trial duration) were assessed in this phase. To study spatial memory in depth, both reference and working memory ratios were analysed over the 4 training days and with respect to the different intertrial intervals (24 h, 45 s, 2 h) present in our protocol. We found that the number of memory errors increased as the trials advanced and fewer rewards remained in the maze. Thus, we also analysed 4 stages within each trial (stage being defined as the finding of 1 reward) that involved increasing difficulty. In the case of working memory, the memory load increased according to the number of rewards previously found within the trial. In the case of reference memory, the memory load (i.e., the 4 spatial locations) theoretically remained constant, but data suggested that mice learned the location of some rewards more easily (i.e., the 2 they searched first) while having more difficulty with the later ones, suggesting that the last half of the trial was a more demanding test of spatial reference memory.

Repeated measures ANOVAs over the 4 days of spatial learning showed a cognitive impairment in maLPA$_1$–null mice in both reference and working memory (effect by 'genotype': reference memory: $F_{(1,35)} = 4.492$, $P < 0.05$; working memory: $F_{(1,35)} = 5.867$, $P < 0.05$). In the case of reference memory, both groups were able to learn the task, as was shown by their improvement over days (LSD on day 2: $P < 0.05$ vs. day 1 for both genotypes, LSD on day 3: $P < 0.05$ vs. day 2 for wild-types; effect by 'day': $F_{(3,105)} = 41.458$, $P < 0.001$). However, maLPA$_1$ knockouts failed to reach the controls' performance at the end (LSD on day 4: $P < 0.05$; 'genotype x day': $F_{(3,105)} = 2.748$, $P < 0.05$) (Fig. 2A). Further analysis of the reference memory ratio considering intertrial intervals showed that wild-type mice were better than knockouts at 24 h and 2 h intervals on day 3 (LSD: $P < 0.05$; effect by 'genotype': $F_{(1,35)} = 9.307$, $P < 0.05$; 'genotype x interval': $F_{(2,70)} = 3.115$, $P = 0.05$) (Fig. 2C). Importantly, on this day wild-types performed worse at 45 s intervals in comparison with 24 h and 2 h intervals (LSD: $P < 0.05$), while knockouts performed equally at all delays. On day 4, knockouts' reference memory impairment was still more notable than wild-type at long intertrial intervals, although this tendency was not statistically significant ('genotype': $F_{(1,35)} = 5.602$, $P < 0.05$; but effects by 'interval' and 'genotype x interval' > 0.05). When reference memory was analysed in terms of different stages within trials, a significant effect according to 'difficulty' proved reference memory errors increased as the trial advanced (days 1 + 2: $F_{(3,105)} = 10.217$, $P < 0.001$; days 3 + 4: $F_{(3,105)} = 6.426$, $P < 0.001$). Decreased reference memory performance was found when mice looked for the third reward (LSD on 3rd reward: $P < 0.05$ vs. 1st reward), except in the case of wild-types on days 3 + 4, when their performance did not get worse until 1 reward remained (LSD on 4th reward: $P < 0.05$ vs. 1st reward). Analysis by difficulty on days 3 + 4 also revealed a reference memory impairment in the null group related to the performance in a more demanding part of the task, corresponding to the finding of the third reward (LSD: $P < 0.05$; effect by 'genotype': $F_{(1,35)} = 4.838$, $P < 0.05$; 'genotype x difficulty': $F_{(3,105)} = 2.580$, $P = 0.058$) (Fig. 2E). Most likely, the last stage of the trial (4th reward) involved a difficulty too high to discriminate between genotypes.

In working memory, a deficit was found in the maLPA$_1$-null group on the first training day (LSD: $P < 0.05$; effect by 'genotype x interval': $F_{(3,105)} = 2.670$, $P = 0.05$), but they significantly improved on day 3 (LSD: $P < 0.05$ vs. day 1; effect by 'day': $F_{(3,105)} = 3.586$, $P < 0.05$) when they reached the performance level of wild-type mice (Fig. 2B). In contrast, no significant change was found on the wild-type group's working memory ratio over the days of the experiment. Analysis of the effect of trial intervals showed no difference between trials in the working memory deficit of null mice on day 1 (effect by 'genotype': $F_{(1,35)} = 7.169$, $P < 0.05$; but effects by 'interval' and 'genotype x interval' were not significant). However, analysis showed a working memory impairment in the null genotype on day 2 that was limited to 24 h and 2 h intertrial delays (LSD: $P < 0.05$; effect by 'genotype': $F_{(1,35)} = 5.997$, $P < 0.05$; 'interval': $F_{(2,70)} = 3.987$, $P < 0.05$; 'genotype x interval': $F_{(2,70)} = 3.081$, $P < 0.05$) (Fig. 2D). Interestingly, this delay-dependent deficit on day 2 was the result of a significant improvement in the working memory ratio of knockouts at the 45 s intertrial interval, in comparison with the longer ones (LSD: $P < 0.05$ vs. 24 h and 2 h). Finally, analysis over the 4 different difficulty stages showed that maLPA$_1$ knockouts failed on days 1 + 2 at the higher demand, when only 1 reward remained (LSD on 4th reward: $P < 0.05$; effect by 'genotype': $F_{(1,35)} = 5.363$, $P < 0.05$; 'genotype x difficulty': $F_{(3,105)} = 3.097$, $P < 0.05$) (Fig. 2F). Effect by 'difficulty' was significant (days 1 + 2: $F_{(1,35)} = 5.363$, $P < 0.05$; days 3 + 4: $F_{(3,105)} = 107.77$, $P < 0.001$), showing an increase in working memory load as fewer rewards remained in each trial. Overall, it was found that task difficulty increased with each reward search for both genotypes (LSD: $P < 0.05$ when comparing the finding of each reward with the next).

Repeated measures ANOVAs on the non-cognitive measures analysed during training revealed that maLPA$_1$-null mice spent more time in the periphery than did controls (LSD on day 1: $P < 0.05$; LSD on days 2–4: $P < 0.001$; 'genotype': $F_{(1,35)} = 59.078$, $P < 0.001$; 'day': $F_{(3,105)} = 2.682$, $P = 0.05$, 'genotype x day': $F_{(3,105)} = 4.221$, $P < 0.05$) (Fig. 1B). This difference was even more notable at the end of the spatial learning phase, as wild-type mice reduced their time in the periphery across days (LSD on day 2: $P < 0.05$ vs. day 1; LSD on day 4: $P < 0.05$ vs. day 2), but no change in this behaviour was found in knockouts. Throughout the spatial learning trials, the null group showed reduced head dipping in comparison to controls ('genotype': $F_{(1,35)} = 19.415$, $P < 0.001$) (Fig. 1D) and a greater latency in finding rewards ('genotype': $F_{(1,35)} = 36.683$, $P < 0.001$; 'day': $F_{(3,105)} = 5.837$, $P < 0.001$). Interestingly, no differences between genotypes were found in rearing and locomotion in this phase; both groups maintained locomotion and drastically reduced rearing across the days ('day' effect on rearing: $F_{(3,105)} = 9.187$, $P < 0.001$) (Fig. 1A and C).

3.3. Relationships among the variables assessed during the hole-board training

PCA was carried out to study the relationships among locomotion, time in periphery, rearing, head dipping, risk assessment, grooming and defecation in each genotype, both in a novel and in a familiar context. PCA with variance maximizing (varimax) rotation in the novel context (i.e., first hole-board habituation day) revealed a 4 components solution in wild-type and maLPA$_1$-null groups accounting for 89% and 91% of the total variance, respectively. In both genotypes, there was reported a strong association between locomotion and rearing that likely represented general activity and exploration of the novel environment, whereas the dimension comprising the time in periphery and defecation suggested anxiety-like behaviour (Table 1). Head dipping, risk assessment and grooming represented different factors, that could be related to directed exploration (head dipping) or arousal (risk

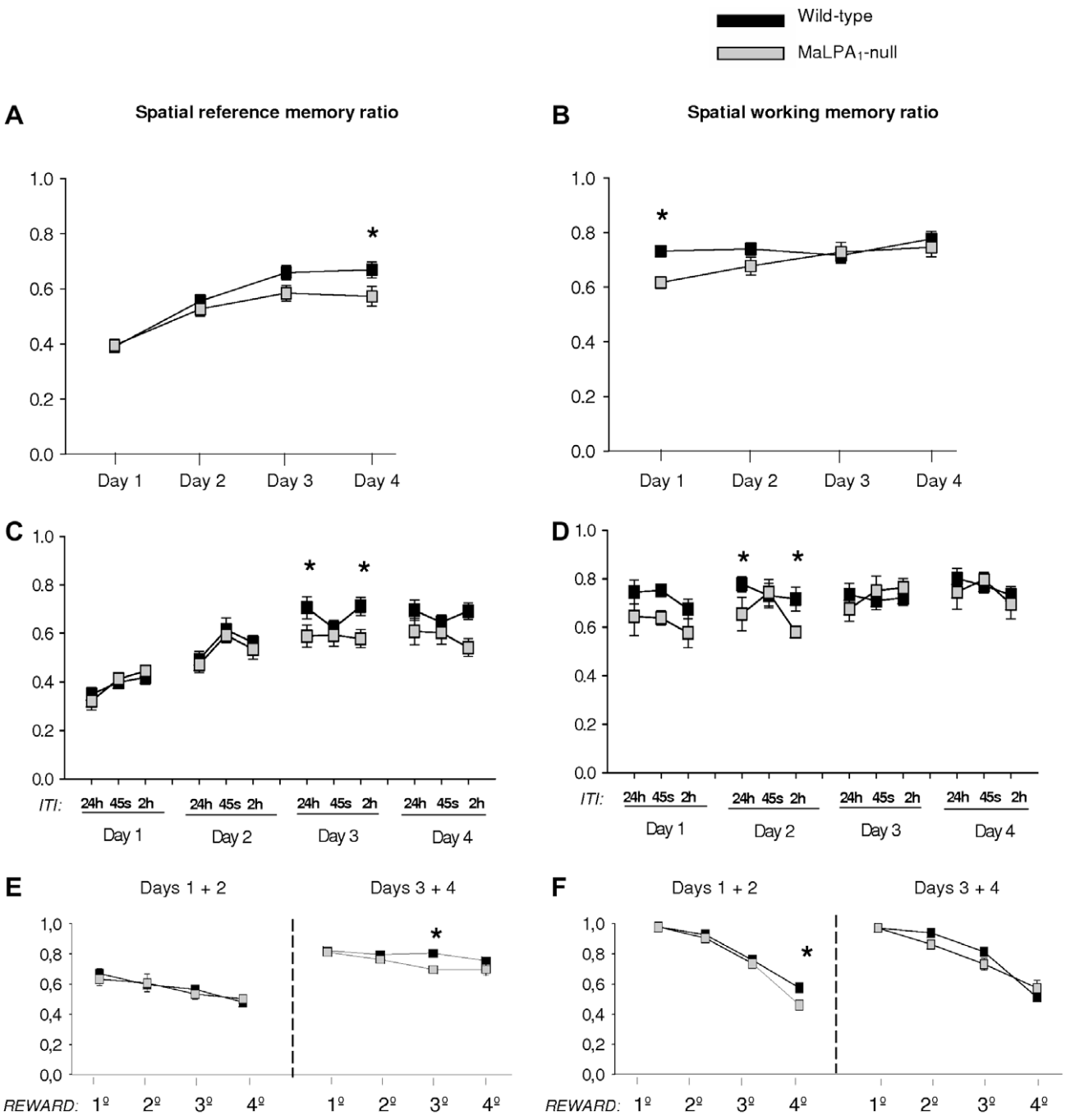

Fig. 2. Spatial reference and working memory deficits in LPA_1-null mice. Both spatial reference and working memory ratios were analysed across days, taking into account both the intertrial interval (the 2 daily trials with interval of 45 s were averaged) and the different difficulty stages within the trials (from the finding of the first reward to the finding of the fourth).Null mice were not able to reach wild-types' reference memory performance on the last training day (A) This deficit was related to the longer intervals between trials (24 h, 2 h) and to a high difficulty level (C and E). Nulls also showed impaired working memory at the beginning of the task, at the higher difficulty (B and F), although they improved with training. This initial working memory deficit was delay-independent on day 1 but related to longer intervals on day 2. Fisher's LSD post hoc comparisons: differences between groups ($^{*}P < 0.05$). ITI: intertrial interval.

assessment, grooming). PCA in the familiar context (i.e., second habituation day) again extracted 4 factors for each genotype explaining 90% of the total variance in the wild-type group and 89% in knockouts. Once again, independent activity and anxiety-related factors were revealed in each group, and they were mainly represented by locomotion and time in periphery, respectively (Table 1). In contrast, defecation no longer seemed to be a reliable anxiety indicator for the null group because it partially contributed to several factors on the second habituation day and, moreover, wild-types did not defecate. Frequency of grooming and risk assessment drastically decreased and also loaded on factors of ambiguous interpretation. Therefore, the aforementioned measures were not further analysed in the spatial learning phase.

In the spatial learning phase, PCA was carried out on the assessed variables (reference and working memory ratios, locomotion, time in periphery, rearing, head dipping and latency to find all rewards) for each spatial learning day and group. Factorial solutions comprised 4 factors and accounted for at least 85% of the total variance in all cases. Overall, no notable differences were found on the factorial solution between genotypes, and independent factors

Table 1
Independent factors representing activity/exploration, thigmotaxis and spatial memory in wild-type (WT) and maLPA$_1$-null mice (NULL) during the hole-board testing.

Day	Hab. 1		Hab. 2		S. Learn. 1		S. Learn. 2		S. Learn. 3		S. Learn. 4	
Genotype	WT	NULL	WT	NULL	WT	NULL	WT	NULL	WT	NULL	WT	NULL
Factor: Exploration/activity												
Locomotion	.927	.950	.961	.882	.931	.861		.926	.654	.831	.668	.922
Head Dipping					.931	.825	.871	.919	.633	.947	.926	.856
Rearing	.897	.835		.845								
Trial duration	–	–	–	–		−.894	−.946	−.828	−.939	−.647	−.899	−.829
% Total variance	43.43	34.90	26.86	39.12	39.80	39.82	45.36	34.49	36.47	39.92	38.08	37.78
Factor: Thigmotaxis												
Periphery (% time)	.909	.918	.754	.936	.768	.971	.962	.929	.882	.910	.870	.867
Defecation	.768	.671			–	–	–	–	–	–	–	–
Rearing			.892				.697		.823	.777	.885	−.667
Head dipping				−.622								
Trial duration	–	–	–	–	.920							
% Total variance	12.05	17.03	30.97	15.90	10.35	9.54	20.63	11.44	23.75	11.46	27.19	15.33
Factor: Reference memory												
Ref. memory	–	–	–	–	.853	.929	−.969	.945	.859	.953	.985	.969
W. memory	–	–	–	–	−.771							
Trial duration	–	–	–	–						−.649		
% Total variance	–	–	–	–	17.66	17.30	13.03	18.65	7.80	31.20	10.00	11.85
Factor: Working memory												
W. memory	–	–	–	–	−.771	.740	−.971	.878	.921	.945	.969	.966
Ref. memory	–	–	–	–	.853							
Rearing						.922		.791				
% Total variance	–	–	–	–	17.66	26.69	12.09	24.85	20.90	9.26	15.15	23.05

Loadings less than 0.6 were not taken into consideration and are not shown. Grooming and risk assessment behaviours were scored on habituation but never loaded on these factors, and thus they do not appear in the table. Hab. = habituation day; S. Learn. = spatial learning day; Ref. memory = reference memory ratio; W. memory = Working memory ratio; % Total variance: Percent of the total variance explained by the factor; (–): measure not assessed on that day.

were revealed for exploration, thigmotaxis, reference memory and working memory (Table 1). Interestingly, the exploration factor was strongly represented by locomotion and head dipping, but not by rearing. In the spatial learning phase, locomotion was likely no longer motivated by context exploration but by the willingness to dip in holes to find the 4 rewards distributed through the maze. Moreover, rearing drastically decreased in frequency across training, perhaps losing its usefulness as the environment became familiar. Its association with the periphery could be related to the fact that all rearings were supported on the maze walls. However, to interpret the thigmotaxis factor, it is important to note that the percent of time in the periphery strongly differed between genotypes in this phase (Fig. 1B). As the periphery in our study consisted of 45% of the total maze area, the wild-type group's percent of time in the periphery (daily means from 40% to 24%) may simply reflect a distributed exploration of the maze surface, whereas time in the periphery in the null group (daily means from 61% to 59%) may reflect anxiety-like behaviour.

4. Discussion

Our results provide reliable and robust evidence of the involvement of LPA through the LPA$_1$ receptor signalling pathway in spatial memory in the absence of either emotional or motor influences. In agreement with a previous report (Santin et al., 2009), maLPA$_1$-null mice exhibited a reduced exploratory behaviour when exposed to a novel environment (i.e., hole-board exploration during the first habituation session). Impaired exploration could be a consequence of the concomitant anxiety levels during the test (Kameda et al., 2007; Ramos & Mormede, 1998). However, the results reported here did not clearly support altered anxiety-like behaviour in knockouts exposed to a novel context, as their residence in the periphery of the maze was not significantly increased. Moreover, PCA of this session confirmed independent activity and anxiety-related factors in each genotype, which resembled frequently reported dimensions (e.g., Fernandes et al., 1999; Gross et al., 2000; Ohl et al., 2003). In this way, exploration of the novel context was not influenced by anxiety-like behaviour. Conversely, thigmotaxis was the result of neither motor function nor motivation to explore, and it likely reflected mice's emotional state (Belzung, 1999; Ohl et al., 2003; Simon, Dupuis, & Costentin, 1994).

It is well known that the rodents' exposure to a familiar environment (i.e., re-exposure 24 h later to the same hole-board in our study), will shift their behavioural response (Holmes & Rodgers, 1998). Accordingly, there was a notable reduction of both activity and anxiety-related measures in the wild-type mice, showing a normal intersession habituation process (Bothe et al., 2004; Leussis & Bolivar, 2006; Thiel, Muller, Huston, & Schwarting, 1999). Head dipping behaviour was an exception to normal habituation indicators but was probably due to the use of baited holes. In contrast, LPA$_1$-null mice did not exhibit a normal intersession habituation for the majority of variables studied. Perhaps one of the most interesting results is the inability of the maLPA$_1$-null mice to reduce thigmotaxis in a familiar context or during spatial memory training. Altered intersession habituation of anxiety has already been pointed out in maLPA$_1$-null mice using the open field test (Santin et al., 2009), but the extreme severity of this habituation impairment is suggested for the first time in the present study. Interestingly, maLPA$_1$-null's normal intrasession habituation revealed the importance of the temporal interval between exposures rather than an absolute inability of the mice to adapt their behaviour, which would indicate an even more dramatic deficit. However, the analyses of the activity related measures (i.e., locomotion and rearing) in relation to the habituation are less conclusive. The absence of habituation in those exploratory variables in the null genotype can be attributed to their low baseline levels more than to a habituation deficit, as animals that exhibit low levels of exploration when exposed to a novel context tend also to show a similar exploration when tested in conditions of contextual familiarity (Thiel et al., 1999).

Deficits in both exploration and habituation of anxiety-like behaviour in the maLPA$_1$-null mice may influence the cognitive performance of this genotype. However, PCA analysis confirmed that working and reference memory ratios weighted independently of exploratory and anxiety scores. The independence of these factors allows us to discuss the results obtained in the context of the relevance of LPA signalling through the LPA$_1$ receptor in memory processing. In regard to reference memory, maLPA$_1$-null mice exhibited a clear deficit during the last day of training, when these mice were unable to achieve the performance level attained by the wild-type mice. We cannot consider this effect a general spatial impairment because maLPA$_1$-null mice were able to learn the task, improving their performance throughout the spatial training. Interestingly, mice exhibited a deficit in increased demand tasks mainly when the intertrial intervals were long (i.e., 2 and 24 h) but not when the intertrial interval was short (i.e., 45 s). This deficit at long intertrial intervals suggests the importance of the LPA$_1$ receptor for long-term, but not for short-term, memory retention. However, some caution should be taken in interpreting the temporal dependence of knockouts' reference memory impairment, as it could also be explained in terms of proactive interference lowering the performance of wild-type mice due to the short intertrial interval but having no further effect on the-null group (Han, Gallagher, & Holland, 1998). The complex design of the hole-board task used in our study, which included different difficulty levels, can explain the absence of a deficit reported by Santin et al. (2009) in a reference memory study using the water maze task with only 1 target location, a test with a relatively low reference memory processing load.

The spatial working memory in maLPA$_1$-null mice is reported for the first time in this experiment. Interestingly, maLPA$_1$-null mice showed a strong deficit during the acquisition of working memory rules, as can be seen during their performance in the first 2 days. This initial deficit during the working memory training is observed when these mice have to increase the working memory load (i.e., when only 1 reward remained in the maze, and the task's difficulty was higher). Furthermore, the memory impairment reported was not associated with any particular intertrial interval during the first training day, but it was clearly related to the longest intervals (i.e., 2 and 24 h) during the second training day. In this sense, it is important to note that this impairment probably does not reflect an inability of the mice to process spatial information in the short term, but, rather, shows a defect in gradual learning of the procedural rule necessary to solve the working memory task (Joel, Tarrasch, Feldon & Weiner,1997; Yoon, Okada, Jung, & Kim, 2008). This idea is supported by the fact that maLPA$_1$-null mice's performance improved over days, in contrast to the wild-type mice, which showed the same performance level throughout the test. Moreover, the presence of a delay-dependent deficit (on day 2) also suggests a long-term component of the working memory task. The correct working memory in wild-type mice from the first training trial is likely due to efficient learning of the rule during the first training exposure or, more probably, during the previous hole-board habituation, which did not require revisiting holes to find the maximum number of rewards.

Taken together, our results contribute to the building evidence that the LPA$_1$ signalling pathway is involved in spatial learning and memory. The reported deficits in both tasks could be a common manifestation of a functional alteration in the hippocampus in the absence of the LPA$_1$ receptor. In support of this hypothesis, these impairments have been consistently reported in animals with hippocampal damage (e.g., Morris, Garrud, Rawlins, & O'Keefe, 1982; Olton & Papas, 1979). The importance of the hippocampus in habituation to a novel environment has been well established (Leussis & Bolivar, 2006), and the role of the hippocampus in spatial memory is also well known (O'Keefe & Dostrovsky, 1971; Oades, 1981). Interestingly, although the spatial working memory also relies on the medial prefrontal cortex (Joel et al., 1997), the working memory deficit reported above in maLPA$_1$-null mice can be specifically linked to the hippocampus. It is known that the long-term components of the working memory task rely on hippocampal functioning (Yoon et al., 2008). Moreover, the initial deficit in the working memory task, followed by improvement at short intertrial intervals, has been related to hippocampal, but not to prefrontal cortex damage (Lee & Kesner, 2003). By extension, our data suggest that the medial prefrontal cortex, which may support a compensatory mechanism at short intervals (Lee & Kesner, 2003), might be functional in the maLPA$_1$-null mice. However, the subsequent performance improvement in trials separated by longer delays, suggests that the hippocampus in mice lacking the LPA$_1$ receptor is functional enough to allow normal working memory after several sessions of spatial training.

Our behavioural outcomes agree with previous studies that suggest a hippocampal malfunction in the absence of the LPA$_1$ receptor (Matas-Rico et al., 2008; Santin et al., 2009). Hippocampal LPA$_1$ expression has been reported (Allard et al., 1998; Pilpel & Segal, 2006; Rhee et al., 2006) in correlation with hippocampal synaptic plasticity (Pilpel & Segal 2004, 2006). Moreover, adult maLPA$_1$-null mice, in the absence of structural abnormalities in the dentate gyrus, show altered regulation of hippocampal neurotrophic factors (BDNF, IGF-1 and NGF) and impaired neurogenesis (Matas-Rico et al., 2008). Both hippocampal BDNF and adult neurogenesis have been widely linked to spatial working and reference memory (Leuner, Gould, & Shors, 2006; Mizuno, Yamada, He, Nakajima, & Nabeshima, 2003; Saxe et al., 2007; Tyler, Alonso, Bramham, & Pozzo-Miller, 2002).

Together, results from previous studies support the role of lysophosphatidic acid through LPA$_1$ receptor signalling in cognitive and emotional processes linked to the hippocampus, as is the case for habituation and spatial memory. In this regard, the cognitive, emotional and exploratory impairments reported in the maLPA$_1$-null mice may be relevant to study the phenotypic characteristics of schizophrenia. To date, three reports using another *lpa*$_1$ mutant mouse showed phenotypic features resembling those found in schizophrenia (Cunningham et al., 2006; Harrison et al., 2003; Roberts et al., 2005). In addition, Bowden et al. (2006) have reported down-regulation of the *lpa*$_1$ gene expression in a cohort of schizophrenic patients, supporting the view that the *lpa*$_1$ gene is involved in this psychiatric disorder. Further research based on LPA$_1$ signalling will likely provide results of notable interest in the study of psychiatric disturbances, such as schizophrenia and hippocampal-dependent emotional and cognitive pathology.

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