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Increased neurosteroids synthesis after brain and spinal cord injury in rats

F. di Michele[a], D. Lekieffre[b], A. Pasini[c], G. Bernardi[a,c], J. Benavides[b], E. Romeo[a,c,*]

[a]IRCCS Santa Lucia, Rome, Italy
[b]Synthelabo Recherche, BP 110 9225 Bagneux, Cedeux, France
[c]Neuroscience Department of Tor Vergata University, Via Tor Vergata 135, 00133 Rome, Italy

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Abstract

We studied the effect of brain and spinal cord injury induced by fluid-percussion on the local synthesis of neurosteroids as measured by a gas-chromatographic/mass-spectrometric method. In the nervous system of sham operated rats i.v. infusion of pregnenolone (PREGN)-sulfate results in a 2–4 fold increase in PREGN, progesterone (PROG), 5alpha-dehydroprogesterone (5α-DHP) and 3alpha-hydroxy-5alpha-pregnan-20-one (3α5α-THP, allopregnanolone) concentrations, as compared to vehicle treated rats. When PREGN-sulfate was infused 1, 3 or 7 days after brain or spinal cord injury it was observed a large time-dependent increase of PROG, 5α-DHP and 3α5α-THP levels in the peri-focal but not in the focal site. This increase in neurosteroids content may be due essentially to the glial cells hyperplasia in the peri-focal area and to an activation of the pathways involved in the metabolism of PREGN-sulfate to PROG, 5α-DHP and 3α5α-THP.

Keywords: Allopregnanolone; Steroids; Neurosteroids; Pregnenolone sulfate; Nervous system; Mass spectrometry

Recent evidences suggest that reactive glia surrounding a lesion can be crucial in nervous system (NS) repair. It has been shown that glia can either constrain or promote neuronal growth and survival, but the mechanisms of these effects are still unclear [4].

Glial cells express nuclear receptors for steroid hormones and, moreover, can synthesize de novo steroids, which are called neurosteroids [1,14]. The major neurosteroids which can be formed within the NS are pregnenolone (PREGN), PREGN-sulfate, progesterone (PROG) and its reduced metabolites 3alpha-hydroxy-5alpha-pregnan-20-one (3α5α-THP, allopregnanolone) and 5alpha-dehydroprogesterone (5α-DHP) [1,2,14].

Neurosteroids can modify NS function either by promoting the synthesis of growth factors or other specific proteins regulating gene expression via intracellular receptors (i.e. 5α-DHP and PROG), or via interaction with membrane receptors, such as $GABA_A$ receptors (3α,5α-THP). Whereas PREGN acts as precursor, presumably [8,10–12,14].

PREGN and PROG play important roles during nerve regeneration as shown in rodents by the reduction of damage after PREGN administration in a paradigm of spinal cord injury [6] and by the remyelinating action of PROG synthesized after injury by Schwann cells in a sciatic nerve injury model [7].

Because neurosteroids may have a beneficial influence on neuronal regeneration both by inhibiting neuronal excitotoxicity through a positive modulation of $GABA_A$ receptors and by a direct or indirect trophic effect, the goal of the present investigation was to determine whether the synthesis of neurosteroids is locally modified following traumatic brain and spinal cord injury.

For the experiments we used male Sprague–Dawley rats (300–320 g body weight) that were anesthetized with pentobarbital (60 mg/kg i.p.) and mounted in a stereotaxic frame before surgery.

For spinal cord injury a laminectomy was performed at T7-T8 and a 2-mm inner diameter Teflon tubing, filled with water, was fixed against the spinal cord. This tubing was connected to a solenoid valve linked to an high pressure liquid chromatography (HPLC) pump. The spinal cord injury was produced by a brief opening of the valve at a pressure of 0.05 kpsi.

For brain injury the traumatic lesion of the rat brain was obtained using a previously described [16] lateral fluid

* Corresponding author. Fax: +39-06-5150-1553.
E-mail address: romeo@uniroma2.it (E. Romeo).

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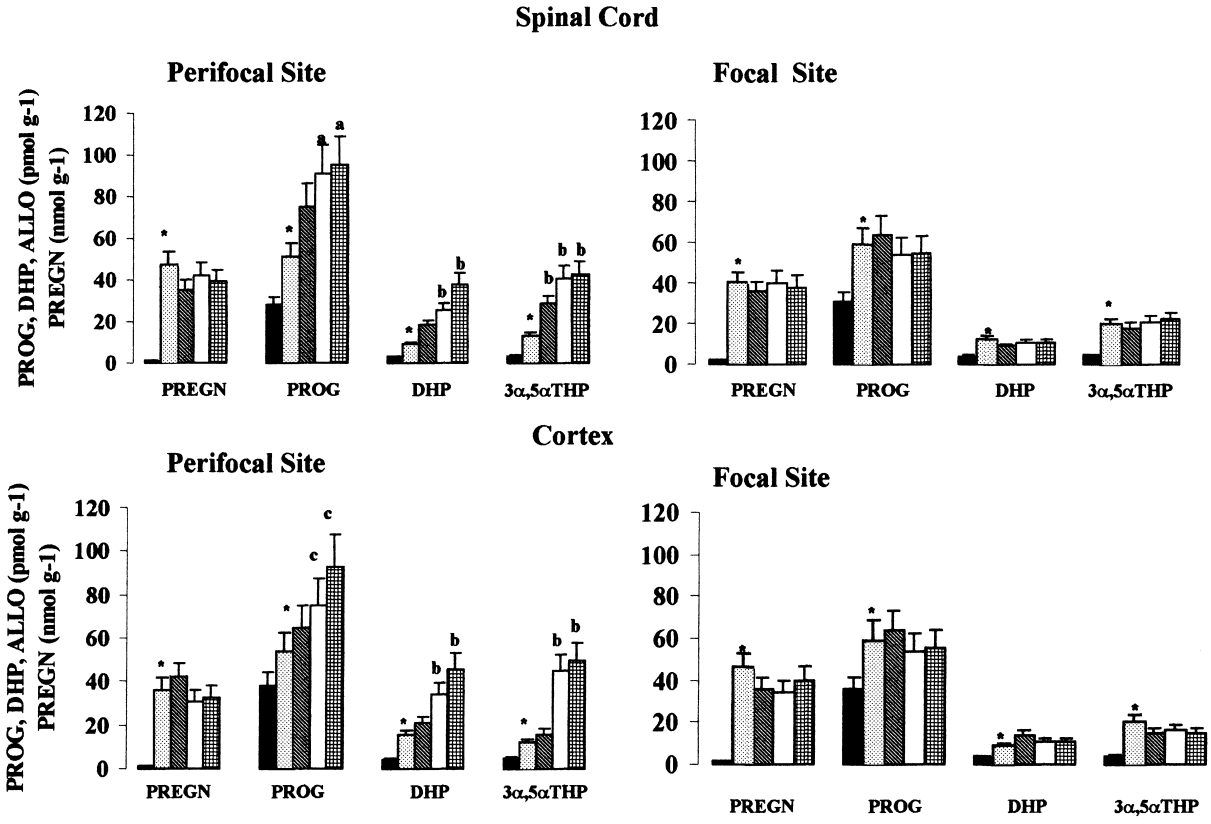

Fig. 1. Time dependent increase of neurosteroids synthesis in the peri-focal site of spinal cord and cortex after injury. Data represent mean ± SEM of three determinations. Statistical analyses were conducted with one-way ANOVA with Multiple Range Tests of Bonferroni. *$P < 0.0001$ when sham operated PREGN-sulfate treated (20 mg/kg, i.v.: 5 min infusion, 1 h before sacrifice) rats were compared with sham operated vehicle treated rats. [a]$P < 0.02$; [b]$P < 0.001$; [c]$P < 0.004$ when injured rats PREGN-sulfate treated were compared with sham operated PREGN-sulfate treated rats. ■ Sham operated vehicle treated; ⁙ Sham operated PREGN-sulfate treated; ▨ PREGN-sulfate treated 1 day after surgery; □ PREGN- sulfate treated 3 days after surgery; ▦ PREGN-sulfate treated 7 days after surgery.

percussion procedure. A craniotomy was performed in the parietal cortex and a tubing was placed over the dura and anchored to the cortex with dental cement. The tubing filled with water was connected to a valve coupled to an HPLC pump, which delivered a short impact pressure.

Following the surgery, the muscle and skin were sutured and rats returned to their home cages until sacrifice at day 1, 3 and 7.

Sham operated rats underwent to the same surgical procedure except for being injured.

One hour before sacrifice, rats were infused via tail vein for 5 min, under chloral-hydrate anesthesia (400 mg/kg, i.p.), with PREGN-sulfate or vehicle at the rate of 0.34 ml/min in a volume of 1 ml/kg. The rats were sacrificed by decapitation and the spinal cords or brains were removed. Spinal cords were cut into two pieces (proximal and centered to the lesion), each of them measured roughly 1 cm length. Brains were removed and the entire cortex was spread out on a dissection plate; a patch (1.3 cm diameter), centered to the impact site, was the focal site. The perifocal site was a patch 1.3 cm diameter of the remaining cortex. The results are expressed as pmol/g-1 of tissue. Samples were frozen on dry ice until the assay.

Neurosteroids were quantified by means of a highly sensitive combined gas-chromatography/mass-spectrometry analysis [13,3]. Briefly, after extraction with 3 × 3 ml of ethyl acetate and separation by thin-layer-chromatography (i.e. carbon tetrachloride/methanol 99:1 v/v, cyclohexane/ethyl acetate 3:2 v/v) the eluate containing steroids was lyophilized and derivatized with heptafluorobutyric acid anhydride (HFBAA). 5α-DHP was derivatized with methoxamine (MO) HCl (2%) in pyridine. HFBAA and MO derivatives were analyzed by gas chromatography-mass spectroscopy using an HP 5971 mass selective detector coupled to an HP 5890A gas-chromatograph equipped with a Hewlett Packard capillary column (HP-5 M.S.; length 30 m; i.d. 0.25 mm; film thickness 0.25 μm). The steroids were assayed in the electron impact mode and the ions at m/z 510, 496, 298 and 343 were selectively monitored. The recovery rate was about 80%. The sensitivity was about 50 pg.

All results are presented as mean ± SEM. Data was

subjected to one-way ANOVA with Multiple Range Test of Bonferroni.

The objective of the present work was to determine if the biochemical mechanisms involved in neurosteroid biosynthesis are up regulated following brain or spinal cord injury. Since the formation of PREGN by the $P450^{scc}$ is known to be the limiting step in steroid biosynthesis, we administered large amounts of PREGN-sulfate in order to provide non-limiting amount of PREGN. PREGN-sulfate readily enters into the central nervous system (CNS) and distributes in a uniform way in brain and spinal cord acting as a precursor for the de novo synthesis of neurosteroids [3,13].

Infusion of sham operated rats with 20 mg/kg of PREGN-sulfate, resulted in an almost 30 fold increase of brain and spinal cord PREGN and a substantial increase in the levels of the neurosteroids studied compared to sham operated vehicle treated animals (Fig. 1).

These increases were even larger in the peri-focal area of the spinal cord, where we found a raise of neurosteroids concentrations that progress from day one (3α5α-THP = 28.6 ± 3.1; 5α-DHP = 18.3 ± 2.8; PROG = 75.4 ± 6.8 pmol g^{-1}) to day seven (3α5α-THP = 42.7 ± 5.4; 5α-DHP = 38 ± 5.8; PROG = 95 ± 4.7 pmol/g). In contrast, no changes were observed on PREGN levels (see Fig. 1). Similarly, following focal traumatic cortical injury, PREGN-sulfate infusion, elicited a significant time dependent increase of neurosteroids content in the peri-focal cortical region. In both paradigms, increase of 3α5α-THP and 5α-DHP concentrations at day seven was of two fold compared to the relatively less pronounced increase of PROG levels.

Interestingly, no increase in neurosteroid was observed in the lesion site either in the cortex or in the spinal cord. It is generally believed that neurosteroid synthesis in the NS occurs mainly in glial cells, although also neurons participate to it [1,2]. Here we reasoned that a corollary of this hypothesis is that in situations in which there is an increased gliosis there must be an increase in neurosteroid synthesis. We have tested this hypothesis in models of traumatic brain and spinal cord injury that are known to produce extensive hypertrophy of reactive glia in the areas surrounding the focal insult [5].

Since PROG, 3α5α-THP and 5α-DHP can be synthesized in the NS from the precursor PREGN-sulfate, we adopted the strategy of infusing rats with PREGN-sulfate to ensure an adequate amount of precursor at the sites of synthesis [3,13].

Using the spinal cord and the brain injury paradigms, we found that the synthesis of PROG, 3α5α-THP and 5α-DHP from PREGN-sulfate increased dramatically 3–7 days after the injury in the areas surrounding the lesion, but not in the focal site. Previous reports [9,16] have shown that in the focal site, most cells are dead and probably this could be the reason why we found a slight but not significant decrease in neurosteroids accumulation compared to sham operated rats.

Since in the perifocal area there is a glia hyperplasia few days after trauma [5], we may infer that the increase in neurosteroids observed at the peri-focal site is mainly due to the activation of glial cells.

Moreover, since 5α-reductase is present either in neurons and in glia, it should be considered that also neurons could play a crucial role in it, by synthesizing the precursor 5α-DHP themselves or regulating glial cells activation.

Because the enzyme 5α-reductase is the rate limiting step for the formation of 5α-DHP and 3α5α-THP from PROG, the fact that in the peri-focal area the increase of 3α5α-THP and 5α-DHP is almost two fold greater than that of PROG, suggests that changes of 3α5α-THP and 5α-DHP cannot be due to modification of plasma levels, but are more likely due to a local difference in biosynthesis.

Pharmacological studies have shown that, at nM concentrations, 3α,5α-THP positively modulates $GABA_A$ receptors function and plasticity, while PROG, 5α-DHP and 3α,5α-THP are able to affect gene expression [14,15].

These effects may be involved in the neuroprotective actions of neurosteroids [17]. The large increase we observed in the peri-focal area of neurosteroids able to potently modulate inhibitory neurotransmission and to induce genomic expression in the CNS, suggests a role for neurosteroids in CNS responses to injury (i.e. expression of neuronal signaling molecules required for regeneration, action as autocrine neurotrophic factors, modulation of neurotransmission).

[1] Baulieu, E.E., Robel, P., Vatier, O., Haug, A., LeGoascogne, C. and Bourreau, E., Neurosteroids: pregnenolone and dehydroepiandrosterone in the rat brain, In K. Fuxe and L.F. Agnati (Eds.), Receptor-Receptor Interaction, A New Intramembrane Integrative Mechanism, MacMillian, Basingstoke, 1987, pp. 89–104.
[2] Baulieu, E.E., Neurosteroids: a novel function of the brain, Psychoneuroendocrinology, 23 (1998) 963–987.
[3] Cheney, D.L., Uzunov, D., Costa, E. and Guidotti, A., Gas chromatographic-mass fragmentographic quantitation of 3 alpha-hydroxy-5 alpha-pregnan-20-one (allopregnanolone) and its precursors in blood and brain of adrenalectomized and castrated rats, J. Neurosci., 15 (1995) 4641–4650.
[4] Fawcett, J.W. and Asher, R.A., The glial scar and central nervous system repair, Brain Res. Bull., 49(6) (1999) 377–391.
[5] Garcia-Segura, L.M., Chowen, J.A., Duenas, M., Parducz, A. and Naftolin, F., Gonadal steroids and astroglial plasticity, Cell. Mol. Neurobiol., 16 (1996) 225–237.
[6] Guth, L., Zhang, Z. and Roberts, E., Key role for pregnenolone in combination therapy that promotes recovery after spinal cord injury, Proc. Natl. Acad. Sci. USA, 91 (1994) 12308–12312.
[7] Koenig, H.L., Schumacher, M., Ferzas, B., Thi, A.N., Ressouches, A., Guennoun, R., Jung-Testas, I., Robel, P., Akwa, Y. and Baulieu, E.E., Progesterone synthesis and myelin formation by Schwann cells, Science, 268 (1995) 1500–1503.
[8] Lambert, J.J., Belelli, D., Hill-Venning, C. and Peters, J.A., Neurosteroids and $GABA_A$ receptor function, Trends Pharmacol. Sci., 16 (1995) 295–303.
[9] Lin, B., Ginsberg, M.D., Busto, R. and Dietrich, W.D.,

Sequential analysis of subacute and chronic neuronal, astrocytic and microglial alterations after transient global ischemia in rats, Acta Neuropathol., 95 (1998) 511–523.
[10] Majewska, M.D., Harrison, N.L., Schwartz, R.D., Barker, J.L. and Paul, S.M., Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor, Science, 232 (1986) 1004–1007.
[11] McEwen, B.S., Non-genomic and genomic effects of steroids on neural activity, Trends Pharmacol. Sci., 12 (1991) 141–146.
[12] Paul, S.M. and Purdy, R.H., Neuroactive steroids, FASEB J., 6 (1996) 2311–2322.
[13] Romeo, E., Cheney, D.L., Zivkovic, I., Costa, E. and Guidotti, A., Mitochondrial diazepam-binding inhibitor receptor complex agonists antagonize dizocilpine amnesia: putative role for allopregnanolone, J. Pharmacol. Exp. Ther., 270 (1994) 89–96.
[14] Rupprecht, R., Reul, J.M., Trapp, T., van Steensel, B., Wetzel, C., Damm, K., Zieglgansberger, W. and Holsboer, F., Progesterone receptor-mediated effects of neuroactive steroids, Neuron, 11 (1993) 523–530.
[15] Smith, S.S., Gong, Q.H., Hsu, F., Markowitz, R.S., Ffrench-Mullen, J.M.H. and Li, X., $GABA_A$ receptor Alpha4 subunit suppression prevents withdrawal properties of an endogenous steroid, Nature, 392 (1998) 926–929.
[16] Toulmond, S., Duval, D., Serrano, A., Scatton, B. and Benavides, J., Biochemical and histological alterations induced by fluid percussion brain injury in the rat, Brain. Res., 620 (1993) 24–31.
[17] Waters, S.L., Miller, G.W., Aleo, M.D. and Schnellmann, R.G., Depletion of endoplasmic reticulum calcium stores protects against hypoxia and mitochondrial inhibitor-induced cellular injury and death, Am. J. Physiol., 273 (1997) 869–876.