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Research report

# Alteration of neonatal Allopregnanolone levels affects exploration, anxiety, aversive learning and adult behavioural response to intrahippocampal neurosteroids

Laura Mòdol[a], Sònia Darbra[a], Monique Vallèe[b], Marc Pallarès[a,*]

[a] Departament de Psicobiologia i Metodologia en Ciències de la Salut, Institut de Neurociències, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain
[b] Institut National de la Santé et de la Recherche Medicale (INSERM), Unité 862, Bordeaux, France

## HIGHLIGHTS

- Neonatal Allop is important for the adult intrahippocampal axiolytic profile of NS.
- Neonatal alteration of Allop levels alters adult exploratory and anxiety behaviour.
- Neonatal alteration of Allop levels affects adult avoidance learning performance.

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## ABSTRACT

Neurosteroids (NS) are well known to exert modulatory effects on ionotropic receptors. Recent findings indicate that NS could also act as important factors during development. In this sense, neonatal modifications of Allopregnanolone (Allop) levels during critical periods have been demonstrate to alter the morphology of the hippocampus but also other brain structures. The aim of the present work is to screen whether the alterations of Allop levels modify adult CA1 hippocampal response to NS administration. For this purpose, pups were injected with Allop (20 mg/kg s.c.), Finasteride (5α-reductase inhibitor that impedes Allop synthesis) (50 mg/kg s.c.) or Vehicle from postnatal day 5 (P5) to postnatal day 9 (P9). NS levels were tested at P5. To test the behavioural hippocampal response to NS in adulthood, animals were implanted with a bilateral cannula into the CA1 hippocampus at 80 days old and injected with Allop (0.2 μg/0.5 μl), Pregnenolone sulphate (5 ng/0.5 μl) or Vehicle in each hippocampus. After injections animals were tested in the Boisser test to assess exploratory behaviour, the elevated plus maze to assess anxiety and the passive avoidance to test aversive learning. Results indicate that alteration of neonatal Allop or pregnenolone levels (by Allop and Finasteride administration, respectively) suppressed intrahippocampal Allop anxiolytic effect in the EPM. Moreover our results also indicate that manipulation of neonatal Allop levels (Allop and Finast administration) alters exploratory and anxiety-like behaviour and impairs aversive learning in the adulthood. These data point out the role of Allop in the maturation of hippocampal function and behaviour.



## 1. Introduction

Neurosteroids (NS) are steroids that are synthesized de novo by the nervous tissue and are well known for exerting modulatory actions on neurons excitability through the modulation of ionotropic receptors [1,2]. In this sense, NS acting as positive modulators of GABAA receptors such as Allopregnanolone (Allop or 3α,5α-tetrahydroprogesterone), have been described to show anticonvulsive [3] and anxiolytic effects when injected systemically [4], into the amygdala [5] or into the hippocampus [6]. Furthermore, ring A reduced pregnanes like Allop have also been described to have a detrimental learning profile when injected systemically [7], directly into the nucleus basalis magnocelularis [8], intraventricularly [9], or into the amygdala [10] in adult animals. On the other hand, NS that act as negative modulators of GABAA receptors such as pregnenolone (PREG), dehydroepiandrosterone (DHEA) and their sulphated esters (PREGS and DHEAS) have been described to act as proconvulsive [11], anxiogenic [4,12] and also to improve memory in several learning tests when injected systemically [7], into the amygdala [10], into the nucleus basalis magnocellularis [13] and also into the hippocampus [6,14]. In this sense, this promnesic effect has been postulated to take place through the potentiation of NMDA receptors located in the pyramidal neurons of the hippocampus [15]. However, it has also been

* Corresponding author. Tel.: +34 93 581 25 42; fax: +34 93 581 20 01.
*E-mail address:* marc.pallares@uab.cat (M. Pallarès).

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suggested that PREGS enhancing profile could be done through the potentiation of the cholinergic neurons via GABAergic inhibition [13,16].

Recent findings indicate NS acting as GABAA positive modulators, such as Allop are important keys during brain development [17]. In fact, it has been described that cortical Allop peak appears before birth and a second peak of Allop occurs during the second postnatal week [18,19]. The specific role that NS play in development, however, has not been yet elucidated. It has been postulated that increased levels of neonatal Allop promote the establishment of neuronal circuitry and supports the survival of developing neurons [20]. Also, previous studies demonstrated that alteration of neonatal Allop levels has a profound effect on the morphology and the structure of several brain areas such as the cortex and the thalamus [19,21,22] but also alters critically the normal development of the hippocampus [23–26]. Accordingly, alterations in adult behaviour have also been reported. In this sense, previous results in our laboratory have shown that manipulation of neonatal Allop levels alters the performance of the elevated plus maze (EPM) and the aversive learning in the passive avoidance test [27]. In addition, Allop administration (from postnatal day 5 (P5) to postnatal day 9 (P9)) has been shown to deteriorate PPI in the adulthood [28], to alter adulthood responses to GABAA modulators (such as benzodiazepines) in the EPM [29], to modify the CA1 response to NS in the open field test [30] and to induce an anxiolytic profile in the EPM [31]. These previous data seem to suggest that alteration of NS during critical developmental periods have important consequences in adult behaviour, however, no studies have been performed to assess the interaction between adult intrahippocampal administration of NS and neonatal injection of an Allop dose (20 mg/kg), that induces anxiolytic-like profile in the adult age [31].

Therefore, given that neonatal Allop levels have been described to play a crucial role in the correct maturation of central CNS, and concretely to the correct formation of hippocampal circuitry, the aims of the present work are to assess the NS hippocampal levels (Allop, THDOC, testosterone, epiallopregnanolone, PREG) at P5 in response to the neonatal treatment (Allop, Finasteride (Finast), Vehicle and No handled (NH)) and to screen whether the alteration of developmental NS levels modifies the effect of adult NS intrahippocampal administration (Allop, PREGS, Vehicle) on exploratory, anxiety and aversive learning behaviour. We have chosen the Boissier test, the EPM and the passive avoidance to test several aspects of the emotional behaviour. We hypothesize that the alteration of the physiological neonatal Allop levels (Allop or Finast administration) can alter adult response to exploration, anxiety and to aversive learning and also, adult behavioural response to NS such as PREGS or Allop when they are administered into the hippocampus.

## 2. Material and methods

### 2.1. Animals and neonatal NS administration

A total number of one hundred eighty seven animals were used in the experiment (see Table 1). Twenty-five animals were used for NS quantification and one hundred sixty-two were used for adult intrahippocampal administration and behavioural evaluation. All animals used were male Wistar rats raised at in-house colony (Laboratori de Psicobiologia, Universitat Autònoma de Barcelona, Barcelona, Spain) allowed with food and water ad libitum. Rats were housed in a temperature-controlled animal room (22–24 °C) on a 12-h light/dark cycle. Experimental sessions were run during the light portion of the cycle (lights on at 08:00 h). The male breeders were separated from the females after 48 h, pregnant females were closely watched and on the day of birth (designed day 0), mothers were removed from the cage and litters were culled to 10 pups. In order to avoid any cohort effects, each litter of the same colony was assigned to different neonatal treatment groups. Pups (males and females) were subcutaneously (s.c) injected with: Finasteride (Finast) (50 mg/kg, $n = 41$), Allop (20 mg/kg, $n = 42$) or Vehicle ($n = 42$), once per day from the fifth to the ninth day alter birth (P5–P9). All products were obtained from SIGMA (Deisenhofen, Germany). In addition, a non-handled group (NH) ($n = 37$) was included in order to avoid the possible effects of the drugs administration (see Table 1 for final experimental groups and number of pairs and litters). Drugs (Allop, Finasteride and Vehicle) were dissolved in 0.9% NaCl by sonication for 10 min and suspended in 10% cyclodextrin ((2-hydroxypropyl)-β-cyclodextrin). As Vehicle, 10%-cyclodextrin dissolved in 0.9% NaCl was used. Injection volume was 0.1 ml/10 g body weight. After injections, pups were immediately returned to the home cage with their mother. After weaning (P21) males were separated into groups of brothers (with a maximum of five subjects per cage), and females were sacrificed. After recovery from surgery, males of each neonatal condition were randomly assigned to each group and they were behaviourally evaluated. All animals were obtained, housed, and sacrificed in accordance with the protocol approved by the Committee of the UniversitatAutònoma de Barcelona for Care and Use of Experimental Animals and the Department of Environment from Generalitat de Catalunya (Regional Government). This protocol follows the guidelines approved by the European Council Directive (86/609/ECC) for care and use of laboratory animals.

### 2.2. Hippocampal NS quantification

The twenty-five animals were sacrificed by decapitation at postnatal day 5 (P5), after 1 h of neonatal administration. Postnatal day 5 was chosen to ensure that NS hippocampal levels were at least altered during the first postnatal injection given that an adaptation effect could result from the repeated NS treatment. Brains were removed and the hippocampus were harvested and frozen in dry ice. Brains were kept stored at −80 °C until they were used for the steroid quantification. Pregnenolone (PREG), Allop, epiallopregnanolone, THDOC and testosterone were determined by gas chromatography/mass spectrometry according to Vallee et al. [32].

### 2.3. Surgery

Surgery was carried out at 80-day-old animals. For permanent implantation of cannula, animals were anaesthetized with ketamine (120 mg/kg) and xylazine (10 mg/kg). Using standard stereotaxic techniques, bilateral 21-gauge stainless steel double guide cannula (model C232G-3.8; Plastics One, Bilaney, Dusserldorf, Germany) was implanted into the CA1 region of the dorsal hippocampus (anteroposterior, 3.6 mm; mediolateral, 1.8 mm; dorsoventral, 1.8 mm from bregma). Each guide cannula was fitted with dummy cannula with no projection. Double guide cannulae were permanently mounted to the skull with four screws and dental cement.

### 2.4. Adult NS administration

After recovery from surgery (2 weeks), the day of behavioural testing freely moving rats were injected with Allop (0.2 μg/0.5 μl), pregnenolone sulphate (PREGS) (5 ng/0.5 μl) (SIGMA, Deisenhofen (Germany)) or Vehicle (10% cyclodextrin) in each hippocampus. See Table 1 for final experimental groups composition. NS doses were determined based on previous studies carried out in our laboratory, according to affect anxiety, learning and memory responses [13,14,27,33]. Allop and PREGS were dissolved in 0.9% NaCl by sonication for 10 min and suspended in 10% cyclodextrin. For injections, double internal 28-gauge stainless steel cannula (model C232I; Plastics One) was inserted extending 1 mm below the guide cannula to a final depth of 2.8 mm from the skull (see Fig. 1). Injection needles were connected with polyethylene tubing to a microsyringe (10 μl) driven by the infusion pump (Harvard 22). Solutions were infused during 1 min at a constant rate of 0.5 μl/min. Control rats received the same volume (0.5 μl) of Vehicle at the same rate of infusion. Injection needles were removed from the guide cannula 2 min after infusions in order to prevent drug reflux. Animals were injected twice: before Boissier test and after passive avoidance acquisition one week later, with the same substance.

### 2.5. Boissier exploration test

A square wooden arena (58 cm × 58 cm × 58 cm) with 16 equidistant holes (5 cm in diameter) was used for the Boissier test. The apparatus was situated in a room lit by a bright light (300 lx mean). This test measures activity and provides a relatively reliable measurement of stimulus-directed exploratory behaviour [34,35]. It was tested for 5 min and was evaluated by means of an activity monitoring system (SMART, Letica, Barcelona, Spain). This system is based on the automated analysis of real-time video-images, recorded by a video camera that is suspended from the ceiling over the arena. The distance moved was recorded for the total arena as locomotor activity, as well as for a virtual 29 cm × 29 cm centre zone. In addition, the number of entries into, and the time spent in the centre zone was also measured as anxiety relevant scores. Moreover, the number of head-dips (into the holes up to the eye line) was recorded as an exploratory measure. After each trial, the apparatus was cleaned with a water solution containing ethanol (20%, v/v) in order to prevent any olfactory-induced behavioural modifications. Rats were tested immediately after intrahippocampal NS infusions (between 09:00 and 11:00 h). Five animals were excluded from the distance-travelled analysis because of detection problems.

### 2.6. Elevated plus maze

The EPM consisted of two open and two closed arms (10 cm wide × 50 cm long) perpendicular to each other and elevated 50 cm from the floor. The walls of the