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**Effects of PACAP-38 and an analog, acetyl-[Ala<sup>15</sup>, Ala<sup>20</sup>] PACAP-38-propylamide, on memory consolidation in the detection of spatial novelty task in rats**

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## **Abstract**

PACAP-38 (P38) is a pleiotropic peptide that exerts multiple peripheral and central actions, including neurotrophic, neuroprotective and anti-inflammatory actions. Previous studies have suggested an improvement of memory in rats that have received a single systemic injection of P38. In a therapeutic perspective, we used an analog, acetyl-[Ala<sup>15</sup>, Ala<sup>20</sup>]PACAP-38-propylamide (ALG), to improve both stability and affinity for PAC1 receptors vs. endogen PACAP. We investigated the effect of P38 and ALG on memory consolidation using a spatial novelty detection (SND) task in which rats had to memorize a configuration of objects to identify that, during a test session, a familiar object has been moved to a new location. Rats received an intravenous injection of P38 or ALG after the last training session. In

1, P38 (30 µg/kg) improved spatial memory consolidation allowing detection of novelty vs. saline injection. In experiment 2, we confirmed this effect and showed that P38 restored the performance similar to what was found using non-injected rats. This suggests that, contrary to ALG, P38 exerted a promesiant rather than an anxiety-related effect whereas ALG did not show similar action. We also examined whether P38 effect involved an interaction with NR2B-containing NMDA receptors (NMDARs) by administrating ifenprodil (IFE; a selective NR2B-containing NMDAR antagonist) alone or in combination with P38 or ALG. The results suggested that P38 action on memory involved NR2B-containing NMDARs. Lastly, brain-derived neurotrophic factor (BDNF) modulation appeared to be not related to the behavioral performance in the SND task. Overall, the results indicate that P38 exerted a beneficial effect on memory consolidation in a non-associative task, whereas ALG did not have this action.

**Key words** : PACAP-38, PACAP analog, spatial memory, ifenprodil, rat

## 1. Introduction

PACAP-38 (P38) is a 38 amino-acid neuropeptide discovered by Miyata et al. (1989). P38 acts through the G protein-coupled receptors VPAC1/2 (with similar affinity as vasoactive intestinal polypeptide) and PAC1 (highest affinity) (Vaudry et al., 2000; Hirabayashi et al., 2018). Binding to these receptors induces activation of the Adenylate cyclase/cAMP/CREB signaling pathway (Vaudry et al., 2000) that is involved in multiple biological functions. P38 is widely distributed in the brain and non-neural tissues. In the central nervous system, P38-immunoreactive cells and P38 mRNA have been localized in the hypothalamic area (Arimura et al., 1991; Köves et al., 1991; Masuo et al., 1992, Piggins et al., 1996). Significant amount of mRNA has been also found in many subcortical regions, for example the nucleus accumbens, substantia nigra, septum, amygdaloid complex, and other nuclei (Masuo et al., 1992; Ghatei et al., 1993; Piggins et al., 1996; reviews: Vaudry et al., 2000; Vaudry et al., 2009). Interestingly, the hippocampal formation - including the dentate gyrus, CA1, CA3 fields - contains P38-immunoreactive cells and P38 mRNA (Hashimoto et al., 1996; Piggins et al., 1996; Hannibal, 2002) although in a greater amount in the embryo brain than in the adult brain (Skoglösa et al., 1999). In addition, several neocortical regions and the cerebellum express P38 (Hashimoto et al., 1996; Hannibal, 2002). Consistent with these properties, PAC1 and VPAC1/2 receptors are found in all these subcortical and cortical regions (Masuo et al., 1992; Shioda et al., 1997; Vertongen et al., 1997). P38 exerts pleiotropic actions that underlie multiple physiological and pathophysiological processes. In particular, it has been largely documented that endogenous and exogenous P38 is a major neuroprotective and regenerative factor that thwarts the deleterious effects of various neurotoxic agents (e.g. NMDA, 6-OHDA, ethanol) and tissue damaging processes (e.g. oxidative stress, ischemic stress, stroke, inflammation, neurodegenerative diseases) (reviews in Dejda et al., 2005; Shioda et al., 2006; Waschek, 2013; Shioda and Nakamachi, 2015; Reglodi et al., 2018). A substantial number of studies have also shown that P38 modulates learning and memory processes (for a review, Ciranna and Costa, 2019). P38 administration induces a beneficial effect on memory in active (Adamik and Telegdy, 2005), and passive avoidance tasks (Telegdy and Kokavsky, 2000; Sachetti et al., 2001), fear conditioning (Meloni et al., 2016; Schmidt et al., 2015) and novel object recognition (Cabezas-Llobet et al., 2018). Additional reports showed that P38- or PAC1-deficient mice exhibit memory alteration in fear conditioning (Otto et al., 2001; Sauvage et al., 2000, Takuma et al., 2014) and novel object recognition task (Ago et al., 2013; Takuma et al., 2014). Finally, P38 administration was shown to allow memory loss rescue in animal

models of degenerative diseases such as Parkinson's (Deguil et al., 2010), Alzheimer's (Rat et al., 2011), and Huntington's (Cabezas-Llobet et al., 2018) diseases.

Based on their properties, P38 and its receptors are therefore being investigated as potential therapeutic targets. Despite its high molecular weight and hydrophilicity, P38 passes the blood-brain barrier through a saturable active transport system called PTS-6 (Peptide Transport System-6; Banks et al., 1993; Mizushima et al., 1999). However, a major limitation for therapeutic applications is the restricted bioavailability and half-life of P38. Thus, an analog has been designed, acetyl-[Ala<sup>15</sup>, Ala<sup>20</sup>]PACAP-38-propylamide (ALG), that exhibits with a four times greater affinity for the PAC1 receptor than P38 and a half-life of 25 minutes in blood plasma instead of less than 5 min for the endogen peptide (Bourgault et al., 2008). Whether this analog possesses similar functional properties is however unclear.

In a recent study, we have shown that an i.v. P38 injection in rats improves learning in a Morris water maze navigation task whereas ALG injection did not (Ladjimi et al., 2019). These results are consistent with the few studies showing an improvement of memory after P38 administration (Telegdy & Kokavszky, 2000; Sacchetti et al., 2001) and suggest that P38 and ALG do not exert similar actions, P38 being more efficient regarding memory processes. However, because these effects may be task-dependent, we investigated in the present study the effect of P38 and ALG in an exploration-based spatial novelty detection task (SND task). In the SND task, the memory of a spatial configuration of objects located in the environment is tested. When exposed to a novel environment containing objects, rodents exhibit spontaneous exploration of these objects. Exploration eventually yields to the formation of an internal representation (O'Keefe and Nadel, 1978) thought to be used for novelty detection in the now-familiar environment. Spatial novelty brought about by displacing a familiar object relative to other objects (spatial novelty test) has been shown to induce a selective re-exploration of the displaced object (Save et al., 1992; Lee et al., 2005; Van Cauter et al., 2013), which reflects novelty detection and updating of the representation. In the SND task, encoding of information occurs during exposure to the initial configuration of objects, consolidation between exposure and the spatial novelty test and retrieval during the test. In the present work, we examined the effect of P38 and ALG on memory consolidation by injecting these compounds after acquisition of the SND task. The effect of P38 in this task was not previously examined and in a first experiment we sought to determine the dose-effect response of an i.v. injection of P38. We then conducted a second experiment including a non-injected control group to address the impact of the saline injection and examined whether ALG produced P38-like effects.

P38 has been shown to modulate NMDA receptors (NMDARs) (Liu and Madsen, 1997; McDonald et al., 2005; Michel et al., 2006; Review in Yang et al., 2010), which plays a crucial role in synaptic plasticity, learning and memory (Wang et al., 2006). In addition, NR2B-containing NMDARs have been suggested to be important for memory consolidation (Ge et al., 2010; Ma et al., 2011; Cercato et al., 2014). It is therefore possible that the beneficial effect of P38 on consolidation in the SND task involves an interaction with NR2B-containing NMDARs. To address this hypothesis, we examined whether (i) NR2B-containing NMDARs affect memory consolidation in the SND task by administering ifenprodil (IFE; a selective NR2B-containing NMDAR antagonist; Reynolds and Miller, 1989; Williams, 1993; Tajima et al., 2016) and (ii) P38 and ALG modulate the performance of IFE-treated rats. It is of note that in this study, we assumed that if NR2B-containing NMDARs are involved in memory consolidation in the SND task, antagonist injection would interfere with the restoring effect found following P38 injection.

In parallel with the behavioral assessment, we investigated some neurobiological consequences of P38 or ALG administration. We therefore studied hippocampal BDNF that may be modulated by P38 and ALG and would possibly mediate their action in the brain. Not only the hippocampus is critically involved in the SND task but also BDNF has been shown to be modulated by P38 in *in vitro* studies (Pellegrini et al., 1998; Yaka et al., 2003; Frechilla et al., 2001; Reichenstein et al., 2008; Dong et al., 2010). In addition, both P38 and BDNF interact with NMDAR (Suen et al., 1997; McDonald et al., 2005; Rittase et al., 2014; Schmidt et al., 2015).

## **2. Results**

### **2.1. Experiment 1**

This experiment was aimed at determining the dose-effect response of an i.v. injection of 3 doses of P38, i.e. 5, 15, and 30 µg/kg. In the SND task, following familiarization with the arena (session 1, S1), rats explored a configuration of 4 objects during 4 successive sessions (learning phase; sessions 2-5, S2-S5). Rats then received their treatment immediately after S5. During a post-treatment session (S6), memory of the configuration was evaluated by the ability to detect a spatial change in the object configuration.

### 2.1.1. Pre-treatment locomotion (S1)

All groups displayed similar locomotor activity (one-factor ANOVA:  $F_{3,16} = 0.1539$ ,  $P = 0.9257$ ) and spent similar time in the peripheral region (one-factor ANOVA:  $F_{3,16} = 0.1746$ ,  $P = 0.9120$ ).

### 2.1.2. Pre-treatment learning (Session 2-5)

As shown in Figure 2A, all groups explored the objects similarly (ANOVA with repeated measures, no effect of group,  $F_{3,63} = 0.547$ ,  $P = 0.6520$ ). The exploration index decreased across sessions for all groups (effect of session,  $F_{3,63} = 12.97$ ,  $P < 0.0001$ , no session x group interaction,  $F_{9,63} = 0.3168$ ,  $P = 0.9666$ ), which indicated habituation.

### 2.1.3. Test (Session 6)

A factorial ANOVA revealed a significant effect of group ( $F_{3,32} = 3.63$ ,  $P = 0.0232$ ), no effect of object ( $F_{1,32} = 1.508$ ,  $P = 0.2284$ ) but an object x group interaction<sub>7</sub> ( $F_{3,30} = 10.94$ ,  $P < 0.0001$ ) (Fig. 2B). Post-hoc Newman-Keuls test showed that only the P38-30 $\mu$ g/kg group explored significantly more the displaced object (DO) than the non-displaced objects (NDO) (DO vs. NDO,  $P < 0.05$ ), a pattern generally found in non-treated rats (Save et al., 1992; van Cauter et al., 2013; Lee et al., 2005). This difference was not found in the two other P38-treated groups (DO vs. NDO,  $P > 0.05$ ). Surprisingly, in the present study, the SAL group exhibited an opposite pattern since they explored more the NDO than the DO ( $P < 0.01$ ). This result suggests a memory impairment in the SAL group.

In addition, comparisons of each re-exploration score (DO and NDO) with 0 value, no difference indicating no re-exploration, revealed that: SAL rats re-explored both DO (T-test comparing scores against 0 value,  $T_4 = 4.57$ ,  $P = 0.010$ ) and NDO ( $T_4 = 6.87$ ,  $P = 0.0024$ ), P38-5 $\mu$ g/kg rats re-explored the NDO ( $T_4 = 6.38$ ,  $P = 0.0031$ ) but not the DO ( $T_4 = 2.23$ , n.s.), P38-15 $\mu$ g/kg rats re-explored the DO ( $T_4 = 3.74$ ,  $P = 0.0201$ ) but not the NDO ( $T_4 = 0.665$ , n.s.), and P38-30 $\mu$ g/kg rats re-explored the DO ( $T_4 = 4.346$ ,  $P = 0.0225$ ) but not the NDO ( $T_4 = 0.549$ , n.s.) (Fig 2B). To summarize, the results show that i) The P38-5 $\mu$ g/kg group re-explored the NDO but not the DO; DO vs. NDO re-exploration was not different, ii) The P38-15 $\mu$ g/kg re-explored the DO and not the NDO; DO vs. NDO re-exploration was not different, iii) Only the P38-30 $\mu$ g/kg rats re-explored the DO and not the NDO in addition to significantly different DO vs. NDO re-exploration.

Thus, the ability to detect and identify spatial novelty was impaired in the SAL group whereas it was fully restored in the P38-30µg/kg group. The beneficial effect of P38 was found to be dose-dependent as the P38-5µg/kg and P38-15µg/kg groups did not show selective detection of the spatial change.

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Figure 2

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#### 2.1.4. Post-treatment locomotion (session 7)

After treatment, locomotion was not different in the 4 groups (one-factor ANOVA:  $F_{3,15} = 0.7755$ , n.s.). Post hoc pairwise tests did not reveal any difference (SAL vs. P38-5µg/kg:  $P = 0.448$ ; SAL vs. P38-15µg/kg:  $P = 0.974$ ; SAL vs. P38-30µg/kg:  $P = 0.560$ ; Fig. 2C).

#### 2.1.5. Conclusion

Unexpectedly, we did not find in the SAL group the object exploration profile generally observed in response to a spatial change in non-treated rats. The P38-30µg/kg group however exhibited this pattern and was therefore able to discriminate the DO and the NDO. Based on these results, we chose to use thereafter the 30 µg/kg dose and included a non-injected group in experiment 2 to address the impact of saline injection.

## 2.2. Experiment 2

This experiment was aimed at examining and comparing the effect of P38-30 µg/kg and ALG (P38 analog PAC1 agonist) on memory consolidation by injecting these compounds after acquisition of the SND task. We also investigated whether the effect of P38 on consolidation in the SND task involved

an interaction with NR2B-containing NMDARs. For this purpose we injected IFE, a selective NR2B-containing NMDA receptor antagonist, after S5.

### 2.2.1. Pre-treatment locomotion (S1)

Before treatments, all groups displayed similar locomotor activity (one-factor ANOVA: pre-IFE groups:  $F_{6,35} = 1.13$ , n.s.) and spent similar time in the peripheral region (pre-IFE groups:  $F_{6,35} = 0.9832$ , n.s.).

### 2.2.2. Pre-treatment learning (Session 2-5)

Before treatment, all groups exhibited similar exploration of the objects (ANOVA with repeated measures: no effect of group ( $F_{6,139} = 1.803$ , n.s.). The object exploration index decreased across sessions in all groups (effect of session:  $F_{3,139} = 22.59$ ,  $P < 0.0001$ ; no trial x group interaction:  $F_{18,139} = 0.5432$ , n.s.), which indicated habituation (Fig 3A).

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Figure 3

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### 2.2.3. Test (Session 6)

A factorial 2-way ANOVA revealed an effect of group ( $F_{6,70} = 2.441$ ,  $P = 0.0336$ ), no effect of object ( $F_{1,70} = 0.4888$ ,  $P = 0.4868$ ) but an object x group interaction ( $F_{6,70} = 2.628$ ,  $P = 0.0235$ ) (Fig. 3B). IFE rats were unable to detect spatial novelty and exhibited a reversed pattern of exploration with the NDO being more explored than the DO (DO vs. NDO,  $P < 0.05$ ). Administration of P38 did not improve spatial novelty detection in IFE-treated rats. Rats did not re-explore more the DO than the NDO (Newman-Keuls post-hoc test, DO vs. NDO,  $P < 0.05$ ). However, considering each kind of object separately, re-exploration (spatial change index) was significantly above 0 value for DO (t test .DO vs. 0 value,  $T_5 = 3.325$ ,  $P = 0.0209$ ) but not for NDO (t test NDO vs. 0 value,  $T_5 = 0.6166$ , n.s.). This latter result indicated that the difference between S5 and S6 was significant for DO only. The ALG treatment also failed to restore this ability (Fig. 3B).

We also measured locomotion during S6 to evaluate general activity following the treatments. More unbiased movement was measured in the empty arena (S7), to be compared with pre-treatment activity (S1)(see below). We found no effect of treatment on distance run during S6 (one factor ANOVA:  $F_{6,35} = 0.4166$ , n.s.; mean (cm)  $\pm$  s.e.m.: CTL,  $10848.66 \pm 1114.13$ ; SAL,  $9110.92 \pm 1254.65$ ; P38,

8189.82 ± 1182.04; ALG, 8662.32 ± 1556.56; IFE, 8396.42 ± 1980.18; P38+IFE, 9790.92 ± 1188.64; ALG+IFE, 8510.18 ± 1770.57)

In summary, consistent with experiment 1, the results showed that SAL rats were not able to properly detect spatial novelty. Detection of spatial novelty was however observed in CTL rats, i.e. rats that did not receive any injection. In this context, P38 induced a beneficial effect as the rats' ability to detect spatial novelty was similar to that found in CTL rats while ALG did not yield similar effect. In addition, the IFE group failed to detect spatial novelty. Finally, co-administration of P38 or ALG with IFE did not restore CTL-like ability although a re-exploration of DO was observed.

#### 2.2.4. Post-treatment locomotion (Session 7)

A significant effect of treatment on locomotion was found (one-factor ANOVA:  $F_{6,35} = 4.977$ ,  $P < 0.0001$ ) (Fig.3C). There was no difference between CTL and SAL groups. P38, but not ALG, treatment reduced locomotor activity (P38 vs. SAL,  $P < 0.05$ ; ALG vs. SAL, n.s.). Administration of IFE did not affect locomotion (IFE vs. SAL, n.s.). Administration of P38 and ALG did not change locomotion in IFE-treated rats (P38+IFE vs. IFE: n.s.; ALG+IFE vs. IFE: n.s.).

### 2.3. BDNF levels in the hippocampus

Figure 4 shows that BDNF content were different between groups (one-factor ANOVA:  $F_{6,24} = 13.91$ ,  $P < 0.0001$ ). The IFE group showed the higher BDNF content vs. the SAL group (Newman-Keuls post hoc test: IFE vs. SAL,  $P < 0.001$ ) while the P38 and ALG groups did not differ from the SAL group ( $P > 0.05$ ). Administration of P38 or ALG to IFE-treated rats produced a decrease in BDNF content vs. IFE treatment alone (P38+IFE vs. IFE and ALG-IFE vs. IFE: both  $P < 0.0001$ ).

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Figure 4

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### 3. Discussion

In the present study we compared the effects of P38 and its analog ALG in a SND task that taxes memory of a configuration of objects. We also examined the effects of administrating IFE, a NMDAR antagonist, after acquisition, alone or in combination with P38 or ALG to address a potential involvement of NR2B-containing NMDARs in P38 and ALG effects.

In the SND task and similar tasks, it has been shown that non-treated rats are able to detect spatial novelty and selectively re-explore the displaced object relative to the non-displaced objects (Save et al., 1992; Lee et al., 2005; Maasberg et al., 2012; van Cauter et al., 2013), this behavior being considered to reflect an updating of the spatial representation. In the present experiments we found that this ability was unexpectedly disrupted in rats that had received saline immediately after learning, 24h earlier. In experiment 1, SAL rats selectively re-explored the non-displaced objects and in experiment 2, they exhibited equivalent exploration of the displaced object and the non-displaced objects. In contrast, rats that did not receive any injection (CTL group) exhibited the well-documented pattern of behavioral response, i.e. re-explored the displaced object more than the non-displaced objects. The difference SAL vs. CTL was probably not due to different learning levels because the two groups exhibited similar object exploration throughout the learning sessions. This suggests that behavior and cognitive processes were influenced by external and/or internal factors that interacted with P38 treatment to produce inconsistent behavior in control conditions. The SND task is probably more likely to be influenced by these factors than other tasks with repeated training trials because it involves spontaneous behavior during a one-shot sequence of sessions including learning and testing. However, in spite of various factors potentially affecting behavior, we were able to obtain a pattern of exploration consistent with previous studies in non-treated rats (Save et al., 1992; Lee et al., 2005; van Cauter et al., 2013). Among these factors, it is possible that the difference between SAL and CTL groups resulted from the i.v. injection procedure which is likely to be a stressful experience that may affect *per se* memory consolidation and/or exploratory behavior during the test session.

Stress has been shown to have a clear impact on memory and hippocampus-dependent learning (reviews: Kim and Diamond, 2002; Schwabe et al., 2012) but the literature does not strongly support this hypothesis on at least two aspects. First, the animals underwent movement restraint for 30 s while in acute stress studies that reveal memory effects, rats are generally submitted to much longer immobilization, i.e. 30 min, 1h and even more (Blank et al., 2002; Conrad et al., 2004; Telegdy and Adamik, 2015; Uwaya et al., 2016; Aguayo et al., 2018). Second, memory consolidation is generally enhanced following stress (Roosendaal et al., 2006; Schwabe et al., 2012). In spite of this, we cannot strictly rule out that immobilization for 30 s is sufficient to induce stress that would affect consolidation for long term memory. The effects of a short-lasting restraint on physiological markers of stress is poorly known. Heath et al., (1971) have shown that a mild 30 s restraint, tail-warming alone or associated with

injection in the tail vein produced peripheral biochemical changes that may underlie physiological impairments and beyond, central effects. Thus, it is possible that the injection conditions we used here yielded some stress that, even weak, occurred within a short time window after learning and eventually disrupted memory consolidation in this task (Schwabe et al., 2012). This needs however to be further investigated. Finally, both SAL and CTL groups exhibited similar locomotor activity during S6 (spatial novelty test session) and S7, suggesting that a general effect on behavior during the test session did not account for this effect.

Because P38 is also implicated in peripheral functions (Vaudry et al. 2009 for a review), another alternative explanation is that effects in the SND task resulted from a peripheral rather than central effect. This possibility appears unlikely however. First, it has been found that P38 crosses the blood-brain barrier (Banks et al., 1993). Second, P38 produced a specific behavioral effect, i.e. increased exploration of the displaced objects but not the non-displaced objects, which reflects cognitive processes (Save et al., 1992; Lee et al., 2005). Such object discrimination has been shown to be affected by brain lesions (van Cauter et al., 2013) and is therefore not likely to result from a general peripheral, e.g. cardiovascular or respiratory, response (Farnham and Pilowsky, 2010; Farnham et al., 2012). Third, locomotor activity that may be affected by P38 peripheral action was not different in all groups after the test session. Thus although we cannot strictly exclude that P38 produced peripheral effects in addition to central effects, we conclude that our results in the SND task could not be accounted for by these peripheral effects. Another issue is that the absence of effect of ALG would be due to the fact that it does not cross the blood-brain barrier. On the contrary, a study by Dejda et al. (2011) suggests that it does cross the blood-brain barrier because an i.v. injection of ALG induced a central effect (decrease of brain infarction in a brain ischemia model). That ALG crosses the BBB remains however to be confirmed in physiological conditions.

We show that injection of P38 allowed restoration of the ability to detect spatial novelty in the SND task. In experiments 1 and 2, rats treated with P38 (30 $\mu$ g/kg) selectively re-explored the displaced object although 5 and 15  $\mu$ g/kg doses did not induce this pattern of response. In contrast, the pattern of response in P38-30  $\mu$ g/kg-treated animals was similar to that in CTL rats, which suggests that P38 thwarted the deleterious effects of a saline injection on memory. As P38 has been shown to elicit anxiety-related effects (Kormos and Gaszner, 2013), a readily hypothesis is that P38 abolishes the stressful effects of the injection due to anxiolytic properties. However, administration of P38 in the central nervous

system produces anxiogenic rather than anxiolytic effects (Hammack et al., 2009; Telegdy and Adamik, 2015) and P38-deficient mice exhibit lower anxiety-related behavior (Hashimoto et al., 2001; Gaszner et al., 2012). In addition, PAC1-deficient mice show decreased anxiety-like behavior (Otto et al., 2001). Thus, an alternative hypothesis is that P38 produces an anxiogenic effect and therefore modulates the hypothalamic-pituitary-adrenal axis and activates the corticotropin-releasing factor (CRF) (Mustafa et al., 2013). Enhancement of memory consolidation may then result from a specific effect on memory through activation of CRF1 receptors in the hippocampus (Wang et al. 1998). However, the anxiogenic hypothesis fails to explain why P38-induced anxiety allowed effective consolidation whereas saline-induced anxiety (due to injection procedure) did not. In addition, this anxiety-related hypothesis cannot really account for the results of other studies showing an implication of P38 in memory. Indeed these studies used various approaches to manipulate the P38ergic system (P38-deficient or PAC1 receptor-deficient mice, intracerebroventricular or systemic P38 injection), used various memory tasks (water maze task, fear conditioning, active or passive avoidance, object recognition, radial maze working memory task, etc.) and targeted various memory processes (acquisition, retrieval) but they all showed an implication of P38 in memory (Telegdy and Kokavszky, 2000; Sauvage et al., 2000; Sacchetti et al., 2001; Otto et al., 2001; Adamik et al., 2005; Takuma et al., 2014; Meloni et al., 2016; Ladjimi et al., 2019). More specifically, some of them have described an improvement of memory following P38 administration. Post-acquisition administration of P38 induces an enhancement of consolidation (Sacchetti et al., 2001) as well as acquisition, and retrieval (Telegdy and Kokavszky, 2000) of a passive avoidance response. Recently, using a more cognitive task, we have found that P38 improved place learning in the water maze when a weak massed-learning procedure was used (Ladjimi et al., 2019). Thus, our current results using the SND task and the literature suggest that P38 administration shortly after acquisition rescues memory consolidation.

The rats treated with IFE exhibited a pattern of object exploration consistent with a deficit in detection of spatial novelty. Not only they failed to specifically re-explore the displaced object like CTL rats did but they actually displayed the opposite response: they re-explored more the non-displaced objects than the displaced one. It is unlikely that this behavior results from anxiogenic effect since IFE has been reported to produce an anxiolytic rather than anxiogenic effect (Fraser et al., 1996; Kincheski and Carobrez, 2010). Our results are consistent with those of Frühauf *et al.*, (2015) who found a similar effect of IFE 10 mg/kg on consolidation in a novel object recognition task. Thus, the fact that IFE rats

did not re-explore the displaced object supports the hypothesis that NR2B-containing NMDARs are important for memory consolidation in this task.

We then found that combining administration of P38 and IFE (P38+IFE condition) yielded a marginal improvement of spatial novelty detection. Indeed, rats significantly re-explored the displaced object. Full rescue of CTL-like response that would involve re-exploration of the displaced object greater than the non-displaced objects, was not obtained. Note that there was no indication that locomotor activity was different in P38+IFE vs. IFE rats. Thus, we did not see a dramatic effect, but it is likely that there was a mild beneficial effect of P38 on memory consolidation in IFE rats. This suggests that P38 mechanism of action on memory consolidation in the SND task involves - but is not specific of - NR2B-containing NMDARs. This is consistent with Yaka et al's study (2003) showing that P38 modulates NR2B-containing NMDARs and produces enhancement of NMDAR-mediated activity in the hippocampus. To further determine the neurobiological events triggered by P38 and ALG treatments in this task, we measured BDNF levels in the hippocampus, a structure required for detection of spatial novelty (Save et al., 1992). *In vitro* studies have shown that P38 stimulates BDNF production in neuron cultures (Frechilla et al., 2001; Pellegrini et al., 2001; Reichenstein et al., 2008; Dong et al., 2010; Brown et al., 2014; Kaneko et al., 2018), and hippocampal slices (Yaka et al., 2003), and increases TrkB receptors in the hippocampus (Pellegrini et al., 1998). Furthermore, PAC1-deficient mice exhibit decreased expression of TrkB receptors in the hippocampus (Zink et al., 2004). In the present study, we did not observe any modulation of BDNF in P38-treated rats. In contrast, IFE-treated rats had increased BDNF levels which was reversed following P38 or ALG administration. This supports our previous results showing similar BDNF content in naïve rats and rats treated with P38 and trained in a place learning task (Ladjimi et al., 2019) and the hypothesis that P38 modulates BDNF expression in the hippocampus. However, BDNF modulations appeared to be not related to behavioral performance in the SND task (at least at the doses we used) because we did not find changes in BDNF content in rats that exhibited preserved memory consolidation following P38 administration vs saline rats. Conversely, an increase in BDNF was measured in rats with IFE administration though they exhibited no detection of spatial novelty.

A major aim of the present study was to compare the effects of P38 and ALG in the SND task. The results show that ALG did not produce similar effects as P38 as they did not re-explore the objects nor were able to discriminate the displaced object from the non-displaced objects during the test session.

When ALG was administrated with IFE, animals did not re-explore the displaced objects and there was no difference between the displaced object and the non-displaced objects, which indicates impaired novelty detection. No effect of ALG on general activity was found. Thus, our results show that the biochemical P38-derived analog ALG did not produce the effect of P38 on memory consolidation in this task despite its high affinity with the PAC1 receptor and its improved metabolic stability vs. P38 (Bourgault et al., 2008). Such properties are essential in a therapeutic perspective. However, whether ALG shows functional properties similar to those of P38 deserves further investigations. Dejda et al. (2011) found that an i.v. ALG injection had a neuroprotective effect in an ischemia model. More recently, we have reported an enhancement of place memory in a Morris water maze navigation task but failed to demonstrate a similar effect of ALG (Ladjimi et al., 2019).

Overall, the present results complement available data showing that P38 modulates memory processes. We showed that P38 improved consolidation of spatial memory of a configuration of objects, an important process for the formation of a spatial representation. In contrast, the analog did not reproduce this effect. Its multiple biological actions (neurotransmitter, neuroprotective and neurotrophic factor, to mention a few) make P38 a potentially promising therapeutic agent. Ideally, P38 analogs such as the acetyl-[Ala<sup>15</sup>, Ala<sup>20</sup>]PACAP-38-propylamide are made not only to improve biological availability but also functional specificity while reducing unwanted peripheral (e.g. cardiovascular) effects (Warren et al., 1992; Vaudry et al., 2000 for a review; Farnham et al., 2012). To this end, it is necessary to seek selective actions of ALG by using various experimental models addressing neuroprotective, memory, anti-inflammatory, etc., aspects of P38 actions. That ALG does not exhibit a P38-like memory effect does not preclude other potential effects that may be developed in a therapeutic perspective.

## **4. Methods and materials**

### **4.1. Chemicals**

P38 and ALG were generously provided by Professor David Vaudry (Laboratory of Neuronal and Neuroendocrine Differentiation and Communication, INSERM U413, Rouen University, France). P38 and its analog were synthesized using the fluorenylmethyloxycarbonyl (Fmoc) chemistry methodology as previously described (Bourgault et al., 2008; Jolivel et al., 2009). Following *in vitro* treatment with dipeptidyl peptidase IV, PACAP-38 initially loses its first two amino acids and becomes PACAP(3-38). It then undergoes cleavage at the Gly<sup>4</sup> of PACAP(3-38) and becomes PACAP(5-38), which behaves as

a PACAP antagonist (Bourgault et al., 2008). We used this analog in our study which was verified in Dr Vaudry's lab using MALDI-TOF mass spectrometry. Spectrum of acetyl-[ALA<sup>15</sup>,,ALA<sup>20</sup>]PACAP38-propylamide shows a peak at 4502,158 Da corresponding to the theoretical mass of the analog (Bourgault et al., 2008). Ifenprodil ( $\alpha$ -(4-Hydroxyphenyl)- $\beta$ -methyl-4-benzyl-1-piperidineethanol tartrate salt) was purchased from Sigma Aldrich (St Louis, US, MO).

## 4.2. Animals

A total of 62 male Wistar rats (Janvier Labs, Le Genest Saint Isle, France) weighing between 200 and 250 g were housed, two in a cage (40 cm long x 26 cm wide x 16 cm high), with water and food *ad libitum* in a temperature-controlled room ( $20 \pm 2^\circ\text{C}$ ) with natural light/dark cycle. Starting 24 hours after delivery, the rats were handled daily by the experimenter for one week. The experiments were performed in accordance with the European guidelines (European Community Council Directive, 2010/63/UE), and National guidelines (Council directive n°87848 of the Direction des Services Vétérinaires de la Santé et de la Protection Animale, permission n° 13.24 from the Ministry of agriculture and fisheries to E.S., local ethic committee and national authorization n° A8-12-12). Behavioral testing was conducted during the light phase.

## 4.3. General protocol

### 4.3.1. Experiment 1: dose-response effect of P38

We first sought to determine a dose that would affect performance in the SND task. On the basis of three studies of Akerman and Goadsby (2015), Deguil et al., (2010) and Hong et al., (1998), the effects of three i.v.-injected doses were tested, 5  $\mu\text{g}$ , 15  $\mu\text{g}$ , and 30  $\mu\text{g}/\text{kg}$  in the SND task. We used 20 rats that were subdivided into 4 groups, P38-5 ( $n = 5$ ), P38-15 ( $n = 5$ ), P38-30 ( $n = 5$ ), and SAL ( $n = 5$ ) before being submitted to the SND protocol. To match the conditions of Experiment 2, the treatment consisted of an i.v. injection followed 5 min later by an intraperitoneal (i.p.) injection (Frühauf et al., 2015). For the i.v. injection, the SAL group received NaCl 0.9 %, and the three P38-treated groups received one of the 3 doses of P38, (5, 10, or 30  $\mu\text{g}/\text{kg}$ ). For the i.p. injection, all groups received a NaCl 0.9 %. The drug administration and behavior protocols were similar in both Experiment 1 and Experiment 2 (see sections 2.4 and 2.5). The treatments were administrated after the last session of the learning phase on day 2. The rats were then submitted to the test phase on day 3.

#### 4.3.2. Experiment 2: effects of P38 and ALG in rats treated with ifenprodil

Following Experiment 1, the 30 µg/kg dose of P38 was chosen to be used in Experiment 2. Forty-two rats were used and were subdivided into 7 groups (n = 6). Immediately after the last session of the learning phase on day 2 of the SND task (see below), all groups were given an i.v. injection followed 5 min later by an i.p. injection (Table 1). On day 3, all rats were submitted to the test phase of the SND task. Table 1 summarizes the groups used in this experiment. An additional control group (CTL) that did not receive any injection but was submitted to the task was added to control for the effect of the injection procedure.

#### 4.4. Drug administration

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Table 1

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For i.v. injections, Microlance BD-23G/30 x 0.6 mm needles were used. The injected volume was 100 µl/100 g. The rat was placed delicately head first in a custom-made intravenous injection apparatus (hereafter termed tailveiner). This tailveiner consisted of a cylindrical portion 20 cm long and 5 cm in diameter (open on one side) of a PVC tube fixed by hose clamps to a wooden board. This board is itself fixed by clamps to the bench. Once inside the tailveiner, the entrance was closed so that the tail of the rat remains outside. The rat's tail was warmed with warm water to reveal the vein and then the injection was done. Contention in the tailveiner lasted about 30 s. For i.p. injections, Microlance BD-25G/16 x 0.5 mm needles were used. The injected volume was 500 µl/100 g. P38, ALG, and IFE were diluted in NaCl 0.9 % (Dejda *et al.*, 2011; Shioda and Nakamachi, 2015). The animal was grasped firmly by the back, blocking the four legs with a slight pressure of the fingers and we proceeded to the injection.

#### 4.5. Spatial novelty detection task (SND task)

The apparatus consisted of a black PVC circular arena (diameter 76 cm diameter, wall height, 50 cm) surrounded by a circle of white curtains so that the environment was visually uniform. Homogenous indirect lighting was provided by 4 x 40W spotlights located outside the curtain and directed toward the ceiling. As in previous studies (Van Cauter *et al.*, 2013) a piece of white cardboard covering 90° was placed on the arena wall to provide a polarizing cue. A digital camera was placed 2 m above the center of arena floor. A radio set was also placed above the arena and produced a background sound (> 70

dB) to mask possible uncontrolled noises. The camera was connected to a computer and a tracking software (Videotrack, Viewpoint, France) was used to track the animal on the video image and analyze the animal's trajectories and spatial location. The general behavioral protocol used for both experiments is shown in Figure 1. The animals were submitted to free exploration sessions on 3 successive days. All behavioral testing was conducted between 6:00 and 10:00 A.M. Between sessions, the floor of the arena was wiped to shuffle olfactory cues and the feces if any were removed. At the end of the session sequence for each rat, the arena was cleaned with a 0.1 % alcohol solution.

On day 1 (familiarization), the arena was empty and the rats were submitted to one 8-min-session (S1). On Day 2 (learning), 4 objects were placed in the arena (Figure 1) and the rats were submitted to four 4-min-sessions (S2-S5; Inter session interval, 4 min, the animals were replaced in their home cage). The objects were all different in color, texture, and shape and included a glass bottle (Ob1), a plastic cylinder (Ob2), a wooden ball (Ob3) and a Rubik's cube (Ob4). Figure 1 shows the initial spatial configuration of the set of objects in S2-S5 (day 2) and after the spatial change (displacement of one object) in S6 (day 3). In S2 the objects at the periphery (Ob1, Ob2, Ob3) were located 15 cm from the wall and the central object (Ob4) was half distance between Ob1 and Ob2. Immediately after the last session of the learning phase, the animals received their treatment. On day 3 (test), Ob4 was moved to the periphery of the arena and was located 15 cm from the wall like the other, non-displaced, objects. The ability to detect a spatial change in the object configuration was evaluated during a post-treatment 4-min-session (S6). Following S6, the animals were submitted to a last 4-min-session (S7) in the empty arena to measure locomotion after treatment. At the end of the experiment, the rats were sacrificed by decapitation and their hippocampi were removed for biochemical analyses. In both experiment 1 and 2, rats from the different groups were trained in pseudorandom order.

## **4.6. Data Analyses**

### **4.6.1. SND task**

All sessions were recorded and the videos were stored on the computer for off line analysis. To evaluate exploratory activity, we measured locomotion and object exploration. Exploration of an object was defined as the approach of the animal's snout to a distance of less than 2 cm from an object while actively exploring it (a contact). Both measures were obtained using the Videotrack system which allows to track the rat's movements as well as the nose position. When the nose was detected in a virtual area

(appearing only on the computer screen) surrounding each object and corresponding to 2cm in the actual arena, a contact was counted. To evaluate object exploration during the learning phase, we calculated a normalized exploration index (Cohen, 1988) for each rat and each session according to the following formula:

$T_{ExpS2-5} \times 100 / mT_{ExpS2}$  where  $T_{ExpS2-5}$  is the time of exploration (sec) averaged for all 4 objects by one rat during each session (S2 to S5),  $mT_{ExpS2}$  is the mean time of exploration (sec) of the objects by all rats of the same group during S2.

To evaluate the exploratory response to the spatial change, we calculated a reexploration score for the displaced object and the non-displaced objects (Parron and Save, 2004; Van Cauter et al., 2008; 2013) according to the following formulas:

Reexploration score for the displaced object (sec) =  $(T_{Ob4S6} - T_{Ob4S5})$ , where  $T_{Ob4S6}$  is the duration of contacts (sec) with the displaced object (Ob4) in S6 (new position), and  $T_{Ob4S5}$  is the duration of contacts (sec) of Ob4 during S5 (initial position).

Reexploration score for the non-displaced objects (sec) =  $(T_{Ob1-3S6} - T_{Ob1-3S5})$  where  $T_{Ob1-3S6}$  is the duration of contacts in sec averaged on the 3 non-displaced objects during S6, and  $T_{Ob1-3S5}$  is the duration of contacts in sec averaged on the non-displaced objects during S5. It has been repeatedly shown that detection of spatial novelty induces an increase in object exploration with the displaced object being more explored than the non-displaced objects (e.g. Save et al., 1992).

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Figure 1

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#### 4.6.2. Quantification of BDNF in the hippocampus

We used the hippocampus removed from the trained rats to measure the content of BDNF by using an enzyme-linked immunosorbent assay kit. The hippocampus was removed and frozen at -80°C. Hippocampal tissues were homogenized under stirring for 1 hour at 4°C in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100 and 1mM EDTA) supplemented with protease inhibitors cocktail (Bio Basic Inc, CA). The tissue lysate was then centrifuged (12 000g, 20 min, 4°C) and the supernatant was stored at -20 °C until use. Protein concentration was assessed by BCA protein assay following manufacturer's instructions (Novagen). BDNF levels were determined using Rat BDNF PicoKine™ ELISA kit purchased from Tebu-bio (Le

Perray-en-Yvelines, France) according to manufacturer's instructions and expressed as picograms per milligram of protein.

#### 4.6.3. Statistical Analyses

Data obtained are presented as means  $\pm$  standard error of the mean (S.E.M). Statistical analyses were performed using ANOVA followed by a Newman-Keuls post-hoc test (GraphPad Prism 7, GraphPad software, San Diego, USA). Statistical significance required a  $P < 0.05$ . As mentioned above all groups were trained and tested at the same time. In addition, we examined whether each group displayed significant re-exploration of DO and NDO by using t-test comparing the re-exploration scores to 0 value (0 value would indicate no re-exploration).

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## 6. Figure legends

**Figure 1. Protocol of the SND task.** Rats explore a circular arena which is empty (S1) or contains 4 different objects (S2-S7). After a familiarization session in an empty circular arena (day1, S1), the rats are submitted to 4 learning sessions with 4 distinct objects placed in the arena (day 2, S2-S5). Animals are injected with saline, P38, ALG (P38 analog PAC1 agonist), IFE, P38+IFE, or ALG+IFE, right after the last learning session (indicated by an arrow). The test session (S6) is conducted 24h after learning. During S6, spatial novelty is produced by the displacement of an object (Obj4) from the center to the periphery. The spatial configuration is therefore different. The 3 other objects remain unchanged. A last session (S7) is made to measure general activity.

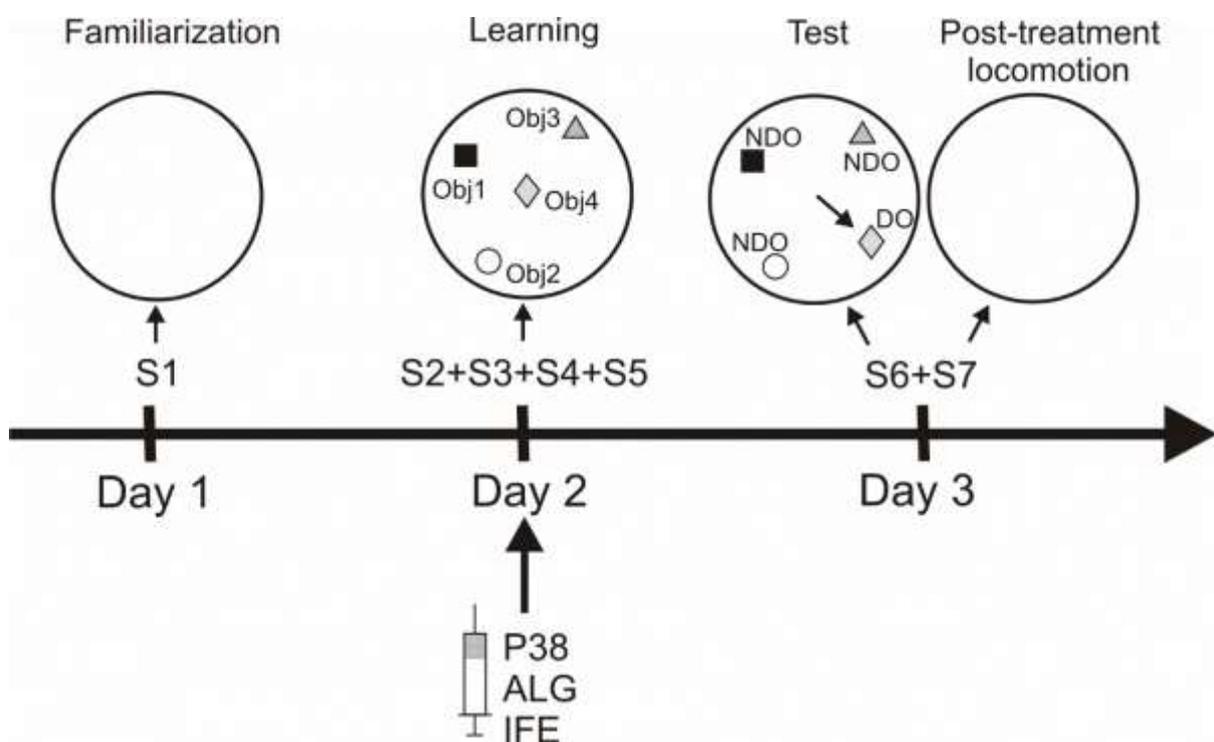
**Figure 2. Experiment 1. A.** Exploration index during learning sessions (S2-S5) for the 4 groups (SAL, P38-5 µg/kg, P38-15 µg/kg, P38-30 µg/kg). **B.** Re-exploration score during the test session (S6) for the displaced object (DO) and the non-displaced objects (NDO). Asterisks show statistically significant differences between DO and NDO for each group, \* P < 0.05; \*\* P < 0.01; Black or white circles represent statistically significant differences for the comparison between re-exploration scores and 0 value for DO and NDO; ° P < 0.05; °° P < 0.01; ns: non-significant P > 0.05. **C.** Total distance run by the rats during S7 (one-factor ANOVA:  $F_{3,15} = 0.7755$ , n.s.; SAL vs. P38-5µg/kg, P = 0.448; SAL vs. P38-15µg/kg, P = 0.974; SAL vs. P38-5µg/kg, P = 0.560).

**Figure 3. Experiment 2. A.** Exploration index during learning sessions (S2-S5) for the 7 groups (CTL, SAL, P38, ALG (P38 analog PAC1 agonist), IFE, P38+IFE, ALG+IFE). **B.** Re-exploration score during the test session (S6) for the displaced object (DO) and the non-displaced objects (NDO). Asterisks show statistically significant differences between DO and NDO for each group, \* P < 0.05, \*\* P < 0.01; Black or white circles represent statistically significant differences for the comparison between re-exploration scores and 0 value for DO and NDO; ° P < 0.05; °° P < 0.01; ns: non-significant P > 0.05. **C.** Total

distance run by the rats during S7. Asterisks show statistically significant differences between groups, \*  $P < 0.05$ .

**Figure 4.** BDNF content in the hippocampus (pg/mg of protein) measured after S7 in all groups. Asterisks show statistically significant differences between groups, \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; ns: non-significant  $P > 0.05$ .

## 7. Figures



**Figure 1**

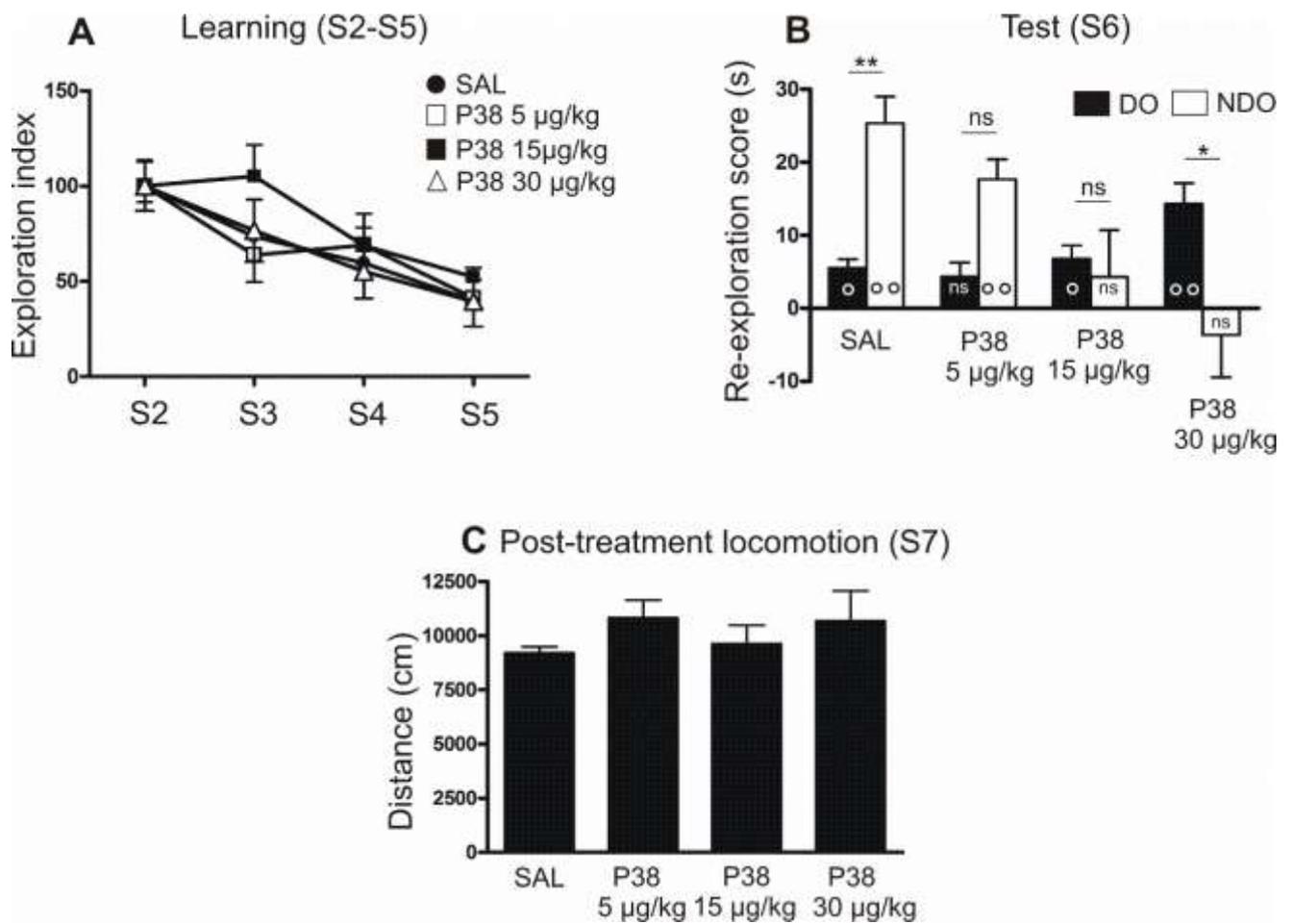


Figure 2

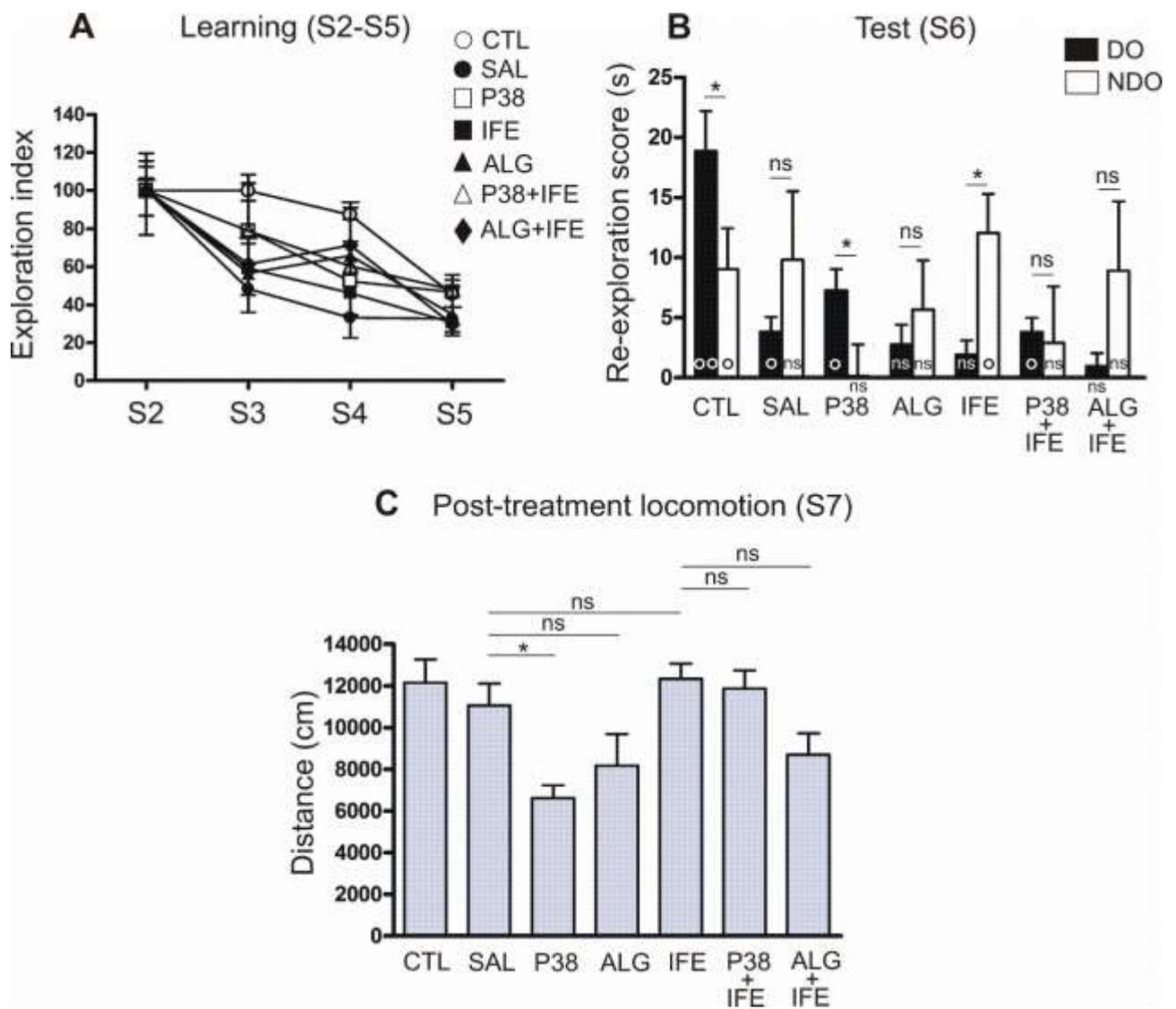


Figure 3

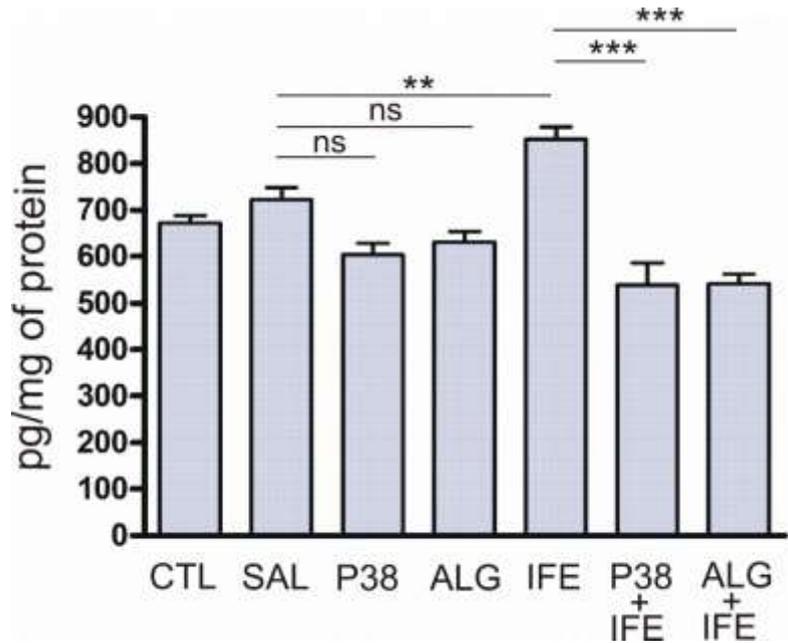


Figure 4

## 8. Tables

Table 1

Group	i.v. injection	i.p. injection	n
CTL	-	-	6
SAL	NaCl 0.9 %	NaCl 0.9 %	6
IFE	NaCl 0.9%	Ifenprodil 3 mg/kg	6
P38	P38 30 µg/kg	NaCl 0.9%	6
ALG	ALG 30 µg/kg	NaCl 0.9%	6
P38+IFE	P38 30 µg/kg	Ifenprodil 3 mg/kg	6
ALG+IFE	ALG 30 µg/kg	Ifenprodil 3 mg/kg	6

**Groups submitted to the SND task.** All groups except the CTL group received an i.v. injection followed by an i.p. injection. The CTL group was submitted to the task only.