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Pregnenolone sulfate and its enantiomer: differential modulation of memory in a spatial discrimination task using forebrain NMDA receptor deficient mice

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Abstract

This study examined the role of forebrain N-methyl-D-aspartate receptors (NMDA-Rs) in the promnesiant effects of natural (+) pregnenolone sulfate (PREGS) and its synthetic (–) enantiomer *ent*-PREGS in young adult mice. Using the two-trial arm discrimination task in a Y-maze, PREGS and *ent*-PREGS administration to control mice increased memory performances. In mice with a knock-out of the NR1 subunit of NMDA-Rs in the forebrain, the promnesiant effect of *ent*-PREGS was maintained whereas the activity of PREGS was lost. Memory enhancement by PREGS involves the NMDA-R activity in the hippocampal CA1 area and possibly in some locations of the cortical layers, whereas *ent*-PREGS acts independently of NMDA-R function.

Keywords

pregnenolone sulfate; steroid enantiomers; memory; Y-maze; NR1 subunit; hippocampus; NMDA receptors

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Contributors

Y. Akwa designed the study, wrote the protocol, managed behavioral experiments and analyses, literature search and wrote the first draft of the manuscript. G.H. Petit and L. Rondi-Reig characterized and provided NR1-KO and control mice. C. Tobin, Y. Moricard and G.H. Petit performed histological location of NR1 subunit location. K. Krishnan and D.F. Covey synthesized *ent*-PREGS. Y. Akwa, C. Tobin, G.H. Petit, D.F. Covey and L. Rondi-Reig contributed to the final manuscript. All authors have approved the final manuscript.

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Conflict of interest

All the authors declare that they have no conflicts of interest.

1. Introduction

N-methyl-D-aspartate receptors (NMDA-Rs) formed by the obligatory NR1 subunit combined with modulatory NR2A-D and NR3A-B subunits (Cull-Candy et al., 2001) play pivotal roles in the mechanisms dependent plasticity underlying learning and memory (Nakanishi, 1992; Riedel et al., 2003). They display diverse pharmacological properties depending on subunit composition and brain region (Yamakura and Shimoji, 1999) and are regulated by several endogenous or exogenous ligands, including glutamate and steroids (Wu et al., 1991; Kemp and McKernan, 2002).

Pregnenolone sulfate (PREGS) is a natural neuroactive steroid that increases neuronal activity by allosterically modulating neurotransmitter receptors including NMDA-Rs (Wu et al., 1991). PREGS potentiates glutamate or NMDA-induced currents in NR1/NR2A-B NMDA-Rs and inhibits those of NR1/NR2C-D NMDA-Rs (Malayev et al., 2002). Moreover, PREGS enhances learning and memory performances in several behavioral paradigms in rodents (Mayo et al., 1993; Mathis et al., 1994; Akwa et al., 2001).

The synthetic enantiomer of PREGS (*ent*-PREGS) has been shown to display promnesiant activity, with potency higher than that of its natural counterpart PREGS (Akwa et al., 2001). Enantiomeric steroids (*ent*-steroids) do not occur in nature and are made by chemical synthesis (Biellmann, 2003) (see Supplementary Figure 1 for PREGS and *ent*-PREGS structures). They may bind differently to neurotransmitter receptors to modulate brain functions differentially (Covey, 2009). Several studies with natural- and *ent*-steroids have shown enantioselective properties depending on the steroid's structure and the biological functions analyzed. For example, in cultured hippocampal neurons, γ -amino-butyric acid type A receptors are inhibited by both natural (+) PREGS and synthetic (–) *ent*-PREGS, whereas they are modulated differently by dehydroepiandrosterone sulfate and its enantiomer (Nilsson et al., 1998). Icv administration of PREGS or *ent*-PREGS reverses scopolamine-induced amnesia in rats, with PREGS being more effective (Vallée et al., 2001). Moreover, *ent*-PREGS promnesiant activity is not inhibited by administration of the NMDA-R antagonist DL-2-amino-5-phosphonovaleric acid (Akwa et al., 2001).

The present experiments aim to investigate the role of forebrain NMDA-Rs in the promnesiant effects of PREGS and *ent*-PREGS, using mice in which the gene for the NR1 subunit of NMDA-Rs is knocked-out (NR1-KO) in the hippocampal CA1 region and decreased in some parts of the deep cortical layers (Tsien et al., 1996; Fukaya et al., 2003; Rondi-Reig et al., 2006).

2. Experimental procedures

All experiments were performed in accordance with the European Communities Council Directive of November 1986 (86/609/EEC).

2.1. Animals

Male NR1-KO mice were generated as previously described (Tsien et al., 1996; Fukaya et al., 2003). They were heterozygous for the viral Cre recombinase gene and homozygous for the floxed NR1 gene. The control group includes male littermates homozygous for the floxed NR1 gene.

2.2. Surgery and treatments

Mice were implanted unilaterally above the lateral ventricle, according to the coordinates: –0.5 mm posterior to bregma, $\pm$ 1.0 mm lateral to the midline, and –1.5 mm below the skull surface, as previously described (Akwa et al., 2001). PREGS (3 β -hydroxy- Δ^5 pregnen-20-

one sulfate) was obtained from Steraloids (Newport, RI, USA). *ent*-PREGS was chemically synthesized as previously described (Nilsson et al., 1998). PREGS (5 nmol), *ent*-PREGS (0.5 nmol) or 0.3 % NaCl (vehicle, VEH) was icv injected, immediately after the acquisition phase, and their effects on memory retention performances analyzed.

2.3. Behavioral protocol and evaluation

Behavioral studies were conducted in 6-12 week old mice. Memory performances were evaluated by using a two-trial arm discrimination task in a Y-maze, a hippocampus relevant spatial task (Conrad et al., 1996) based on the innate tendency of rodents to explore novelty (Dellu et al., 2000). This test has already been applied to steroid modulation of spatial memory performances in rats and mice (Mayo et al., 1993; Ladurelle et al., 2000; Akwa et al., 2001).

The procedure was based on previous studies in rodents (Dellu et al., 1992; Conrad et al., 1996; Ladurelle et al., 2000; Akwa et al., 2001). Briefly, the test consisted of two trials that lasted for 5 min, separated by an inter-trial interval (ITI) of either 2 h (study 1) or 6 h (study 2). During the acquisition trial, mice were allowed to visit two arms (familiar arms 1 and 2) of the Y-maze, and during the retention phase, they had free access to the three arms *i.e.* the familiar arms and the “novel arm” that was previously closed (see Supplementary Figure 2).

Two independent studies were carried out. Study 1 was designed to compare the basal memory performances of NR1-KO mice (n=10) to those of controls (n=12) after a 2 h-ITI, in order to determine if the mutant mice performed this spatial discrimination task despite non-functional NMDA-R in their CA1 hippocampal region. This short ITI was chosen because control mice are known to preferentially visit the novel arm at this time interval (Ladurelle et al., 2000). Study 2 examined the effects of steroid and VEH on memory performances of NR1-KO mice (PREGS, n=14; *ent*-PREGS, n=12; VEH, n=11) and control mice (PREGS, n=13; *ent*-PREGS, n=13; VEH, n=14) after a 6 h-ITI, at which time control rodent show no more arm discrimination (chance level, 33.3 %) and display enhanced memory performances following PREGS or *ent*-PREGS treatment (Akwa et al., 2001).

Different parameters were analyzed: the number of visits and/or rearings in all arms during the acquisition and retention phases as they reflected locomotion and exploratory behavior; the amount of time in the novel arm relative to the total time (*i.e.* the percentage of time in the novel arm) during the retention phase, as an index of memory performance, and the amount of time in the novel arm and in the familiar arms.

2.4. Statistical analysis

Data were expressed as mean $\pm$ SEM. In study 1, unpaired Student t-test was used to compare between NR1-KO mice and controls, the number of visits or rearings in all arms during the acquisition and retention phases. One sample t-test was used to compare the percentage of time in the novel arm to chance level (33.3 %) during retention phase and unpaired t-test was used to compare between NR1-KO and control mice. Two-way analysis of variance (ANOVA) was used to compare the amount of time in each of the three arms of the Y-maze according to genotype.

In study 2, unpaired Student t-test was used to compare, between NR1-KO mice and controls, the number of visits and rearings in all arms during the acquisition trial. Two-way ANOVA was applied to analyze the effects of genotype and treatment for the number of visits in all arms, the percentage of time in the novel arm, and the time in novel and familiar arms during the retention trial. When ANOVA was significant, it was followed by Student-Newman-Keuls post-hoc test. The level of significance was $p < 0.05$.

2.5. Histological control of NR1 subunit location

Histological verification was carried out as previously described (Rondi-Reig et al., 2006). Sagittal sections were incubated with rabbit polyclonal antibody against the rat NR1 subunit (AB 1516; Chemicon, Euromedex France) and immunoreactivity was visualized with avidin-biotin peroxidase complex (Vector Laboratories).

3. Results

3.1. NR1 subunit location

NR1 subunit expression was absent in the CA1 area of the hippocampus in mutant mice of 6 to 12 weeks of age and decreased in deep layers of the cerebral cortex at 10 and 12 weeks (see Supplementary Figure 3), as previously reported (Fukaya et al., 2003; Rondi-Reig et al., 2006).

3.2. Behavioral analysis

In both study 1 and study 2, mutant mice displayed a similar number of visits or rearings in all arms during the acquisition and retention phases as control mice, indicating that they exhibited normal locomotion and exploratory behavior (see Supplementary Table 1).

3.2.1. NR1-KO mice display normal spatial retention performances (study 1)—

After a 2 h-ITI, both control (39.9 ± 2.0 vs 33.3 %, $p=0.011$) and NR1-KO (40.0 ± 3.0 vs 33.3 %, $p=0.049$) mice performed significantly above chance level (33%) (one sample t-test) with no significant difference observed between the two groups ($t=-0.031$, d.f. 20, $p=0.975$, unpaired t-test) (Figure 1A). The amount of time spent in each arm is presented in Figure 1B. Two-way ANOVA showed a significant arm effect [$F(2, 60) = 15.266$; $p<0.001$]. Student-Newman-Keuls post-hoc test revealed that control mice and NR1-KO mice spent significantly less time in the familiar arm 1 ($p=0.018$ and $p=0.037$, respectively) and in the familiar arm 2 ($p=0.006$ and $p=0.002$, respectively) than in the novel arm, while no significant difference was noted between the two familiar ones for each group (control mice, $p=0.723$ and NR1-KO mice, $p=0.294$).

3.2.2. The promnesiant effect of PREGS is lost in NR1-KO mice, whereas that of *ent*-PREGS is maintained (study 2)—

The effects of VEH, PREGS or *ent*-PREGS on the retention performances, *i.e.* on the percentage of time spent in the novel arm, of control mice and NR1-KO mice are presented in Figure 2A. Two-way ANOVA indicated a significant interaction between genotype and treatment [$F(2,71) = 3.253$, $p=0.045$] meaning that the treatment effect depends on the genotype. Student-Newman-Keuls post-hoc analysis between control and NR1-KO mice indicated a significant difference in the PREGS-group ($p=0.008$) but not in the VEH-group ($p=0.550$) and the *ent*-PREGS group ($p=0.931$). In control mice, significant differences were obtained between PREGS- and VEH-groups ($p=0.010$), and between *ent*-PREGS- and VEH-groups ($p=0.006$). In NR1-KO mice, a significant difference was obtained between *ent*-PREGS- and VEH-groups ($p=0.002$) but not between PREGS- and VEH-groups ($p=0.730$).

The amount of time mice spent in the novel arm is presented in Figure 2B. Two-way ANOVA indicated significant effects of both treatment [$F(2,71)=14.319$, $p<0.001$] and genotype [$F(1,71) = 4.206$; $p=0.044$]. In control mice, significant differences were observed between PREGS- and VEH-groups ($p=0.015$) and, between *ent*-PREGS- and VEH-groups ($p=0.006$). In NR1-KO mice, a significant difference was found between *ent*-PREGS- and VEH-groups ($p=0.001$), but not between PREGS- and VEH-groups ($p=0.262$).

The amount of time spent in the familiar arms is shown in Figure 2C. Two-way ANOVA revealed no interaction between genotype and treatment [$F(2,71)=1.268$, $p=0.288$], no effect of treatment [$F(2,71)=1.787$, $p=0.175$] or genotype [$F(1,71)=0.535$, $p=0.467$].

4. Discussion

The main goal of the present work is to investigate the possible implication of NMDA-Rs in the promnesiant effects of PREGS and *ent*-PREGS by studying mice without CA1 hippocampal NMDA-R in the Y-maze arm discrimination test, a relevant hippocampal spatial task. When we examined the basal retention performances of NR1-KO at 2 h-ITI, we showed that they were comparable to the good performances of control mice (study 1). These results demonstrate that memory performances for spatial information in the Y-maze does not require NMDA-Rs located in the CA1 region, although NMDA receptor activation is mandatory for some forms of learning and memory (Riedel et al., 2003). They corroborate the proposal that hippocampal subregions other than CA1 play a major role in rapid encoding of novel information for fast learning of a one-time experience (Nakazawa et al., 2003).

We then assessed the influence of PREGS and *ent*-PREGS on the spatial retention performances in NR1-KO mice as compared to controls (study 2). Our results showed that NR1-KO injected with VEH performed closed to chance level similarly to VEH-control mice. In addition, in control mice, both PREGS and *ent*-PREGS significantly increased the percentage of time spent in the novel arm during the retention phase as compared to the VEH-injected group. This confirmed the promnesiant effects of these steroids previously seen in young adult rodents using similar behavioral tasks (Mayo et al., 1993; Darnaudéry et al., 2000; Ladurelle et al., 2000; Akwa et al., 2001).

The intriguing novelty of this work is the distinct actions of PREGS and of its enantiomer upon memory retention in NR1-KO mice: whereas *ent*-PREGS was active and improved memory performances, PREGS lost its promnesiant effect. These results indicate that PREGS memory performance effect is mediated through forebrain NMDA-Rs, whereas that of *ent*-PREGS is not. The promnesiant effect of PREGS on spatial memory is due, at least in part, to increased NMDA-R activity and long-term potentiation of field excitatory postsynaptic potentials at CA1 synapses necessary for spatial memory (Slivinski et al., 2004; Sabeti et al., 2007). In contrast, our data show that CA1 NMDA-Rs are not involved in the enhancement of spatial memory by *ent*-PREGS in the Y-maze test. The mechanism whereby *ent*-PREGS has its memory-enhancing effect remains to be identified. It is unlikely that this effect is caused by non-specific membrane effects. As enantiomers, PREGS and *ent*-PREGS have identical physicochemical properties, and would be expected to modulate membrane properties in a similar manner (Covey, 2009). Thus, if non-specific membrane effects were the cause for the memory-enhancing effect of *ent*-PREGS, then PREGS should have the same non-specific membrane effects and it should have retained its memory-enhancing effects in the forebrain NMDA knockout animals.

Is there a receptor in the mouse brain for *ent*-PREGS that underlies this *ent*-steroid's memory-enhancing effect? Our results suggest this possibility. A future challenge will be to identify this putative *ent*-PREGS receptor. A characteristic of this receptor would be its ability to be more potently modulated by *ent*-PREGS than by PREGS. The fact that γ -aminobutyric acid type A receptors are more potently modulated by *ent*-androgens than by androgens (Katona et al., 1998) suggests a receptor that is selective for *ent*-PREGS is possible.

In conclusion, our enantioselectivity results demonstrated that functional NMDA-Rs located in the forebrain (predominantly in the hippocampal CA1 area) are required for PREGS, but not for *ent*-PREGS memory enhancement of spatial memory. These findings may be relevant under physiopathological conditions in which dysregulation or loss of NMDA-Rs occurs, and in this context, *ent*-PREGS has the potential to reduce memory deficits because its promnesiant effect does not require forebrain NMDA-Rs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PREGS	pregnenolone sulfate
<i>ent</i>-PREGS	pregnenolone sulfate enantiomer
NMDA-R	N-methyl-D-aspartate receptor
NR1-KO	NR1 knock-out
ITI	inter-trial interval
icv	intracerebroventricular
VEH	vehicle

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Figure 1.

Basal retention performances of control and NR1-KO mice in the Y-maze test (study 1). Data were expressed as mean $\pm$ SEM. The percentage of time in the novel arm (panel A) in control and NR1-KO mice was significantly different from chance level (one sample t-test). The amount of time in each arm (panel B) was analyzed by two-way ANOVA which indicated a significant arm effect ($p < 0.001$). *, $p < 0.05$; **, $p < 0.01$; Student-Newman-Keuls post-hoc test. Control, $n = 10$; NR1-KO, $n = 12$.

Figure 2.

Effects of PREGS and *ent*-PREGS on retention performances of control and NR1-KO mice in the Y-maze test (study 2). Data were expressed as mean $\pm$ SEM. For the percentage of time in the novel arm (panel A), two-way ANOVA indicated a significant interaction between genotype and treatment ($p < 0.05$). For the amount of time in the novel arm (panel B), two-way ANOVA showed significant effects of both treatment ($p < 0.001$) and genotype ($p < 0.05$). The amount of time in the familiar arms (panel C) was not statistically different according to treatment or genotype (two-way ANOVA; $p > 0.05$). *, $p < 0.05$; **, $p < 0.01$; Student-Newman-Keuls post-hoc test. Control: VEH, $n = 14$; PREGS, $n = 13$; *ent*-PREGS, $n = 13$; NR1-KO: VEH, $n = 11$; PREGS, $n = 14$; *ent*-PREGS, $n = 12$. VEH: Vehicle.