UNIVERSITA' degli STUDI di

NAPOLI FEDERICO II

DOTTORATO DI RICERCA

IN

"SCIENZA DEL FARMACO"

XXII CICLO

Indirizzo Farmacologia, Farmacognosia e Tossicologia

PALMITOYLETHANOLAMIDE, AN ENDOGENOUS PPAR-ALPHA AGONIST, MODULATES NEUROSTEROIDS *DE NOVO* SYNTHESIS

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2006-2009

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Introduction.

Palmitoylethanolamide (PEA), a member of fatty-acid ethanolamide family, is an endogenous neuromodulator with a broad spectrum of pharmacological properties, including analgesic (Calignano et al., 1998, 2001), anti-inflammatory (Costa et al., 2002; D'Agostino et al., 2007), anticonvulsant (Lambert et al., 2001) and antiproliferative (Di Marzo et al., 2001) effects.

Less investigated are the functions of PEA in the central nervous system (CNS), where PEA is present in detectable levels (Cadas et al., 1997), showing diurnal variation (Rodriguez et al., 2006). Despite its molecular target remain debate, PEA failed to exert its analgesic and anti-inflammatory properties in mice lacking peroxisome proliferator-activated receptor alpha (PPAR-α) (LoVerme et al., 2005; D'Agostino et al., 2007). Although PPAR-α is a well characterized transcription factor (Lemberger et al., 1996), it seems to be pivotal for other effects not strictly related to its transcriptional activity, such as the effect on calcium-activated K^+ channels, mediating rapid analgesia (Lo Verme et al., 2006). Likewise PEA, PPAR-α has been localized in discrete areas of the CNS with a peculiar distribution (Kainu et al., 1994; Moreno et al., 2004; Benani et al., 2004), although its role in these areas is unclear and poorly defined.

In the last ten years it has emerged that neurosteroids are strongly involved

in several physiological cognitive and emotive functions of the CNS (Jung-Testas and Baulieu, 1998). Neurosteroids are known to exert several rapid effects, including modulation of hypnosis, through activation of $GABA_A$ receptors, these studies have been mainly conducted with the action of pregnenolone-like neuroactive steroid (Mendelson et al., 1987), and metyrapone, a blocker of the enzyme 11β-hydroxylase, which is essential for the biosynthesis of corticosteroids (Burade et al., 1996). Keller et al. (2004) have shown that 5α-reduced neurosteroids act on $GABA_A$, as well as peripheral benzodiazepine receptor (PBR), modulating the GABA-induced Cl^- currents that result in neuron hyperpolarization. Among 5α-reduced neuroactive steroids, the 3α-hydroxy-5α-pregnan-20-one (allopregnanolone, ALLO) displays anxiolytic, sedative, analgesic and anaesthetic properties (Rupprecht et al., 2001), causing a great pharmacological interest. The main pharmacological properties above reported are shared with those of PEA.

Particularly, it was previously reported that N-acylethanolamines of unsaturated fatty acid (N-linoleoyl-, N-oleoyl-) and of saturated fatty acid (PEA), significantly prolonged pentobarbital-induced hypnosis in mice (Watanabe et al., 1999).

Here, we hypothesize that exogenous administration of PEA, through a PPAR-α-dependent mechanism, participates in neurosteroid formation increasing their levels and leading to a positive modulation of $GABA_A$ receptor. For this aim, we study the effect of PEA *in vitro* using C6 glioma

cell line and primary murine astrocytes, which, as other glial cells and neurons, have the enzymatic machinery for *de novo* synthesis of neurosteroids and *in vivo* using pentobarbital induced LORR duration and EEG recordings in mouse. In particular, Fast Fourier Transformer (FFT) power spectral analysis was applied to reveal the time change of slow wave activity in the EEG, which would provide some quantitative measures of LORR EEG.

The demonstration of the capacity of a neural centre to synthesize neurosteroids requires the localization in that centre of active forms of key steroidogenic enzymes, and local increase of neurosteroids. Therefore, we evaluated the ALLO content and the expression of StAR and P450scc, two proteins implicated in the early step of neurosteroidogenesis in mitochondrial fraction, supernatant and brainstem. As well known, this area serves integrative functions, including, pain sensitivity control, alertness, and consciousness, and shows appreciable and functional levels of PEA (Petrosino et al., 2007; Melis et al., 2008), as well as of PPAR-α (Moreno et al., 2004; Melis et al., 2008).

Discovery of PEA.

The discovery of naturally occurring fatty acid ethanolamides (FAEs) (fig. 1) stems from an interesting clinical finding in the early 1940s, when investigators noted that supplementing the diets of underprivileged children with dried chicken egg yolk prevented recurrences of rheumatic fever, despite continued streptococcal infections (Coburn and Moore, 1943).

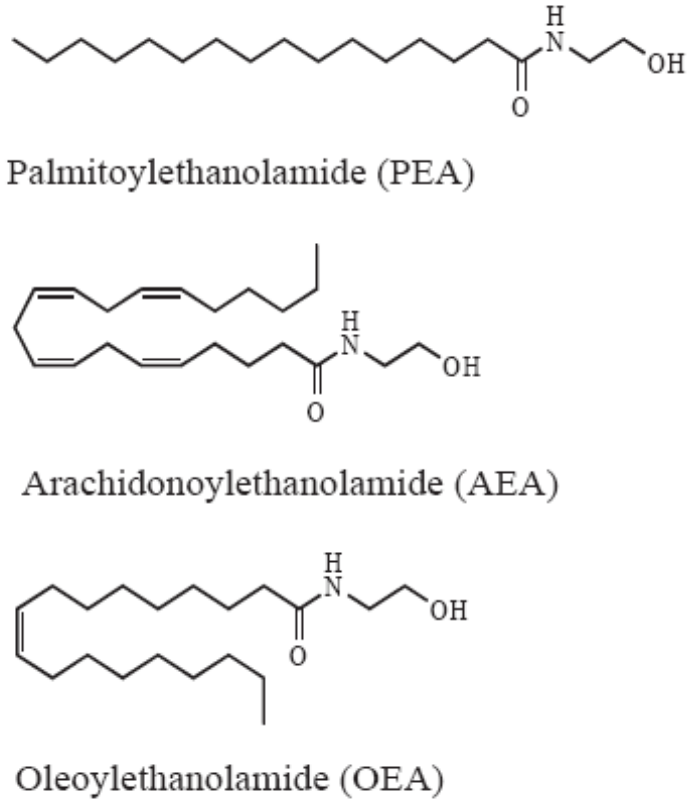


Fig. 1. Chemical structures of various fatty acid ethanolamides, palmitoylethanolamide, oleoylethanolamide, and arachidonoylethanolamide (anandamide).

In a continuation of this research, it was demonstrated that lipid fractions purified from egg yolk (Coburn et al., 1954; Long and Martin, 1956), as well as peanut oil and soybean lecithin (Long and Miles, 1950), exerted anti-allergic effects in the guinea pig (see fig. 2 for a timeline of events). Soon thereafter, PEA (N-(2-hydroxyethyl)hexadecanamide, N-palmitoylethanolamide, LG 2110/1) was isolated as the agent responsible for these anti-inflammatory properties (Kuehl et al., 1957). This work ultimately led to its identification in mammalian tissues in 1964 (Bachur et al., 1965).

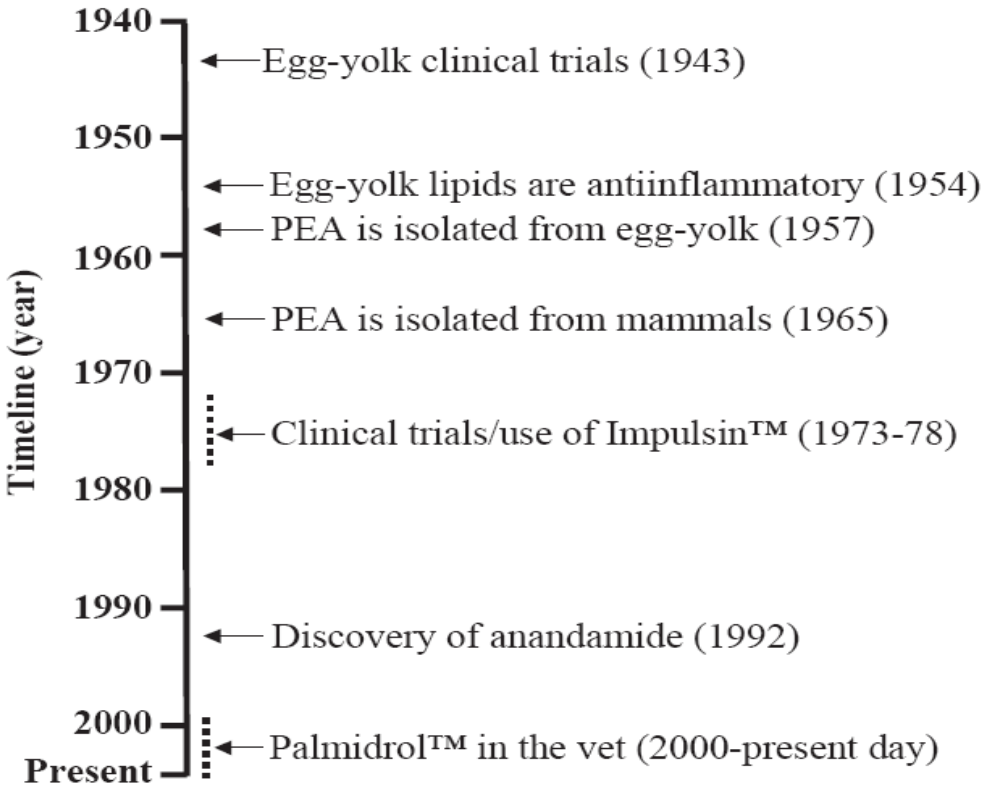


Fig. 2. PEA: a historical perspective. The discovery of PEA dates back to 1957, when it was found to be the active anti-inflammatory constituent of egg yolk. PEA was identified in mammalian tissues in 1965 and was used in the clinic during the 1970s for the treatment of influenza. PEA is currently used as a veterinary anti-inflammatory medication as part of a nutritional composition sold under the trade name of Redonyl®.

Pharmacological properties of PEA.

Since the discovery of anandamide, the properties of PEA have been explored with growing interest (fig. 3).

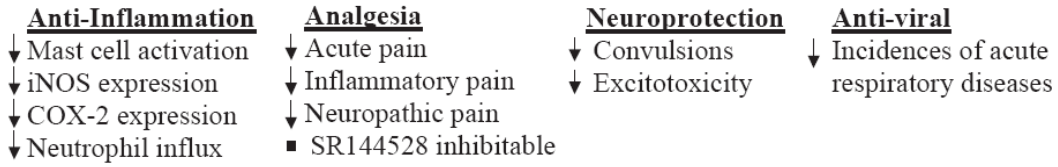


Fig. 3. Pharmacological consequences of PEA administration. PEA produces a broad range of actions including anti-inflammation, analgesia, neuroprotection, and anticonvulsant activity. It may also exert prophylactic actions towards respiratory tract infections.

In addition to its known anti-inflammatory activity, PEA also produces analgesia (Calignano et al., 1998, 2001; Jaggar et al., 1998), anti-epilepsy, and neuroprotection (Franklin et al., 2003; Lambert et al., 2001; Sheerin et al., 2004; Skaper et al., 1996). PEA also inhibits food intake (Rodríguez de Fonseca et al., 2001), reduces gastrointestinal motility (Capasso et al., 2001) and cancer cell proliferation (De Petrocellis et al., 2002; Di Marzo et al., 2001), and finally protects the vascular endothelium in the ischemic heart (Bouchard et al., 2003).

Following the early experiments in the 1950s, a series of more recent studies has shown that PEA inhibits mast cell degranulation (Aloe et al.,

1993; Mazzari et al., 1996) and pulmonary inflammation in mice (Berdyshev et al., 1998). These effects have been tentatively linked to reduced nitric oxide production by macrophages (Ross et al., 2000) and/or neutrophil influx (Farquhar-Smith and Rice, 2003). For example, in one study, animals treated with PEA for 3 days (10 mg kg^{-1}, orally, once daily) following a carrageenan-induced inflammatory insult displayed significantly lower levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) (Costa et al., 2002) a genomic action reminiscent of steroidal anti-inflammatory drugs. Other studies have demonstrated that PEA reduces inflammation in a matter of hours (Conti et al., 2002; LoVerme et al., 2005), implying that a non-genomic mechanism of action may also exist.

Broad-spectrum analgesia by PEA has been documented in a variety of pain models. PEA reduces pain behaviors elicited by formalin (Calignano et al., 1998; Jaggar et al., 1998), magnesium sulfate (Calignano et al., 2001), carrageenan (Conti et al., 2002; Mazzari et al., 1996), nerve growth factor (Farquhar-Smith and Rice, 2003), and turpentine (Farquhar-Smith and Rice, 2001; Jaggar et al., 1998).

Moreover, PEA was found to inhibit hyperalgesia after sciatic nerve ligation, a model of neuropathic pain (Helyes et al., 2003). Because PEA-induced analgesia is rapid and precedes the compound’s anti-inflammatory actions, it has been suggested that PEA may function as an endogenous regulator of nociception (Calignano et al., 1998).

Less understood are the functions of PEA in the central nervous system (CNS), where PEA is present at high levels (Cadas et al., 1997). Increasing evidence points to an antiepileptic and neuroprotective action of PEA (Lambert et al., 2001; Sheerin et al., 2004). For example, in one study, intraperitoneal administration of the compound was shown to inhibit electroshock-induced and chemically induced seizures with a half-maximally effective dose (ED50) of 9 mg/kg, i.p. (Lambert et al., 2001). Other neuroprotective actions have also been reported; for example, in a separate study, PEA dose-dependently protected cultured mouse cerebellar granule cells from glutamate toxicity (Skaper et al., 1996). In yet another study, PEA reduced histamine-induced cell death in hippocampal cultures (Skaper et al., 1996).

Finally, PEA was more recently shown to enhance microglial cell motility (Franklin et al., 2003).

PEA biosynthesis and inactivation.

Unlike classical neurotransmitters and hormones which are stored in and released from intracellular secretory vesicles, the production of FAEs occurs through on-demand synthesis within the lipid bilayer (Cadas et al., 1996; Schmid et al., 1990). In mammalian tissues, two concerted and independent biochemical reactions are responsible (fig. 4).

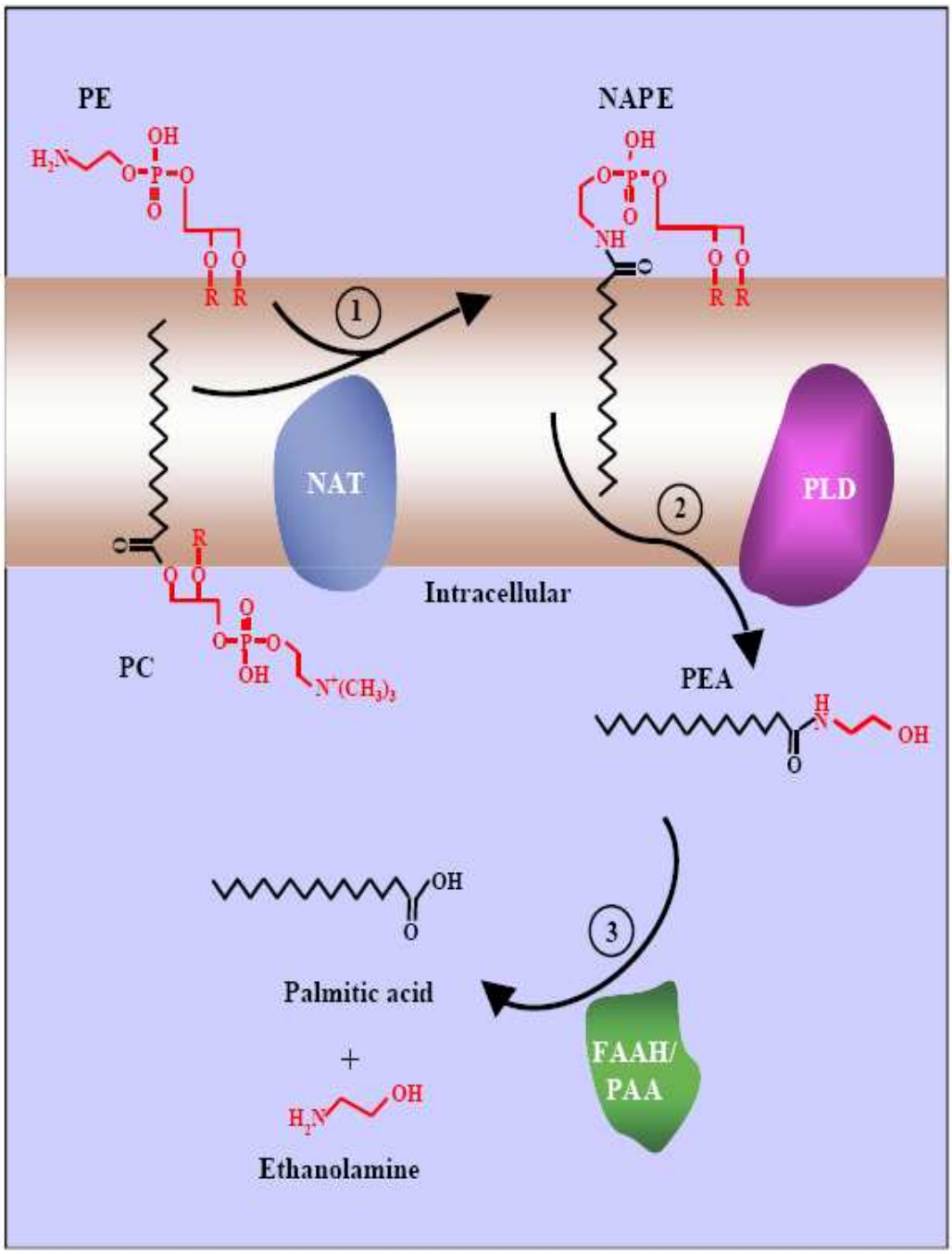


Fig. 4. PEA biosynthesis and inactivation. The biosynthesis of PEA occurs in a two steps. (1) The calcium- and cAMP-dependent transfer of palmitic acid from phosphatidylcholine (PC) to phosphatidylethanolamine (PE) to form N-acylphosphatidylethanolamine (NAPE). (2) The cleavage of NAPE to release PEA, mediated by a NAPE-specific phospholipase D (PLD). (3) PEA is hydrolyzed by fatty acid amide amidohydrolase (FAAH) or PEA-preferring acid amidase (PAA) to form palmitic acid and ethanolamine.

The first is the transfer of a fatty acid from membrane-bound phospholipids to phosphatidylethanolamine (PE), catalyzed by a calcium ion and cyclic-AMP regulated N-acyltransferase (NAT), to form the FAE precursor N-acyl phosphatidylethanolamine (NAPE). Different FAE precursors are generated according to which fatty acid is initially transferred to PE (i.e., the initial transfer of palmitic acid will yield a PEA precursor, while that of arachidonic acid will yield an anandamide precursor). The second step in FAE synthesis is the cleavage of membrane-bound NAPE to release free PEA, which is mediated by a NAPE-specific phospholipase D (PLD). This lipid hydrolase shares little sequence homology to other members of the PLD family and recognizes multiple NAPE species, producing PEA along with other FAEs (Okamoto et al., 2004).

Alternatively, a separate mechanism of synthesis has been proposed involving a similar two-step reaction: (1) the hydrolysis of NAPE to N-palmitoyl-lysoPE (lyso-NAPE) by soluble phospholipase A2 (sPLA2) and (2) the subsequent cleavage of lyso-NAPE by a lysophospholipase D (lyso-PLD) (Natarajan et al., 1984). The activities of these two enzymes are highest in the stomach, brain, and testis (Sun et al., 2004). The relative contribution of each of these synthetic pathways is unknown at present.

PEA inactivation primarily consists of its intracellular hydrolysis by lipid hydrolases (Fig. 4) (Schmid et al., 1985). One of these enzymes, called fatty acid amide hydrolase (FAAH), has been molecularly cloned (Cravatt et al., 1996) and extensively characterized (Bracey et al., 2002), and

selective inhibitors that block its activity in vivo have been developed (Kathuria et al., 2003). A second enzyme, referred to as PEA-preferring acid amidase (PAA), has also been identified (Ueda et al., 2001).

FAAH, a membrane-bound intracellular serine hydrolase, for which PEA is an excellent substrate (Désarnaud et al., 1995; Hillard et al., 1995; Ueda et al., 1995), is present in all mammalian tissues, but is particularly abundant in brain and liver (Cravatt and Lichtman, 2002). In fact, mice lacking the faah gene have dramatically reduced PEA hydrolysis and increased PEA levels in brain and liver tissues (Cravatt et al., 2004; Lichtman et al., 2002; Patel et al., 2004). In contrast to FAAH, PAA activity is most abundant in the rodent intestine, spleen, and lung (Ueda et al., 2001). PAA recognizes all FAEs, suggesting that it may play a broad role in the deactivation of these compounds by intact cells; however, in the presence of detergent, this activity displays a marked preference for PEA as a substrate (Ueda et al., 2001).

The PEA receptor.

Despite its potential clinical significance, the cellular receptor responsible for the actions of PEA has long remained unidentified, and a great deal of controversy has surrounded its identity. The structural and functional similarities between anandamide and PEA first suggested that these two lipid mediators might share the same receptor. In support of this idea, PEA was initially reported to displace the binding of the high-affinity cannabinoid agonist [3H]WIN55,212-2 with a half-maximal inhibitory concentration (IC50) of 1.0 nM from RBL-2H3 cell membranes, which are known to express CB2 mRNA (Facci et al., 1995). However, these results have not been subsequently replicated (Jacobsson and Fowler, 2001; Lambert et al., 2002; Lambert and Di Marzo, 1999; Showalter et al., 1996; Sugiura et al., 2000) and it is now generally accepted that PEA does not bind to CB2 receptors. Adding to the PEA mystery, the CB2 antagonist/inverse agonist SR144528 inhibits many, but not all, of the pharmacological actions of PEA (Calignano et al., 1998, 2001; Conti et al., 2002; Jaggar et al., 1998). In particular, the analgesic actions of PEA are blocked by SR144528 (Calignano et al., 1998; Farquhar-Smith and Rice, 2001, 2003; Helyes et al., 2003), whereas its anti-peristaltic effects are not (Capasso et al., 2001). Further confounding the issue, it appears that the anti-inflammatory actions of PEA are only sensitive to SR144528 in acute models of inflammation (Conti et al., 2002; Costa et al., 2002; LoVerme et

al., 2005).

In light of these apparent discrepancies, several possible scenarios have been proposed to describe the mechanism of action of PEA (fig. 5).

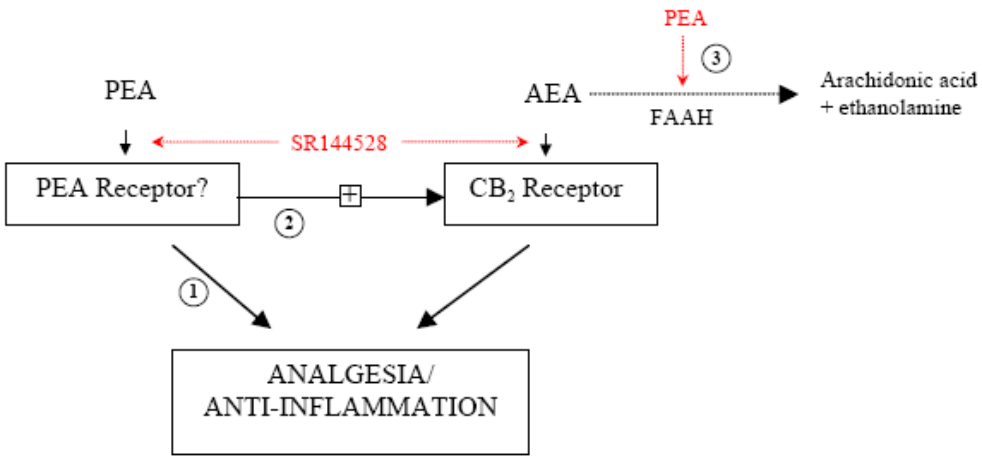


Fig. 5. Proposed mechanisms of action for PEA. PEA may exert its effects through an unknown receptor, which may either be sensitive to SR144528 (1) or engage CB2 receptors as downstream effectors (2). (3) PEA may act as an "entourage compound", effectively inhibiting FAAH activity, and thus increasing local anandamide levels (see text for further details).

The first possibility is that PEA binds to an unidentified receptor for which SR144528 is a functional antagonist (i.e., a compound that either directly interacts with the receptor, or indirectly blocks a downstream effector of this receptor (CB2 receptors)). A second possibility is that PEA acts through a so-called "entourage effect" (Ben-Shabat et al., 1998; Lambert and Di Marzo, 1999). According to this theory, PEA may increase anandamide levels in tissues by competing with this compound for FAAH-

mediated hydrolysis, resulting in a potentiation of anandamide's actions. However, "entourage effects" have only been observed in vitro. In contrast, administration of PEA in vivo has the opposite result of decreasing endogenous anandamide levels (LoVerme et al., 2005). Finally, Di Marzo et al. (2001) suggested that in human breast cancer cells, PEA may work by inhibiting FAAH expression, which should lead to an increase in anandamide levels. This hypothesis does not account, however, for the very rapid actions of PEA, which occur within minutes of its administration.

PEA: An Independent Endocannabinoid?

Results showing that PEA interacts with a distinct non-CB1/CB2 receptor suggest that this lipid might constitute a unique "parallel" endocannabinoid signaling system. Providing support to this concept is the evidence that PEA production and inactivation can occur independently of AEA and 2-AG production and inactivation. Specifi cally, in rodent cortical neurons, general activity-dependent production of AEA, 2-AG, and PEA occurs concomitantly (Di Marzo V et al., 1994; Cadas et al., 1997; Hansen et al., 1997). Yet, subsequent studies showed that 2-AG production can be increased independently when N-methyl-D-aspartate (NMDA) receptors are activated, while increased AEA and PEA production requires addition of carbachol (Stella and Piomelli, 2001). A more detailed pharmacological

study showed that activation of nicotinic receptors increases AEA production, while activation of muscarinic receptors increases PEA production. Thus, although biosynthesis of all endocannabinoids in this model system is contingent on NMDA-receptor occupation, increased AEA production requires the co-activation of α7 nicotinic receptors, while increased PEA production requires the co-activation of muscarinic receptors. This finding suggests that glutamate and acetylcholine may elicit the biosynthesis of different endocannabinoids depending on the complement of cholinergic receptors expressed in their target neurons. Additional evidence for independent production of AEA, 2-AG, and PEA comes from experiments performed on mouse astrocytes in culture. In this model, the calcium ionophore ionomycin and the peptide endothelin-1 increase the production of both AEA and 2-AG, while PEA levels remain unchanged (Walter et al., 2002; Walter and Stella, 2003). The notion that PEA might be independently produced also holds true in vivo (Franklin et al., 2003). For example, in the case of focal cerebral ischemia, PEA levels in ischemic cerebral cortex increase ~25-fold compared with sham-operated animals, while AEA levels increase by barely 3-fold and 2-AG levels remain unchanged.

Novel evidence shows that PEA inactivation can also occur independently from that of AEA and 2-AG. The laboratory of Natsuo Ueda discovered the existence of a unique enzyme capable of hydrolyzing PEA to much greater extent than AEA and 2-AG. The original observation was obtained with

homogenates prepared from human megakaryoblastic cells (CMK), in which AEA hydrolysis occurred with low activity and a strikingly different pH profi le from that of FAAH (Ueda et al., 2001).

Specifically, while FAAH is known to maximally hydrolyze AEA at pH 9 (with this activity dropping by 70% at pH 5), CMK cells were shown to maximally hydrolyze AEA at pH 5 (with this activity dropping by 95% at pH 9). Using an elegant 4-step purifi cation approach, Ueda and colleagues were able to purify this novel enzymatic activity by 760-fold, obtain partial protein sequence, and clone a cDNA encoding this protein, which was named N-acylethanolamine-hydrolyzing acid amidase (NAAA) (Tsuboi et al., 2005).

When assessing its substrate specifi city, it became clear that this enzyme preferred PEA over AEA (having hydrolytic activities toward these substrates of 8 and 0.25 nmol/min/mg, respectively). Besides a distinct pH profi le and substrate specificity, NAAA has additionally very interesting properties. It is highly expressed in spleen and thymus, as well as in macrophages homing to the lungs and small intestine, highlighting its potential importance in regulating PEA signaling in the context of immunobiology. Here, it should be emphasized that NAAA expression and activity are quite low in healthy brain (Tsuboi et al., 2005).

The laboratory of Didier Lambert developed a competitive inhibitor of NAAA, N-cyclohexanecarbonylpentadecylamine, which has an IC50 of 5 μM and is inactive against FAAH at 100 μM (Ueda et al., 2005).

When considering that methyl arachidonoylfluorophosphonate (MAFP) and URB597 inhibit FAAH with nanomolar IC50s and are both inactive against NAAA at a concentration of 1 μM, experiments designed to use these compounds in combination may be useful to distinguish the biological importance of either PEA or AEA hydrolysis in various biological responses (Sun et al., 2005; Ueda et al., 2005).

This series of studies raises many fascinating questions: for example, "What is the subcellular location of NAAA and does it differ from FAAH? "The pH profile of NAAA is quite intriguing. With maximal NAAA activity occurring at pH 5 and only 10% of this activity remaining at pH 7 (i.e., the cytosolic pH), one wonders if NAAA might be active only in lysosomes. Accordingly, NAAA-GFP (green fluorescent protein) fusion protein localized to lysosome-like vesicles (Tsuboi et al., 2005).

This result is quite interesting when considering that FAAH is also abundant in intracellular organelles such as mitochondria and the smooth endoplasmic reticulum (Gulyas et al., 2004).

Clearly, elucidating the exact biological role of NAAA will be facilitated by genetic studies similar to those performed on FAAH. Finally, while AEA and 2-AG hydrolysis give rise to new bioactive lipids (ie arachidonic acid and eicosanoids), PEA hydrolysis gives rise to 2 relatively inactive products, palmitic acid and ethanolamine, suggesting that the role of NAAA is to truly stop biological responses initiated by increases in PEA production.

Acylethanolamides and PPAR-α.

The first evidence of acylethanolamide interactions with PPARs came in 2002 in a study by Kozak et al., who showed that lipoxygenase (LOX) metabolism of the endocannabinoid, 2-arachidonoylglycerol (2-AG), produced a metabolite (15-hydroxyeicosatetraenoic acid glyceryl ester, 15-HETE-G, 1-10 mM) that increased the transcriptional activity of PPAR-α, as shown in a reporter gene assay. In 2003, it was then shown by Fu et al. that oleoylethanolamide (OEA, 0.1-10 μM) bound to and increased the transcriptional activity of PPAR-α, a finding later confirmed by Sun et al. (2006). OEA is a naturally occurring amide of ethanolamine and oleic acid, produced through biosynthetic pathways similar to those of anandamide but, despite the structural and metabolic similarities with anandamide, OEA does not bind to cannabinoid receptors. Fu et al. (2003) showed that the appetite-suppressing and weight-reducing effects of OEA (10 mg kg^{-1}) were absent in PPAR-α knockout mice, and that daily treatment with OEA (5 mg kg^{-1}, 2 weeks) reduced serum cholesterol levels in rat and mouse models of obesity. Guzman et al. (2004) then showed that the stimulatory effect of OEA (5 mg kg^{-1}, 4 weeks) on lipolysis in vivo was absent in PPAR-α knockout mice, and that a single dose of OEA (10 mg kg^{-1}) in rats increased the mRNA levels of a number of PPAR- α target genes (PPAR-α, fatty acid-binding protein and uncoupling protein 2). Further work has now shown that the anti-inflammatory effects of OEA in 12-O-tetradecanoyl-

phorbol-13-acetate-induced oedema in mice (LoVerme et al., 2005) and the neuroprotective effects of OEA in a mouse model of cerebral artery occlusion (Sun et al., 2006) were also absent in PPAR-α knockout mice. Together, these studies suggest that many of the physiological responses to OEA are mediated by PPAR-α activation. A number of structural analogues of OEA have also been shown to have a high affinity for PPAR-α, with similar reductions in food intake when administered in vivo (Astarita et al., 2006).

Like OEA, another fatty acid ethanolamide, palmitoylethanolamide (PEA) is reported to have actions that cannot be attributed to traditional cannabinoid receptor sites of action. After demonstrating that OEA, which is structurally related to PEA, activates PPAR-α (Fu et al., 2003), Lo Verme et al. (2005) went on to show that PEA (1-30 μM) similarly activates PPAR-α transcriptional activity, causing anti-inflammatory actions in both 12-O-tetradecanoylphorbol-13-acetate-induced and carrageenan-induced oedema that were absent in PPAR-α knockout mice (at 10 mg kg^{-1}; LoVerme et al., 2006). Further studies showed that PEA (50 μg, intraplantar injection) caused analgesic effects in vivo in several models of pain behaviour, which were also absent in PPAR-α knockout mice (LoVerme et al., 2006). Other acylethanolamides shown to activate and bind to PPAR-α include anandamide, noladin ether and virodhamine (Sun et al., 2006), suggesting that PPAR-α activation is common to all acylethanoamides, or at least all those tested to date. It is of note that the concentrations of

acylethanolamides required to activate PPARs are in the same range as those reported for fatty acids (Kliewer et al., 1997). However, it has not yet been established whether combinations of fatty acids/endocannabinoids (as would occur intracellularly) may act synergistically at PPARs.

The majority of research to date has focused on the effects of acylethanolamides on PPAR-α, and the effects of synthetic or phytocannabinoids at this receptor are yet to be investigated. The synthetic CB1/CB2 agonist, WIN55212-2, is reported to bind to and increase the transcriptional activity of PPAR-α (Sun et al., 2006), although whether WIN55212-2 causes similar effects (anti-inflammatory actions, anorexia, lipolysis and analgesia) through this site, as reported for OEA and PEA, remains to be established.

Peroxisome proliferator-activated receptors.

PPARs belong to the nuclear hormone receptor superfamily and three different subtypes have been identified, PPAR-α, PPAR-β (also called PPAR-δ or β/δ, NUC-1 or FAAR), and PPAR-γ and these receptors have been found in teleosts, amphibians, rodents, and humans (Dreyer et al., 1992; Schmidt et al., 1992; Sher et al., 1993; Kliewer et al., 1994; Greene et al., 1995; Escriva et al., 1997; Fajas et al., 1998). Alternate promoter usage and splicing occur and three PPAR-γ variants have been

characterized, PPAR-γ 1, 2 and 3. PPAR-γ 2 has 30 additional N-terminal amino acids. A third promoter in the human sequence encodes a protein, PPAR-γ 3 (Fajas et al., 1998). The PPAR subtypes are encoded by distinct single-copy genes and have a structure characteristic of the nuclear hormone receptors. Each receptor has an N-terminal “A/B” domain that has a ligand-independent activation function and is poorly conserved between the isoforms. The “C” domain encodes the DNA binding region of the receptor, is highly conserved, includes two zinc-finger-like structures with α-helical DNA binding motifs and is followed by the “D” or hinge region. The C-terminal ligand binding domain (LBD), the “E/F” domain, contains the ligand-dependent activation function 2 (AF-2) and is also important for RXR heterodimerization. The ligand-binding domains (LBD) are highly conserved and the mouse PPAR-γ 2 LBD sequence is 70% and 68% similar to mouse PPAR-β and PPAR-α LBDs, respectively (Escher and Wahli, 2000). The rat and mouse PPAR-α proteins share 92% identity with the human receptor. The rat and mouse PPAR-β are 91% and 92% homologous with the human PPAR-β amino acid sequence. Rat and mouse PPAR-γ 1 and 2 have 95–98% homology with the human receptor (Borel et al., 2008). PPARs regulate gene expression by binding to specific DNA sequences, peroxisome proliferator response elements (PPRE), in the promoter regions of target genes (fig. 6).

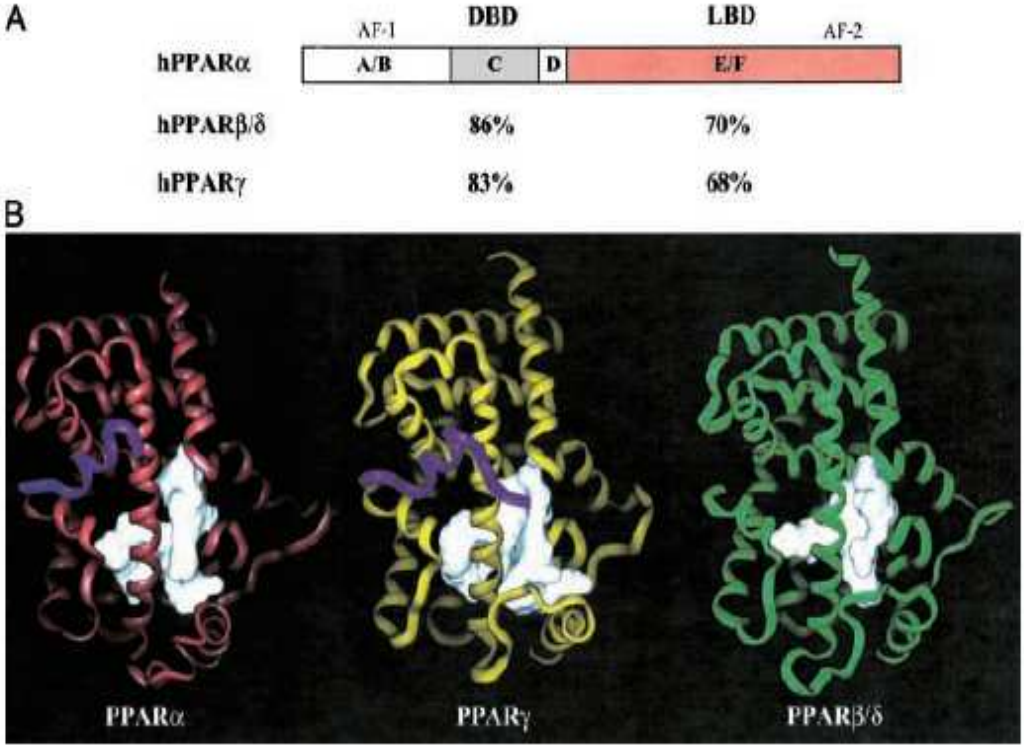


Fig. 6. Structure of PPARs. (*A*) Schematic view of the domain organization of PPARs. Structural and functional domains are depicted.The A/B domain contains the activation function 1 (AF-1), which has a ligand-independent transcriptional activity. The C domain corresponds to the DNA binding domain (DBD). The E/F domain contains the ligand binding domain (LBD) and carries the activation function 2 (AF-2), which has a ligand-dependent transcriptional activity. The percentage of amino acid identity of the DBDs and LBDs of human PPAR-β and PPAR-γ compared with PPAR-α is indicated. (*B*) Three-dimensional structure of ligand binding domains of PPARs. A comparison of the X-ray crystal structures of PPAR-α (red worm), PPAR-γ (yellow worm) and PPAR-β (green worm) is shown. Each PPAR is complexed to a high-affinity ligand (not pictured). PPAR-α and PPAR-γ are shown associated to LXXLL peptides (purple worms), the signature motif of the receptor coactivators. For each PPAR, the solvent-accessible ligand binding pocket is displayed as an off-white surface.

The initial characterization of the PPRE identified it as a direct repeat 1 motif (DR1), which consists of two copies of a core motif sequence, AGGTCA, separated by one nucleotide. The determinants of the PPRE differ from those for response elements of other members of the TR/RAR class, however there is considerable variation in the sequences with which PPARs interact (Keller et al., 2000). Prior to DNA binding PPAR forms a heterodimer with the retinoid X receptor (RXR), another member of the nuclear receptor super family (Tan et al., 2005; Tien et al., 2006). The endogenous ligand for RXR is 9-cis-retinoic acid and RXR is a partner for several other nuclear receptors (retinoic acid receptor, thyroid hormone receptor, and vitamin D receptor). Upon ligand binding, conformational changes are induced in the LBD that involve the AF-2 C-terminal helix and the PPAR-RXR heterodimer subsequently interacts with the PPRE, recruits co-activators/co-factors, and activates transcription (Tan et al., 2005; Tien et al., 2006; Zoete et al., 2007). The co-activators known to interact with the complex include the steroid receptor co-activator 1 (SRC-1) which interacts with the E/F and A/B domains of all three isoforms, and CREB-binding protein (CBP/p300) which associates with the helical AF region of PPAR-α and γ (fig. 7).

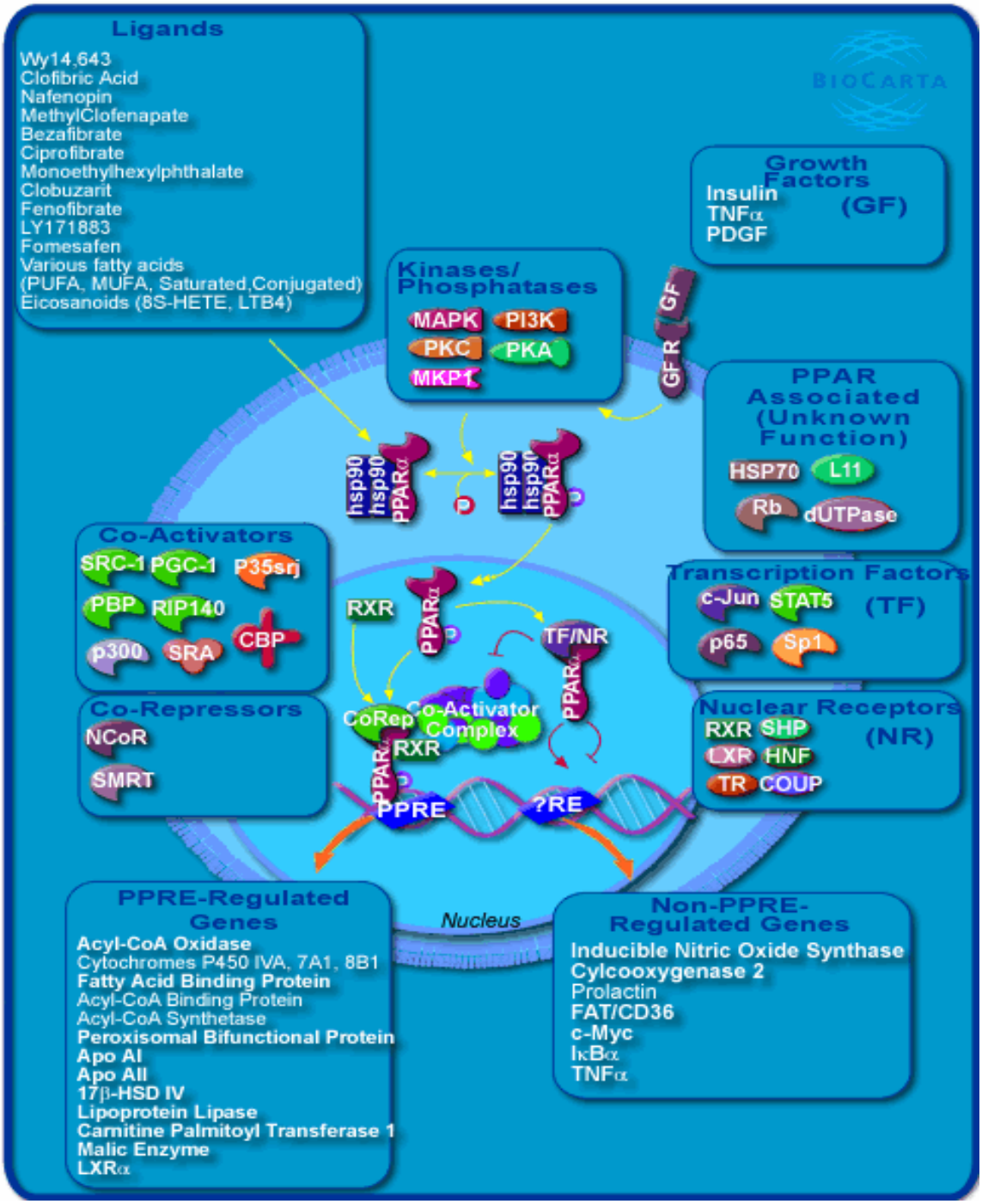


Fig. 7. The most recognized mechanism by which peroxisome proliferators regulated gene expresssion is through a PPAR/RXR heterodimeric complex binding to a peroxisome proliferator-response element (PPRE) (classical mechanism). However, there are the possibility of several variations on this theme: 1). The peroxisome proliferator interacts with PPAR that preexists as a DNA complex with associated corepressors proteins. The interaction with ligand causes release of the corepressor and association with a coactivator, resulting in the classical

mechanism. 2). The peroxisome proliferator interacts with PPAR as a soluble member of the nucleus. The binding of ligand results in RXR heterodimerization, DNA binding and coactivator recruitment. 3). In this scenario, PPAR exists in the cytosol, perhaps complexed to heat shock protein 90 and/or other chaperones. Binding of peroxisome proliferator causes a conformational change and translocation into the nucleus. Scenarios 4 and 5 require regulation of gene expression via non-classical mechanisms: 4). PPAR is capable of interacting with, and forming DNA binding heterodimers with, several nuclear receptors including the thyroid hormone receptor. The binding site for this non-RXR heterodimer need not be the classic DR-1 motif found in the PPRE. 5). PPAR may participate in the regulation of gene expression witout binding to DNA. By association with transcription factors such as c-jun or p65, PPAR diminishes the ability of AP1 or NFB to bind to their cognate DNA sequences, respectively. Also shown in this scheme are two means to modify the peroxisome proliferator response. Most importantly, growth factor signaling has a pronounced affect on PPAR via post-translational modification. PPAR is a phosphoprotein and its activity is affected by insulin. Several kinase pathways affects PPAR-α's activity, although the specific kinases and phosphorylation sites have not been conclusively determined.

Other co-activators are PPAR-γ co-activator 1 (PGC-1) and PGC-2 which interact with PPAR-γ, the ubiquitously expressed PPAR binding protein (PBP/TRAP220/DRIP230) which interacts with PPAR-α and γ, the androgen receptor associated protein 70 (ARA70), and receptor interacting protein (RIP140) an inhibitor of PPAR-α and γ activity. The interactions may also result in release of co-repressors, such as the silencing mediator for retinoid and thyroid hormone receptors (SMRT) and the nuclear receptor corepressor (NCoR) which dissociates from PPAR-α and γ in the presence of agonists. As can be deduced from the names of some of these co-factors, the same proteins interact with other steroid hormone receptors (glucocorticoid receptor, thyroid hor- mone receptor, estrogen receptor, vitamin D receptor, retinoic acid receptor, etc.). PPARs can regulate gene expression via multiple modes of action (Tan et al., 2005). PPARs may repress expression of genes by interacting with co-repressors and with RXR to suppress expression of target genes and this repression could be released upon activation with either a PPAR or an RXR ligand followed by recruitment of co-activators (Tan et al., 2005; Tien et al., 2006; Zoete et al., 2007). PPARs may also influence gene expression through interactions with or modulation of other transcription factors and cell signaling pathways (Glass and Ogawa, 2006).

A number of endogenous ligands have been identified for each PPAR isoform and include long chain fatty acids, polyunsaturated fatty acids such as linoleic and arachidonic acid, saturated fatty acids such as

palmitoylethanolamide, and eicosanoids. A variety of synthetic ligands have been developed for pharmaceutical purposes, including the fibrate class of hypolipidemic drugs that activate PPAR-α (clofibrate, benzafibrate, ciprofibrate), the anti-diabetic thiazolidinediones that activate PPAR-γ (rosiglitazone), PPAR-β agonists designed to treat metabolic syndrome (GW501516) and non-steroidal anti-inflammatory drugs (indomethacin, ibuprofen, fenoprofen). Other chemicals that may occur in industrial processes and as environmental contaminants are known to activate PPAR, e.g. phthalates, tri- and dichloroacetic acid, trichloroethylene, perfluoroalkyl acids such as PFOS and PFOA (Desvergne and Wahli, 1999; Escher et al., 2000; Klaunig et al., 2003; Peraza et al., 2006). Ligands may not be specific to a single PPAR isoform. There are examples of both endogenous and exogenous ligands with the ability to bind and activate multiple or specific isoforms (Zoete et al., 2007). However, it is also important to note that there are PPAR-dependent effects which require expression of the receptor, but for which ligand is not required.

Genes regulated by PPAR isoforms are involved in important physiological processes that have impacts on lipid homeostasis, inflammatory responses, adipogenesis, reproduction, wound healing, and carcinogenesis (Escher et al., 2000; Michalik et al., 2002; Mandard et al., 2004; Kilgore and Billin, 2008).

PPAR regulation impacts cellular processes such as proliferation, differentiation and cell death. PPAR-α regulates peroxisome proliferation,

lipid catabolism, inflammatory responses, skin wound healing, inhibition of arachidonic acid metabolism and inhibition of ureagenesis. PPAR-β activation influences cell proliferation, myelination, embryonic implantation, adipocyte differentiation, skeletal muscle fatty acid catabolism, glucose metabolism, inflammation, and skin wound healing. PPAR-γ regulates lipid storage, macrophage maturation, embryonic implantation, adipocyte differentiation and inflammation control.

Biosynthesis and actions of neurosteroids.

Discovery and definition of neurosteroids.

A major finding in the research on neuroactive steroids was the identification of the ability of neurons and glial cells to synthesize bioactive steroids, also called neurosteroids (Baulieu et al., 1999). This important discovery stemmed from a series of pioneer works showing the persistence of substantial amounts of PREG, DHEA and their sulfated derivatives in the rodent brain after gonadectomy and adrenalectomy (Corpechot et al., 1981 and 1983). However, the consolidation of the concept of neurosteroids has required several investigations performed in various laboratories using different animal species (Baulieu et al., 1999; Mensah-Nyagan et al., 1999; Compagnone and Mellon 2000; Schumacher et al., 2003). These investigations, which significantly increased the basic knowledge on neurosteroids, have also allowed a strict definition of the term neurosteroid with specific identification criteria. The consensual definition that emerged considers these molecules as endogenous steroidal compounds synthesized in neurons or glial cells of the CNS and PNS. To be qualified as a neurosteroid, the candidate steroidal molecule must persist in substantial amounts in the nervous system after removal of the peripheral or traditional

steroidogenic glands such as the adrenals and gonads (Baulieu et al., 1999). Furthermore, it has been demonstrated that neurosteroids act as paracrine or autocrine factors, regulating the activity of classical nuclear steroid receptors and through the modulation of membrane receptors, including steroidal membrane receptors coupled to G proteins (Zhu et al., 2003a and b), $GABA_A$ and T-type calcium channels (Majewska, 1992; Belelli and Lambert, 2005; Pathirathna et al., 2005; Hosie et al., 2006) or via NMDA (Wu et al., 1991; Bowlby, 1993), P2X (De Roo et al., 2003) and sigma (Monnet et al., 1995; Maurice et al., 2006) receptors.

Evidence for neurosteroid biosynthesis.

The demonstration of neurosteroid biosynthesis requires the observation of the expression and biological activity of key steroidogenic enzymes in nerve cells (Baulieu et al., 1999).

Neurosteroid formation has been evidenced in the nervous system by various molecular and biochemical studies, which have revealed in neurons and/or glial cells the expression of TSPO, the steroidogenic acute regulatory protein (StAR) and of several key steroid-synthesizing enzymes such as cytochrome P450 side chain cleavage (P450scc; CYP11A), 3β-hydroxysteroid dehydrogenase (3β-HSD), cytochrome P450c17 (P450c17; CYP17), 5α-reductase (5α-R), 3α-hydroxysteroid oxido-reductase (3α-

HSOR), 17β-hydroxysteroid dehydrogenase (17β-HSD) and aromatase (CYP19) (Baulieu et al., 1999; Mensah-Nyagan et al., 1999; Compagnone and Mellon 2000; Schumacher et al., 2003).

TSPO, StAR and cytochrome P450scc.

Cholesterol conversion to PREG, the first step in the biosynthesis of all classes of steroid hormones (fig. 8), is catalyzed by the mitochondrial enzyme P450scc. However, there is a previous step that is rate limiting and hormonally regulated: the transfer of cholesterol from the outer to the inner mitochondrial membrane, where the P450scc enzyme is located. In this mechanism, two proteins located in the mitochondrial membranes, TSPO (Papadopoulos et al., 2006) and StAR (Lavaque et al, 2006), exert an active role.

TSPO is expressed in the nervous system, predominantly in glial cells of CNS and PNS. Neural injury and different neuropathological conditions result in the induction of the expression of this molecule. TSPO basal expression is up-regulated in gliomas, in neurodegenerative disorders (e.g., Alzheimer's disease), as well as in various forms of brain injury and inflammation induced by neurotoxins (Papadopoulos et al., 2006).

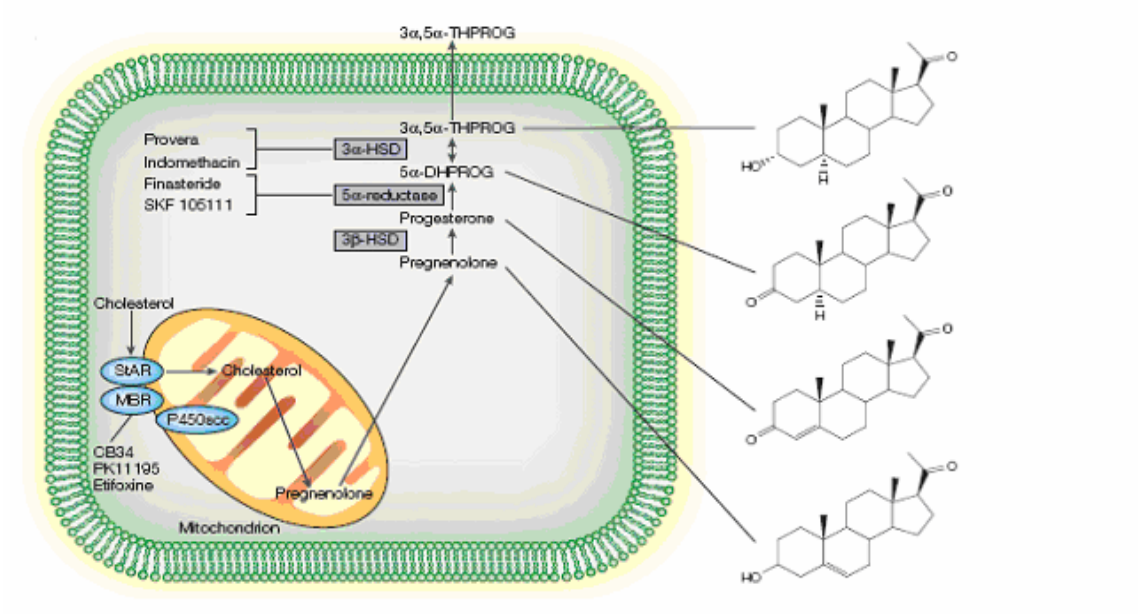


Fig. 8. Structure and synthesis of neurosteroids. Biosynthesis of GABA (γ-aminobutyric acid)-modulatory neurosteroids. The pathway for the synthesis of 5α-pregnan-3α-ol-20-one (3α,5α-THPROG) from cholesterol is shown. Also indicated is the site of action of drugs that have been used to evaluate the influence of endogenous 3α,5α-THPROG on inhibitory neurotransmission. Steroidogenic acute-regulatory protein (StAR) might interact with the mitochondrial benzodiazepine receptor (MBR) to facilitate the transport of cholesterol across the mitochondrial membrane. 3α-HSD, 3α-hydroxysteroid dehydrogenase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 5α-DHPROG, 5α-dihydroprogesterone; P450scc, P450 side-chain cleavage.

The induction of TSPO expression after injury in the CNS is mainly restricted to microglia and astrocytes (Kassiou et al., 2005).

In the PNS, the expression of TSPO in Schwann cells is increased after nerve lesion, and return to normal levels when regeneration is completed (Lacor et al., 1999).

StAR is formed as a 37-kDa protein, which is rapidly transported into mitochondria where it is cleaved, generating a mature 30-kDa intramitochondrial StARprotein that is inactive. StAR appears to be widely distributed throughout the brain, although different levels of expression have been detected between different brain areas. For instance, StAR expression seems to be restricted to very specific neuronal and astroglial populations in each brain area, although it is predominantly expressed by neurons (Sierra et al., 2003). In the PNS, StAR is expressed by Schwann cells (Benmessahel et al., 2004). Molecular cloning revealed the existence of a single gene encoding P450scc in the human and rat genome (Chung et al., 1986; Morohashi et al., 1987; Oonk et al., 1990). Immunoreactivity for P450scc was detected in the white matter throughout the rat brain and biochemical experiments have shown the conversion of cholesterol to PREG by primary culture of glial cells (Le Goascogne et al., 1987; Jung-Testas et al., 1989). However, these studies, which were focused only on the brain, did not investigate the presence and activity of P450scc in the spinal cord. Initial data suggesting P450scc gene expression in the spinal cord were provided by Mellon and coworkers who have mainly used

developing rodent embryos (Compagnone et al., 1995). These authors reported the expression of P450scc mRNA and immunoreactivity in sensory structures of the PNS, including the dorsal root ganglion, the trigeminal and facio-acoustic preganglia, and mentioned P450scc existence in the rodent embryo spinal cord ventral horn as data not shown. Expression of this enzyme has also been located in the glial component of peripheral nerves (i.e., Schwann cells) (Schumacher et al., 2004). The first anatomical and cellular distribution of P450scc in the adult rat spinal cord has been provided by Patte-Mensah and coworkers (2003). The highest density of P450scc immunolabeling was found in superficial layers laminae I and II of spinal cord dorsal horn, where sensory neurons are located (Patte-Mensah et al., 2003). Double-labeling experiments revealed that most of the P450scc-positive fibers in the dorsal horn were also immunoreactive for microtubule-associated protein-2, a specific marker for neuronal fibers (Patte-Mensah et al., 2003). Motor neurons of the ventral horn also expressed immunoreactivity for P450scc, suggesting a possible role of the enzyme or its steroid products in the control of motor activity (Patte-Mensah et al., 2003). Moreover, P450scc immunostaining was detected in ependymal glial cells bordering the central canal in the spinal cord, an observation which suggests a possible release of neurosteroids in the cerebrospinal fluid and their involvement in volume transmission mechanisms in the CNS (Patte-Mensah et al., 2003). Furthermore, P450scc-immunoreactive cell bodies were localized in brainstem (fig. 9).

Biochemical experiments showing that homogenates from the adult rat spinal cord and brainstem are capable of converting cholesterol into PREG have demonstrated that P450scc-like immunoreactivity detected in the adult rat brain and spinal tissue corresponds to an active form of the enzyme (Patte-Mensah et al., 2003).

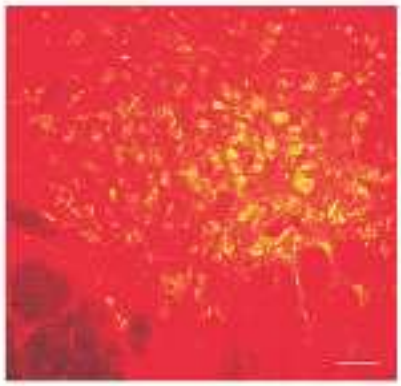


Fig. 9. P450 side chain cleavage-immunoreactive cell bodies in the mice brainstem. Scale bar, 100 μm.

5α-reductase.

The enzyme 5α-R is responsible for the transformation of testosterone and PROG into DHT and DHP, respectively (fig. 8). In a similar manner, 5α-R converts 11-deoxycorticosterone to dihydrodeoxycorticosterone (DHDOC). Two isoforms of 5α-R, designated type 1 (5α-R1) and type 2 (5α-R2), have been cloned in humans and rats (Andersson and Russell, 1990; Andersson et al., 1991; Berman et al., 1993). The genes encoding 5α-R1 and 5α-R2 are located on chromosome 5 and 2, respectively, and the two isoenzymes have different optimal pH and sensitivity to substrates (Normington and Russell, 1992; Wilson et al., 1993). In humans, the 5α-R1gene is predominantly expressed in the skin, notably in the pubic skin and scalp (Andersson and Russell, 1990; Jenkins et al., 1992). The 5α-R2 gene is mainly expressed in the prostate and gonads and its deletion provokes male pseudohermaphroditism (Andersson et al., 1991; Thigpen et al., 1993). In rats, 5α-R1 and 5α-R2 cDNAs have been cloned from a prostate library but the two genes are transcribed in distinct cells: mRNAs encoding 5α-R1 are found in the basal epithelial cells, while 5α-R2 mRNAs are localized in stroma cells (Andersson and Russell, 1990; Andersson et al., 1991; Berman et al., 1993).

The expression of 5α-R in the brain has been extensively studied (Saitoh et al., 1982; Melcangi et al., 1993; Pelletier et al., 1994; Stoffel-Wagner, 2003). It has been suggested that 5α-R1 essentially plays a catabolic and

neuroprotective role whereas 5α-R2 participates in sexual differentiation of the CNS; however, the neurophysiological significance of these two isoenzymes remains a matter of speculation (Celotti et al., 1992; Poletti et al., 1998 a and b; Torres and Ortega, 2003). In contrast to that observed in the brain, the quantity of 5α-R2 mRNAs extracted from the whole adult rat spinal cord is higher than that of 5α-R1 (Pozzi et al., 2003). Immunoreactivity for 5α-R1 and 5α-R2 has been detected in the white matter of the spinal cord, from the cervical to sacral regions. However, the intensity of 5α-R1 immunostaining was low and cell bodies as well as fibers containing this isoenzyme were observed mainly in the white matter of the cervical and thoracic segments. 5α-R2 immunofluorescence, which was moderate in the white matter, was intense in the dorsal and ventral horns of the gray matter (Patte-Mensah et al., 2004). Doubllabeling identification with specific markers for nerve cells revealed that the 5α-R1 immunostaining was mainly expressed in oligodendrocytes and astrocytes of the white matter, whereas 5α-R2-immunolabeling colocalized with neurons and glial cells in the gray and white matters (Patte-Mensah et al., 2004). The observation of a restricted localization of 5α-R1 to the spinal cord white matter is in agreement with its localization in myelinated structures of the female and male rat brain (Melcangi et al., 1988; Poletti et al., 1997).

3β-hydroxysteroid oxido-reductase.

The enzyme 3α-HSOR also called 3α-hydroxysteroid dehydrogenase converts in a reversible manner DHT and DHP into the respective neuroactive steroids 3α-diol and THP (fig. 8). Similarly, DHDOC is converted to tetrahydrodeoxycorticosterone (THDOC). 3α-HSOR is a member of the aldo-keto reductase superfamily which includes aldehyde reductase, aldo reductase and dihydrodiol dehydrogenase (Jez et al., 1997; Jez and Penning, 2001). There are four human 3α-HSOR isozymes, but, to date, only one isoform has been cloned in rats (Pawlowski et al., 1991; Penning et al., 2003). 3α-HSOR enzymatic activity and its encoding mRNA have been detected in the brain (Krieger et al., 1984; Krieger and Scott 1989; Khanna et al., 1995; Stoffel-Wagner, 2003). In the spinal cord, intense immunoreactivity for 3α-HSOR has been detected in the white and gray matters throughout the cervical, thoracic, lumbar and sacral regions. However, the highest density of 3α-HSOR-immunostaining is found in sensory areas of the dorsalhorn (Patte-Mensah et al., 2004). This study also revealed that 45% of 3α-HSOR immunofluorescence was localized in oligodendrocytes, 35% in neurons and 20% in astrocytes. A comparative analysis of 5α-R1, 5α-R2 and 3α-HSOR-positive elements in the spinal cord reveals three different situations: (i) cell bodies and fibers that express both 3α-HSOR and 5α-R; (ii) cell bodies that express either 5α-R1 or 5α-R2 and (iii) cell bodies that express 3α-HSOR only (Patte-Mensah et al.,

2004). Consequently, it appears that certain glial cells and neurons of the spinal cord contain both 5α-R and 3α-HSOR enzymatic proteins, which could catalyze the biochemical reductions required for the biosynthesis of 5α/3α-reduced steroids that control, through allosteric modulation of $GABA_A$ receptors, neurobiological mechanisms including nociception, pain and locomotion (Majewska, 1992; Thomas et al., 1999; Gonzalez Deniselle et al., 2002; Patte-Mensah et al., 2004, 2005 and 2006). The production of 5α/3α-reduced steroids may involve the cooperation of neurons, astrocytes and oligodendrocytes, which contain only one of the two enzymes, i.e., 3α-HSOR or 5α-R. This cooperation may occur during physiological and pathological situations (Melcangi et al., 1998). In addition, 5α-R1 or 5α-R2 may convert PROG or testosterone from peripheral sour ces into DHP or DHT, metabolites that act via genomic receptors, the existence of which has been demonstrated in spinal tissues (Lumbroso et al., 1996; Matsumoto, 1997; Labombarda et al., 2000). In a similar manner, 3α-HSOR may convert, in the spinal cord, DHP, DHT or DHDOC into THP, 3α-diol or THDOC, respectively, for the modulation of $GABA_A$ receptors (Majewska, 1992; Frye, 2001; Reddy, 2003; Lambert et al., 2003). The fact that the rat spinal tissue homogenates are capable of converting [3H]cholesterol into various metabolites including THP clearly indicates that 5α-R1, 5α-R2 and 3α- HSOR detected in the spinal cord correspond to active forms of these enzymes (Patte-Mensah et al., 2003 and 2004). The capability to convert PROG and testosterone into their 5α-(by 5α-

reductase) and 5α/3α-reduced metabolites (by 3α-HSOR) is not restricted to CNS but is also present in peripheral nerves and in Schwann cells (Melcangi et al., 1990; Caruso et al., 2007).

Neuroactive steroid 3α-hydroxy-5α-pregnan-20-one (3α,5α-THP)

Neuroactive steroid 3α-hydroxy-5α-pregnan-20-one (3α,5α-THP) is a potent positive allosteric modulator of $GABA_A$ receptors, synthesized in brain, adrenals and gonads by successive metabolism of progesterone and 5α-dihydroprogesterone (5α-DHP). Physiologically, 3α,5α-THP plays a vital role in stress, pregnancy and CNS neurotransmission (Hiemke et al., 1991; Morrow et al., 1995; Concas et al., 1998). Systemic administration of 3α,5α-THP induces anxiolysis, loss of righting reflex (hypnosis) and anesthesia in rats (Mok et al., 1993; Korneyev and Costa 1996; Brot et al., 1997; Bitran et al., 2000). Recently, it was suggested that 3α,5α-THP also plays an important role during sleep (Damianisch et al., 2001; Muller-Preuss et al., 2002) (fig. 10).

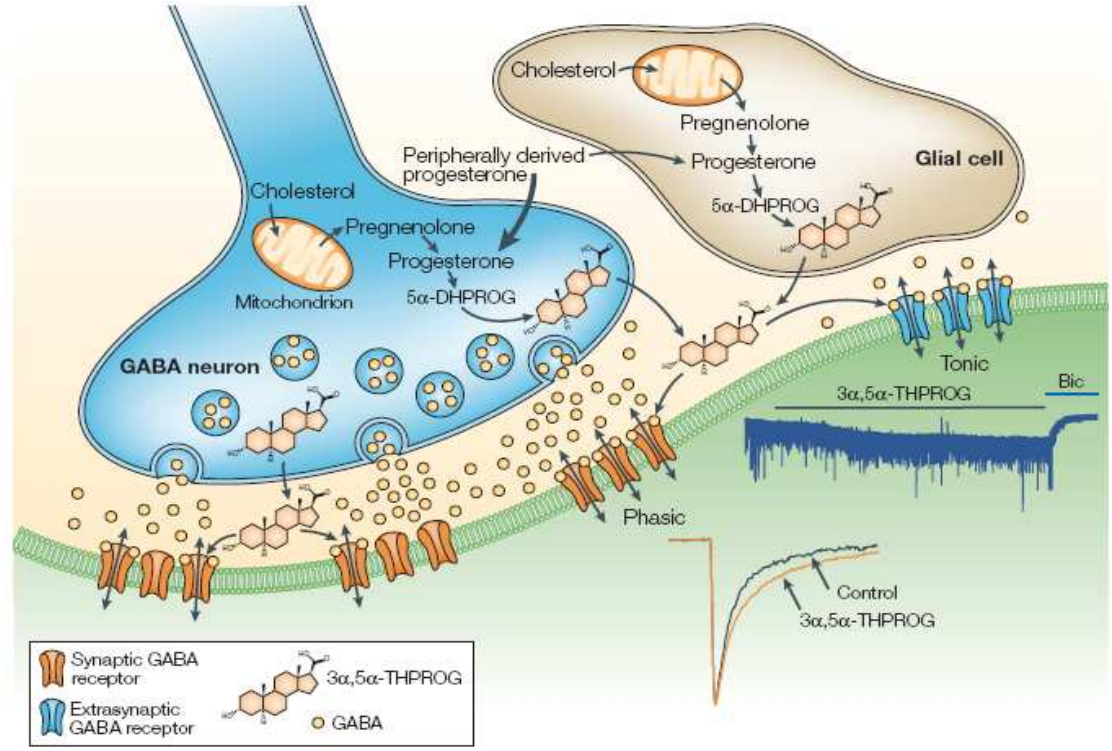


Fig. 10. A diagrammatic representation of an inhibitory synapse and the modulation of neuronal inhibition by locally released neurosteroids. GABA (γ-aminobutyric acid) that is released from vesicles rapidly activates a family of postsynaptic $GABA_A$ (GABA type A) receptors, which gives rise to a transient miniature inhibitory postsynaptic current ('phasic' response). Neurosteroids that are released locally from neurons or glia prolong the decay of such responses, thereby enhancing synaptic inhibition. In addition, certain neurons contain extrasynaptically-located receptors that are activated by low levels of ambient GABA to cause a 'tonic' background current. This manifests under voltage-clamp as a 'noisy' baseline and is uncovered by the application of the $GABA_A$-receptor antagonist bicuculline (Bic), which induces the closure of these extrasynaptic receptors. The current might have a considerable influence on neuronal firing and, in some neurons, is selectively enhanced by low concentrations of neurosteroids that have little effect on the phasic response that is mediated by synaptic receptors. 3α,5α-THPROG, 5α-pregnan-3α-ol-20-one; 5α-DHPROG, 5α-dihydroprogesterone.

Furthermore, an active role of GABAergic neurosteroids in sleep induced by pentobarbital (Matsumoto et al., 1999) and muscimol (Pinna et al., 2000) has been demonstrated. Ethanol administration produces neuropharmacological effects similar to GABAergic neuroactive steroids. Recent studies have demonstrated that acute ethanol administration remarkably increases plasma and cerebral cortical levels of 3α,5α-THP (VanDoren et al., 2000). Similarly, Barbaccia et al. (1999) showed ethanol-induced increases in the cerebral cortical and hippocampal levels of 3α,5α-THP and 3α,21-dihydroxy- 5α-pregnan-20-one (3α,5α-THDOC) in Sardanian alcohol preferring rats compared to non-alcohol preferring rats. This increase in the 3α,5α-THP levels induced by ethanol is sufficient to enhance GABA-mediated neurotransmission in the CNS (Morrow et al., 1987 and 1990). Furthermore, prior administration of the 5v-reductase inhibitor finasteride, that inhibits bio- synthesis of 3α,5α-THP in brain, partially reduced ethanol- induced 3α,5α-THP levels (VanDoren et al., 2000; Dazzi et al., 2002), and prevented anticon- vulsant and inhibitory effects of ethanol on spontaneous neural activity in medial septum/diagonal band of Broca neurons (VanDoren et al., 2000). Various lines of evidence suggest the increase in 3α,5α-THP induced by ethanol contributes to several behavioral effects of ethanol including anticonvulsant properties, loss of righting reflex (VanDoren et al., 2000), reinforcing properties (Janak et al., 1998), discriminative stimulus (Hodge et al., 2001), loss of spatial memory (Matthews et al., 2002), anxiolysis (Morato et al., 2001)

and antidepressant action (Hirani et al., 2002). Differential levels of neuroactive steroids are reported in alcoholic subjects (Romeo et al., 1996). The levels of plasma 3α,5α-THP and THDOC were markedly lower than those of control subjects during the early withdrawal phase (day 4 and 5), when anxiety and depression scores were higher. In contrast, 3α,5α-THP and THDOC plasma levels did not differ from those of control subjects during the late withdrawal phase when anxiety and depression scores were low (Romeo et al., 1996). Hence altered levels of neuroactive steroids might contribute to ethanol withdrawal symptoms.

Sleep-related problems are more common among alcoholics than non-alcoholics. During both drinking periods and withdrawal, alcoholics commonly experience problems falling asleep and decreased total sleep time (Aldrich, 1998; Ehlers, 2000), and high doses of acute ethanol administration can reduce the latency to induce sleep and produce loss of righting reflex (LORR). Acute ethanol administration acts like a hypnotic for insomniacs and appears to be associated with its reinforcing effects (Roehrs, et al., 1999). Since administration of GABAergic neuroactive steroid 3α,5α-THP induces sleep (Lancel et al., 1997; Muller-Preuss et al., 2002) and ethanol administration increases 3α,5α-THP levels, it is possible that elevated 3α,5α-THP may contribute to loss of righting reflex induced by ethanol. Although loss of righting reflex does not represent natural sleep, it has recently been shown that the sedative-hypnotic component (LORR) of GABAergic drugs is mediated by $GABA_A$ receptors in an

endogenous sleep pathway (Nelson et al., 2002). A rapid tolerance to sedative–hypnotic effects develops upon chronic ethanol consumption (Roehrs et al., 2001). Similarly, tolerance to ethanol-induced elevation of 3α,5α-THP in the cerebral cortex has been demonstrated following 14 days of ethanol consumption (Morrow et al., 2001a).

Several studies have demonstrated that systemic administration of 3α,5α-THP potentiates ethanol-induced LORR (Vanover et al., 1999; Czlonkowska et al., 2000 and 2001). We have previously shown that waking cerebral cortical levels of 3α,5α-THP correlate with the duration of ethanol-induced LORR (VanDoren et al., 2000). However, the role of 3α,5α-THP in the hypnotic effects of ethanol is controversial because there are equivocal reports on the effect of 3α,5α-THP biosynthesis inhibitors on ethanol-induced sleep time. Finasteride had no effect on ethanol- induced LORR and did not alter cerebral cortical 3α,5α-THP levels at hypnotic ethanol doses in rats (Morrow et al., 2001b). Recently, Guidotti et al. showed that systemic administration of (17β)-17-[bis(1-methylethyl)aminocarbonyl)]androsta-3,5-diene-3-carboxylic acid (SKF-105,111) a 5α-reductase type-1 inhibitor to mice significantly reduced the duration of LORR induced by ethanol (Guidotti et al., 2001). In contrast, Atzori et al. presented evidence that SKF-105,111 did not modulate ethanol-induced LORR in mice (Atzori et al., 2001). In view of these conflicting reports, the present study systematically examined the effect of adrenalectomy and 5α-reductase inhibition on ethanol-induced increases in

3α,5α-THP levels and LORR in rats. Furthermore, the ability of 5α-DHP to reverse the effect of adrenalectomy on the effects of systemic ethanol administration was studied to determine the role of circulating steroids in ethanol-in- duced LORR. The results of this study clearly demonstrate that GABAergic neurosteroids contribute to the LORR induced by ethanol.

In Vitro Experiments.

Materials.

DMEM F-12, fetal bovine serum (FBS), cell culture media, and supplements were purchased from Cambrex Bio Science Verviers (B-800 Verviers, Belgium). Protease and phosphatase inhibitors (leupeptin, trypsin inhibitor, phenylmethylsulfonylfluoride, Na_3VO_4), trypsin-EDTA solution, anti-β-actin antibody (clone AC-15) were purchased from SIGMA (SIGMA-Aldrich, St. Louis, MO, USA).

GW6471 and PEA were purchased from Tocris Bioscience (Ellisville, Missouri). PEA was first dissolved in absolute ethanol and then diluted with DMEM. The final ethanol concentration was less than 0.5%.

Cell cultures.

The rat C6 cells, originating from a rat brain glioma, was a generous gift from Dr. Jan Cornelis, INSERM 375, Deutsches Krebsforschungszentrum, Heidelberg, Germany.

C6 glioma cells were maintained in DMEM containing 10% FBS supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified incubator under 5% CO2 and 95 % air. Cells were

passaged at confluence using a solution of 0.025% trypsin and 0.01% EDTA and used between passage 54-58.

Primary astrocyte cultures were prepared from new-born BALB/c mice (up to 2 days old) by the method published by Marriot et al., 1995, and adapted from Burudi et al., 1999.

The level of purity was confirmed by performing Western blot analysis on proteins from astrocytes lysates. Antibodies against the scavenger receptor (specific marker for microglia) and glial fibrillary acidic protein (specific marker for astrocytes) were used to detect the relative amount of contaminating microglia in astrocyte cell lysates. More than 95% pure astrocyte cultures were ensured.

Cell treatments and preparation of mitochondrial extracts.

C6 glioma cells or astrocyte primary culture were incubated in serum-free DMEM at 37°C for at least 24 h before each experiment. Then, cells were treated with PEA (10μM), in the presence and absence of GW6471 (10μM) added 30 min prior to ethanolamide treatment. Then cells were incubated for 24 h in serum-free medium. Preparation of mitochondrial protein extracts from C6 or astrocytes was carried out as described by Hartl et al. (1986).

Western blot analysis.

The protein concentration of the extracts were estimated by the Bio-Rad protein assay using bovine serum albumin as standard. Equal amounts of protein (20 or 40 μg) of nuclear and mitochondrial extracts were dissolved in Laemmli's sample buffer, boiled for 5 min, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8% polyacrylamide). Western blotting was performed by transferring proteins from slab gel to a sheet of nitrocellulose membrane at 240 mA for 40 min at room temperature. The filters were then blocked with 5% non fat dried milk in phosphate-buffered saline for 45 min at room temperature and incubated with the specific anti-StAR (1:500; Santa Cruz Biotechnology), or anti-cytochrome P450scc (1:1,000; Chemicon), or anti-PPAR-α (1:500; Santa Cruz Biotechnology), antibody in 1X PBS, 5% non-fat dried milk and 0.1% Tween 20 (PMT) overnight at 4°C. Thereafter, the filters were incubated with the appropriate secondary antibody (anti-mouse or anti-rabbit IgG-horseradish peroxidase conjugates, 1:2,000, Jackson Laboratories) for 1 h at room temperature.

The antibody-reactive bands were revealed by chemiluminescence (ECL). To ascertain that blots were loaded with equal amounts of protein mitochondrial and nuclear lysates were analysed for the expression of hsp60 (Stressgene, 1:10,000) or lamin B1 protein (1:5,000; Sigma), respectively. The protein bands on X-ray film were scanned and

densitometrically analyzed by Model GS-700 Imaging Densitometer.

Quantitative Analysis of Neurosteroids in C6 cell supernatant.

Pregnenolone and allopregnanolone in C6 cell culture supernatants were first isolated by solid-phase extraction (SPE) on C18 columns (Varian) preconditioned with 2 ml methanol and 2 ml of 5% methanol in water. The loaded columns were eluted with 2 ml methanol. Eluates were dried under a nitrogen stream and then derivatized. For the evaluation of recovery, reproducibility and the generation of calibration curves, 500 µl of serum-free DMEM medium used for C6 cell culture was used for each analysis. The response was linear in the range of 0.5 to 32 pg steroids in standard curve. Quantification of neurosteroid in samples is based on chromatographic peak areas of pregnenolone and allopregnanolone relative to their deuterated internal standards at 20pg. Derivatized samples and standard courve were analysed by gas chromatography-mass spectrometry, according to the method of Vallée (2000) with slight modifications. Briefly, neurosteroids were reacted by adding 40µl pyridine and 20µl Florox at 5 mg/ml to the evaporated C6 cell supernatant samples at 70°C for 45 min. The reaction mixture was evaporated off under a stream of nitrogen at 50°C and trimethylsilyl derivatives were formed by adding 15 µl of ethyl acetate and 15 µl of BSTFA and reacted for 20 min at 70°C.

The derivatized samples were injected directly into a Shimadzu GCMS-QP2010 (Shimadzu Corporation, Japan) via autosampler. The instrument was employed in negative ion chemical ionization (NICI) mode with methane as reactant gas (Air Products). A 15-m Rtx-5Sil MS (Restek, Bellefonte, PA) with a 0.25-mm inside diameter and 0.1-µm film thickness was employed for analyte resolution. Helium (ultra-high purity, Linde Gas) at linear velocity set at 60 cm/sec at 160°C was employed as carrier gas. Splitless injections of 1µl volumes were made at injector temperature of 260°C. Splitless injection conditions were maintained for 0.5 min following sample injection. The capillary column temperature was maintained at 160°C for 1 min and the injector contents were split at a ratio of 25:1. One minute after sample injection, the column oven was heated to 230°C at a rate of 60°C/min. The column oven temperature was subsequently raised to 260°C at a rate of 4°C/min and finally to 290°C at 60°C/min. The transfer-line temperature was maintained at 290°C. Derivatized neurosteroids were first analyzed qualitatively by full scan in the mass range of 150-600. For quantification, the mass spectrometer was operated in the selected ion monitoring mode.

Monitoring ions at *m/z* 411 and *m/z* 407 for allopregnanolone-d4/allopregnanolone and *m/z* 566 and *m/z* 563 for pregnenolone-d3/pregnenolone, respectively. The limit of quantification of GC/MS (LOQ) is defined as the signal equivalent to 10 times the noise (S/N of 10:1).

siRNA Treatment.

siRNA-mediated silencing of endogenous expression of PPAR-α in C6 cells was performed using 21 nucleotide siRNA desalted duplexes (Eurogentec, Liege, Belgium). The sequence of sense and antisense oligonucleotides were 5′-GGGUGACAAGUUCAAGCUGtt-3′ and 5′-CAGCUUGAACUUGUCACCCtc-3′, respectively. PPAR-α siRNA was transfected with ICAFectin™ 442 according to the manufacturer's instructions (Invitrogen). Briefly, one day before transfection, C6 cells were resuspended in 12-well plates in appropriate growth medium and grown overnight. Then, the medium was changed to Opti-MEM (Invitrogen) 20 min before transfection. The siRNA complexes were added (20-80 pmol/L) while gently rocking the 12-well plates. Cells were transfected with PPAR-α siRNA for at least 4 h at 37°C before switching to fresh Opti-MEM containing 20% FCS, and subsequently incubated overnight. The medium was then changed to DMEM supplemented with 3% glutamine, antibiotics (see above) and 10% FCS. As a control for PPAR- α, Silencer Negative Control #1 siRNA (Ambion) was also used.

Statistics.

Data are presented as mean ± SEM values of 3 independent determinations. All experiments were done at least three times, each time with three or

more independent observations. Statistical analysis was performed by one-way ANOVA test and multiple comparisons were made by Bonferroni's test. A value of $P<0.05$ was considered significant.

Results.

PEA induces StAR activation and P450scc expression in mitochondrial fraction of C6 cells.

Positioned at the cytosolic aspect of the outer mitochondrial membrane, StAR is functional and exhibit high activity, as 37-kDa preprotein. After its cleavage in mitochondrial membrane contact site, the 30 kDa “mature” StAR is produced to promote cholesterol transfer (Bose et al., 2002 a and b) from the outer to the inner membrane. The cleavage terminates the activity of the protein. Here, we showed that the stimulation of C6 cells with PEA leads to an increase of the mature form of StAR (fig. 11), an indirect rate of cholesterol transfer into the mitochondria.

The reverting effect by a pre-incubation with GW6471, a PPAR-α antagonist, suggests the involvement of this receptor on PEA-induced StAR increase. No effect on StAR was observed when C6 cells were incubated with GW6471 alone.

The net steroidogenic capacity is also determined by the expression of P450scc, which is the first and rate-limiting enzyme in steroidogenesis. PEA induces a significant increase in P450scc expression in the mithocondrial fraction of C6 cells and GW6471, which was unable itself, completely blunted PEA effect (fig. 12).

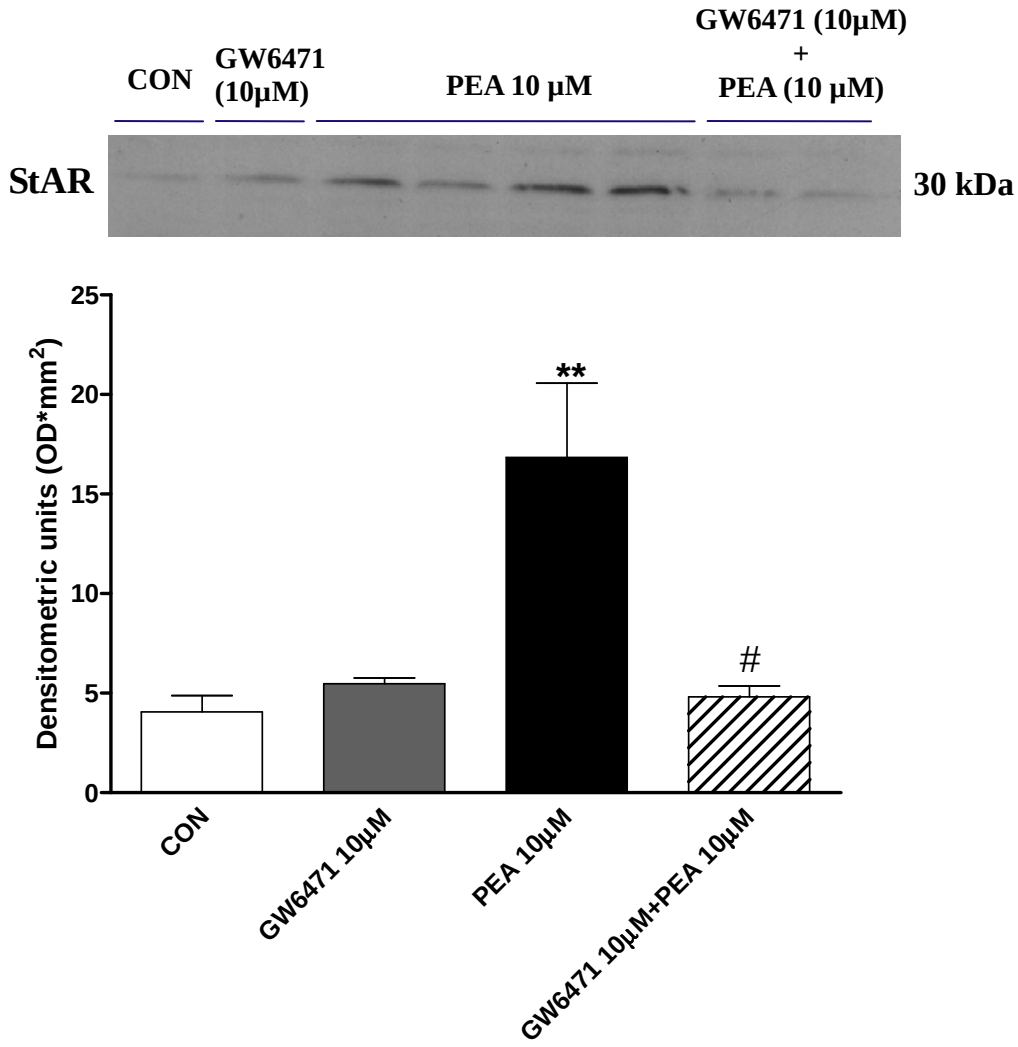


Fig. 11. Effect of PEA (10 µM) on StAR mature form expression in C6 cells pre-treated or not with GW6471 (10 µM). Treatment of cells was performed as described in Methods. Representative immunoblot is shown. The densitometric quantification of all determinations of three independent experiments is also reported. All data are expressed as mean ± SEM. Basal level of protein expression was also reported (CON). **p<0.01 vs CON; #P<0.05 vs PEA alone.

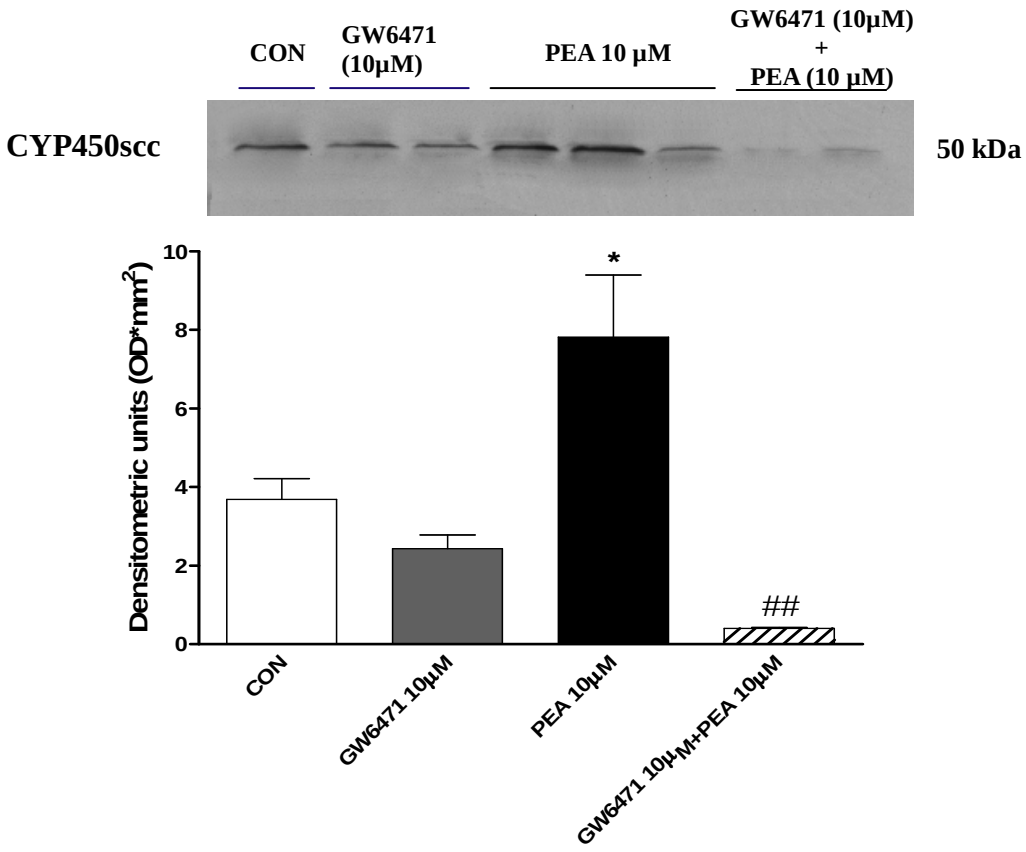


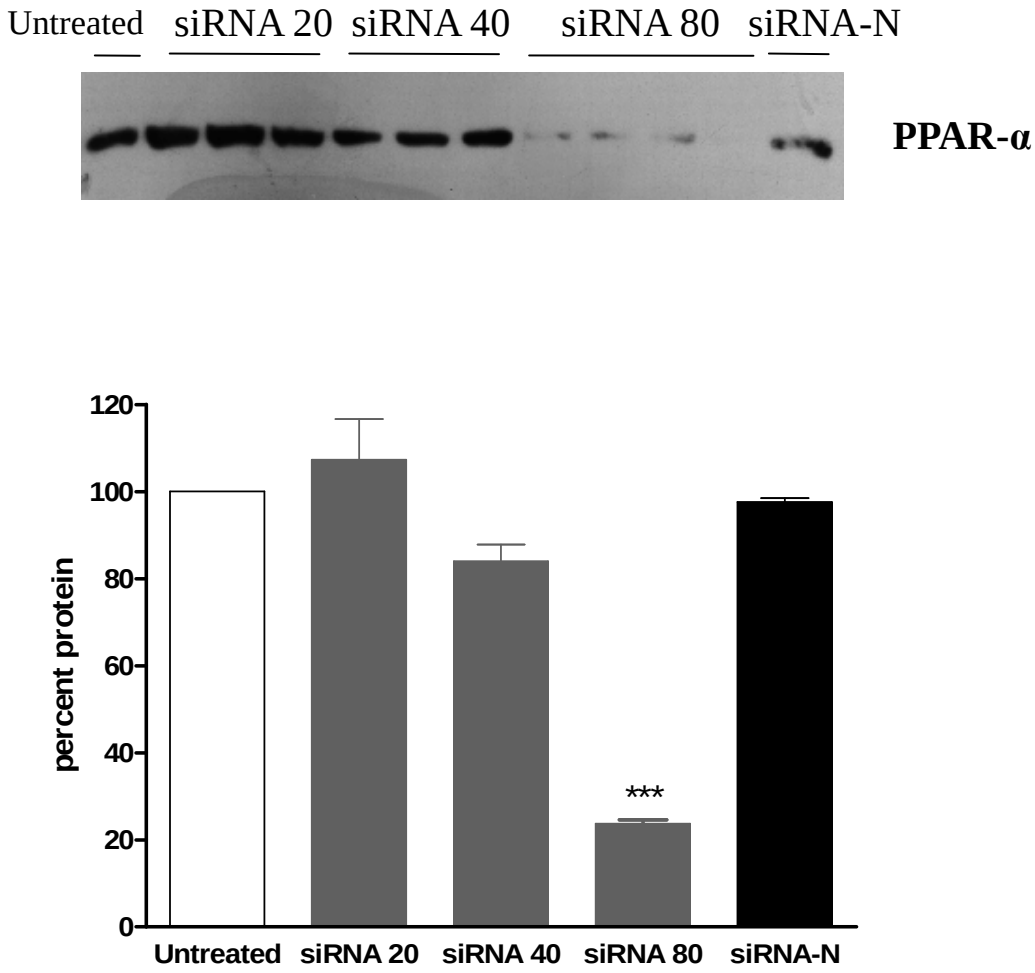
Fig. 12. Effect of PEA (10 μM) on CYP450scc expression in C6 cells pre-treated or not with GW6471 (10 μM). Treatment of cells was performed as described in Methods. Representative immunoblot is shown. The densitometric quantification of all determinations of three independent experiments is also reported. All data are expressed as mean ± SEM. Basal level of enzyme expression was also reported (CON). *P<0.05 vs CON; ##P<0.01 vs PEA alone.

PEA failed in StAR and P450scc modulation in PPAR-ά silenced C6 cells.

To further confirm the involvement of PPAR-ά in PEA modulating effect of steroidogenic proteins StAR and P450scc, this receptor was silenced in C6 cells. As depicted in fig. 13 A, Western blot showed that siRNA concentration-dependently inhibited the constitutive expression of PPAR-α, that was significantly reduced (~80%) at the highest concentration used (80 pmol/L). Silencing negative control did not modify PPAR-α expression.

In PPAR-άknock-down cells the increased amount of StAR and P450scc induced in control cells by PEA, was reduced about 70% and 77%, respectively (fig. 13 B and C).

A


Untreated
siRNA 20
siRNA 40
siRNA 80
siRNA-N
PPAR-α
percent protein
120
100
80
60
40
20
0
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Untreated
siRNA 20
siRNA 40
siRNA 80
siRNA-N

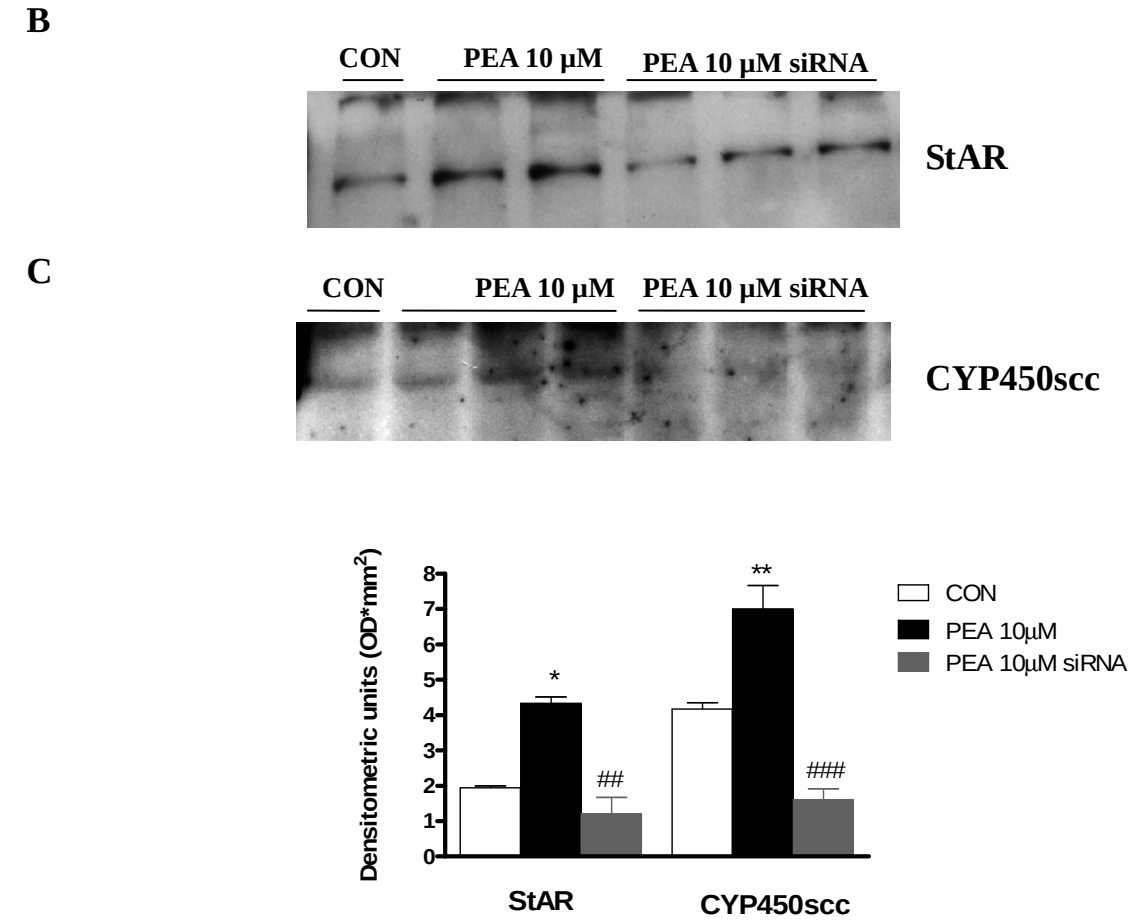


Fig. 13. siRNA knock-down of PPAR-α in C6 cells (panel A). PEA-induced StAR activation (panel B) and CYP450scc expression (panel C) in cells silenced or not for PPAR-α expression.

In panel A the constitutive PPAR-α expression was measured by Western blot analysis in lysates of C6 cells transfected with PPAR-α siRNA at increasing concentrations (20-80 pmol/L). Silencer Negative Control (siRNA-N) was also analysed. Densitometric data are expressd as % of control untreated cells. ***P<0.001 vs untreated cells.

Cells transfected or not with PPAR-α siRNA (80 pmol/L) were then stimulated with PEA (10µM) and StAR mature form (panel B) and CYP450scc (panel C) were analysed. Densitometric units of all determinations are reported. Data represent mean ± S.E.M. *P<0.05 and **P<0.01 vs CON; ##P<0.01 and ###P<0.001 vs PEA.

PEA induces StAR and P450scc in mitochondrial fraction of primary murine astrocytes.

PEA stimulation was also performed on murine primary astrocytes. As expected, PEA induced an increase of both proteins, StAR and P450scc, in the mitochondrial fraction of astrocytes and this effect was reverted by the pre-treatment with GW6471 (fig. 14 A and B). No significant modulation of both steroidogenic proteins was observed after GW6471 incubation alone.

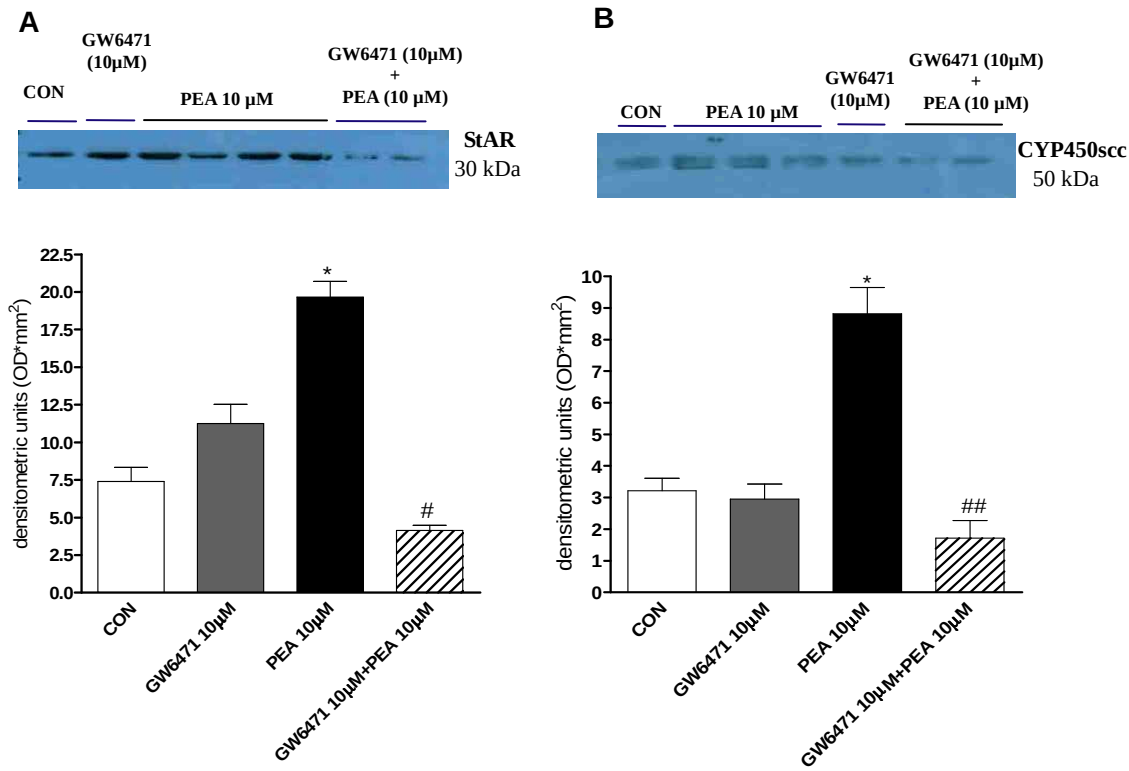


Fig. 14. Effect of PEA (10 μM) on StAR (panel A) and CYP450scc (panel B) expression in primary murine astrocytes pre-treated or not with GW6471 (10 μM). Treatment of cells was performed as described in Methods. Representative immunoblots are shown. The densitometric quantification of all determinations of three independent experiments are also reported. All data are expressed as mean ± SEM. *P<0.05 vs CON; #P<0.05 and ##P<0.01 vs PEA.

PEA induces ALLO synthesis in C6 cell or primary astrocyte supernatant.

Once inside the mitochondrion, cholesterol is the starting material for the synthesis of steroid hormones, oxysterols, and vitamin D, depending on cell type. Here, we measured the amount of ALLO synthesized after 24h PEA stimulation. While pregnenolone is the first metabolite of cholesterol, ALLO is synthesized from progesterone via two enzymatic reactions: 5α reduction of progesterone, yielding dihydroprogesterone, mediated by 5α reductase, followed by 3α reduction of the C3 ketone, mediated by 3α hydroxysteroid dehydrogenase.

ALLO was significantly increased in the supernatant of PEA-stimulated glioma cells or primary astrocytes, and its synthesis was reverted by GW6471 pre-treatment (fig. 15 A and B). No significant effect was induced by GW6471 alone.

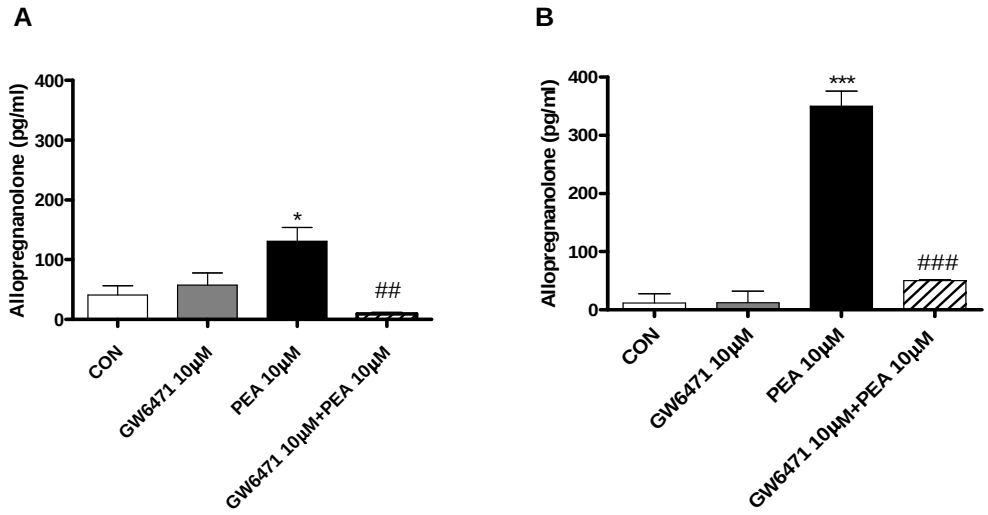


Fig. 15. Effect of PEA (10 μM) on ALLO production evaluated in the supernatant of C6 cells (panel A) or primary murine astrocytes (panel B) pre-treated or not with GW6471(10 μM). Treatment of cells was performed as described in Methods. All data expressed in pg/ml are mean ± SEM of three independent experiments. *P<0.05 and ***P<0.001 vs CON; ##P<0.01 and ###P<0.001 vs PEA.

In Vivo Experiments.

Materials and methods.

Animals.

Male Swiss mice weighing 20 to 25 g were purchasedfrom Harlan (Udine, Italy). They were housed in stainless steelcages in a room kept at 22 ± 1°C on a 12/12-h light/darkcycle. The animals were acclimated to their environment for1 week, and they had ad libitumaccess to tap water and standardrodent chow.

Mice (4–5weeks old; 20–22 g) with a targeted disruption of thePPAR-α gene (PPAR-α knockout) and their wild-type littermate controls (PPAR-α wild type) were purchased from Jackson Laboratories (HarlanNossan, Italy). Mice homozygous for the Ppara^{tniJGonz}-targetedmutation are viable, fertile, and they seem normal in appearanceand behaviour (Genovese et al., 2005). Animal care was in compliancewith Italian regulations on protection of animals used for experimentaland other scientific purposes (D.M. 116192) as well as with European Economic Community regulations (O.J. of E.C. L 358/112/18/1986).

Materials and treatments.

Pentobarbital sodium was purchased from Carlo Sessa S.p.A. (Italy), palmitoylethanolamide (PEA), GW7647, GW0742 and ciglitazone were purchased from Tocris Cookson Ltd. (UK); aminoglutethimide (AMG), 3α-hydroxy-5α-pregnan-20-one (allopregnanolone, ALLO), charybdotoxin and iberiotoxin were purchased from Sigma-Aldrich (Italy); finasteride (FIN) was procured from Steraloids (Newport, RI); Rimonabant, CB1 antagonist, was a generous gift from Sanofi (France). For quantitative analysis of allopregnanolone, deuterium-labeled standards (≥ 98% chemical purity) 3α-hydroxy-5α-pregnan-20-one-d4 (allopregnanolone-d4) was a gift from Dr. Purdy (The Scripps Research Institute, La Jolla, USA). Derivatizing agents including pyridine, O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (Florox) and N,O-bis(trimethylsilyl)trifluoroacetamide with 1% Trimethylchlorosilane (BSTFA+TMCS) were purchased from Sigma-Aldrich, (Italy).

Pentobarbital (20 mg/kg) was dissolved in saline and injected into the caudal vein (i.v.). Just before pentobarbital injection, PEA, GW7647, GW0742, ciglitazone, rimonabant, FIN and AMG or vehicle were dissolved in apirogen and distilled water with PEG (10%)/Tween 80 (5%). Charybdotoxin and iberiotoxin were dissolved in distilled water. Drugs were injected intracerebroventriculary (i.c.v.) in a constant volume of 2µl per mouse, using a 25-µl glass Hamilton which needle was modified with a

shaft, so to control injection depth at 2 mm, according to the method of Haley and McCormick (1957) (2mm lateral, 2mm caudal to bregma and 2 mm deep).

Measurement of pentobarbital-induced LORR.

In order to evaluate the potentiation effect of hypnosis induced by pentobarbital, mice were randomly divided into groups of 10 animals and each group was i.c.v. injected with drugs tested just before the sodium pentobarbital (20 mg/kg) administration into the tail vein. The control group animals were i.c.v. injected with 2µl of vehicle. LORR time was defined as the loss of righting reflex, while time to recovery of this reflex determined the hypnosis endpoint. The time from the loss of righting reflex to spontaneous recovery was considered as the LORR duration (Matsumoto et al., 1991).

Electroencephalographic recording (EEG).

Mice were chronically implanted with five electrodes for EEG recordings and a guide cannula for i.c.v. administration under chloral hydrate anaesthesia (40 mg/kg i.p.; Carlo Erba, Milan, Italy), using a Kopf

stereotaxic instrument (according to the brain atlas of Paxinos and Franklin, 2001).

Electrode leads were soldered to a miniature connector, which was head-mounted with cranioplastic cement and mounting screws. After surgery, animals were allowed to recover for at least one week before EEG recording. To habituate animals to the recording conditions, mice were connected to the recording cables for at least 3 days before the experiments. Animals were attached to a multichannel amplifier (Astro-Med, West Warwich, USA) by a flexible recording cable and an electric swivel, fixed above the cages, permitting free movements of the animals.

Mice were randomly divided into groups of 6-8 animals. Two groupsreceived PEA (1 or 3µg/2µl i.c.v.), while the control group was i.c.v. injected with 2µl of vehicle, than, 30 min after, all groups received the i.p. injection of pentobarbital (40 mg/kg).

Every recording session lasted from a minimum of 2 to a maximum of 5 hours: 1h baseline without injection, and after LORR induction the recordings always lasted up to 1 hour following awakening; in the groups pre-treated with PEA 30 extra minutes were recorded after baseline recordings and before pentobarbital.

EEG was high-pass filtered with a 0.5-Hz half-amplitude filter (6 dB/octave) and low-pass filtered with a 35-Hz half-amplitude filter (12 dB/octave). EEG channels were analog-to-digital converted to a samplingrate of 200 Hz.

Rotarod test.

To evaluate motor neurological deficits of mice, we used the rotarod test, which measures balance, coordination and motor control. The rotarod apparatus (Ugo Basile, Comerio, Italy) consists of a suspended rod able to run at constant or accelerating speed. All mice were exposed to a 5-min training period, at constant speed (4.5 rpm), immediately before the test to familiarize them with the apparatus. Treated mice were randomly divided into three groups of 6 animals and each group was i.c.v. injected only with PEA (1, 3 and 5 µg/2 µl). Control group were i.c.v. injected with 2µl of vehicle. During the test the mice had to remain on the rod for as long as they could. The length of time that the animal remained on the rod was recorded (a 60 s maximal trial was used for the test) 30 and 60 min after administration of PEA.

Preparation of total tissue protein extracts from brainstem.

In another set of experiments, brainstem obtained from 3 animals at different time points (15min. And 30min. after PEA and pentobarbitaladministration), were homogenized on ice in lysis buffer (10 mM Tris-HCl, 20 mM pH 7.5, 10 mM NaF, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulphonyl fluoride, 1 mM Na3VO4, leupeptin and

trypsin inhibitor 10 µg/ml; 0.25 ml per 50 mg tissue). After 1 h, tissue lysates were centrifuged at 100,000xg for 15 min at 4°C, and supernatant was stored at -80° until use. All the extraction procedures were performed on ice using ice-cold reagents.

Western blot analysis.

Protein content was measured using bovine serum albumin (Sigma Aldrich) as a standard. Total extract (for StAR and P450scc) containing equal amount of protein were separated on sodium dodecyl sulfate-polyacrylamide minigels and transferred onto nitrocellulose membranes (Protran Nitrocellulose Transfer Membrane Schleicher & Schuell Bioscience, Dassel, Germany), blocked with phosphate-buffered saline (PBS) containing 5% non-fat dried milk for 45 min at room temperature, and incubated at 4°C, overnight in the presence of commercial antibodies for StAR (Santa Cruz Biotechnology; dilution 1:500); or P450scc (Chemicon International Antibodies; dilution 1:500) in PBS containing 5% non-fat dried milk, 0.1% Tween 20. The secondary antibody (anti-mouse IgG, or anti-rabbit IgG, or anti-goat peroxidase conjugate) was incubated for 1 h at room temperature. Blots were washed with PBS, developed using enhanced chemiluminescence detection reagents (Amersham Pharmacia, Piscataway, NJ) following manufacturer's instructions, and exposed to X-

Omat film (Eastman Kodak Co., Rochester, NY). Protein bands for StAR (~30 kDa) and P450scc (~50 kDa), were quantified using a model GS-700 imaging densitometer (Bio-Rad Laboratories). alpha-tubulin protein (dilution 1:1000; Sigma-Aldrich, Milan, Italy) was performed to ensure equal sample loading.

Quantitative Analysis of Neurosteroids.

ALLO in brainstem samples was isolated by solid-phase extraction (SPE). Briefly, 10 to 50 mg of tissue samples were spiked with 20 pg of deuterium-labelled standards and applied onto 100 mg C18 columns (Varian C18 cartridges) preconditioned with 2 ml methanol and 2 ml of 5% methanol in water. The loaded columns were washed with 2 ml of 5% methanol in water followed by 2 ml of 50% methanol in water. ALLO was eluted with 2 ml methanol into 5 ml glass vials. The methanol eluate was dried under a nitrogen stream prior to derivatization. For the evaluation of recovery, reproducibility and the generation of calibration curves, 200 µl of a brainstem (20 mg of tissue) was used for each analysis. The pool containing increasing known quantities of ALLO standard ranging from 0.5 to 32 pg was spiked with 20 pg of deuterated standard and SPE extraction procedure was performed as described above. The response was linear in the range of 0.5 to 32 pg, standard curve generated with 20 pg of internal

standard was interpolated for quantization of neurosteroid in samples. Dried methanol extracts of biological samples and standards were derivatized for capillary gas chromatography-mass spectrometric analysis according to the method of Vallée (2000) with slight modifications. Briefly, neurosteroids were reacted by adding 40µl pyridine and 20µl Florox at 5mg/ml to the evaporated brain and spinal cord samples at 70°C for 45 min. The reaction mixture was evaporated off under a stream of nitrogen at 50°C and trimethylsilyl derivatives were formed by adding 15µl of ethyl acetate and 15 µl of BSTFA and reacted for 20min at 70°C.

The derivatized saples were injected directly into a Shimadzu GCMS-QP2010 (Shimadzu Corporation, Japan) via autosampler. The instrument was employed in negative ion chemical ionization (NICI) mode. The ion source temperature was mantained at 200°C, and the ion source pressure was maintained at 0.7-0.8 Torr with CH4 as reactant gas methane (research grade 5.0, 99.9995% Air Products). The emission current was 60µA, and the electron energy set at 70eV. A 15-m Rtx-5Sil MS W/Integra Guard capillary columns (Restek, Bellefonte, PA) with a 0.25-mm inside diameter and 0.1-µm film thickness was employed for analyte resolution. Helium (ultra-high purity, Linde Gas) at linear velocity set at 60 cm/sec at 160°C was employed as carrier gas. Splitless injections of 1µl volumes were made at injector temperature of 260°C. Splitless injection conditions were maintained for 0.5 min following sample injection. The capillary column temperature was maintained at 160°C for 1 min and the injector contents

were split at a ratio of 25:1. One minute after sample injection, the column oven was heated to 230°C at a rate of 60°C/min. The column oven temperature was subsequently raised to 260°C at a rate of 4°C/min and finally to 290°C at 60°C/min. The transfer-line temperature was maintained at 290°C. Derivatized neurosteroids were first analyzed qualitatively by full scan in the mass range of 150-600. For quantification, the mass spectrometer was operated in the selected ion monitoring mode.

The limit of quantification of GC/MS (LOQ) is defined as the signal equivalent to 10 times the noise (S/N of 10:1). However, the 10:1 S/N definition of LOQ does not address accuracy. We then, include accuracy in the LOQ by measuring the lowest amount of neurosteroid by GC/MS with a minimum error of +/- 20% in triplicate standard samples (Vallée, et al 2000).

Statistical Analysis

Data experiments were analyzed with t-test or one-way analysis of variance (ANOVA) followed by Dunnett's test. The significance of differences among groups was determined by two-way analyses of variance (ANOVA) followed by Bonferroni post hoc test for multiple comparisons.

The EEG signals were amplified and converted into digital values using a (FFT); spectral powers were then digitally calculated in real time and their

densities integrated and averaged every 30 min (60 epochs). From FTT Discrete Fourier Transform (DFT) was efficiently and rapidly calculated for analysis of dominant frequency. Quantitative changes in EEG power for each of 4 frequency bands, [δ (0.5-4 Hz), θ (4-8 Hz), α (8-13 Hz) and β (13-18Hz)] were analyzed in the power spectrum of each 30 min from mice administrated with various treatment with respect to the their control period (Deboer et al., 2007). To quantify the changes induced by pentobarbital, or its combination with PEA, of total voltage power and preselected bands of frequency, the values obtained as V2/sby FFT, during periods of 30 min, were converted as percentage of change in comparison to the same interval referred to the baseline period. In addition, to reduce possible inter-animal variations of the baseline electrocortical activity and single frequency bands in the same group, the percentage changes after treatment with drugs were compared to the values for the corresponding period before treatment, using paired Student's t-test. (De Sarro et al., 1990; 1995; 2000). Difference with $P<0.05$ was considered statistically significant.

Results.

Effects of PPAR-α agonists on pentobarbital-induced LORR.

In order to evaluate the effect of endogenous PPAR-α agonists (PEA) and synthetic (GW7647) on hypnosis induced by pentobarbital LORR time was determined. Both agonists increase LORR time in a dose-dependent manner. In fact, this effect was significant in a range of 2-5 μg/mouse ($p<0.01$; fig. 16 A and B), in particular at 3 μg/mouse, the LORR duration was two fold increased respect to vehicle. Thus to determine the selective involvement of PPAR-α in the increase of LORR duration, we used ciglitazone, a PPAR-γ agonist, and GW0742, a PPAR-δ agonist, even at higher doses than those used for PPAR-α agonists. Both drugs at all doses tested (1-10 μg/2 μl/mouse), did not shown effect on pentobarbital-induced LORR (fig. 16 C and D), indicating the pivotal role of PPAR-α receptors.

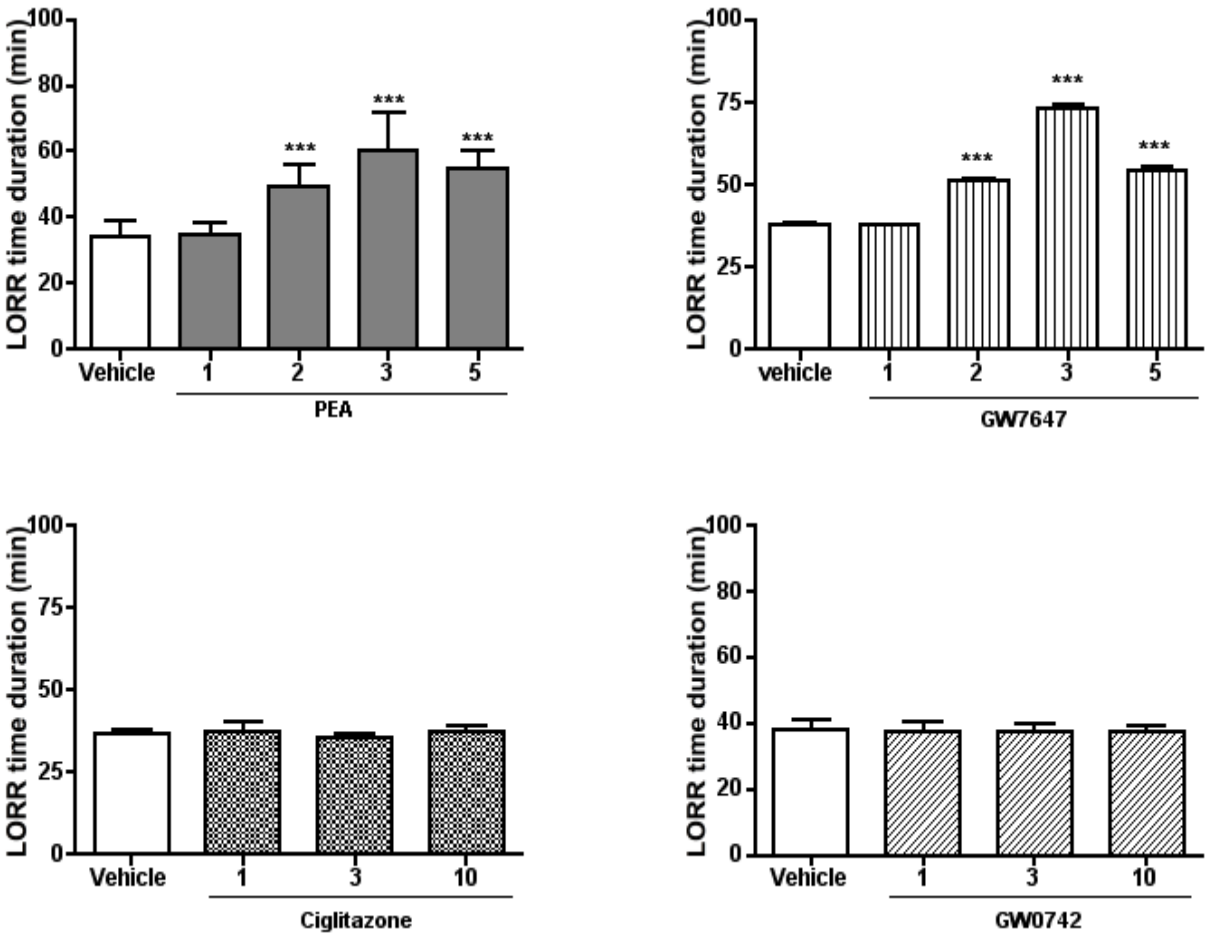


Fig. 16. Effect of PPAR agonists on pentobarbital-induced LORR: PEA (PPAR-α endogenous agonist, 1-5 μg/2μl, panel A), GW7647 (PPAR-α synthetic agonist, 1-5 μg/2μl, panel B), ciglitazone (PPAR-γ agonist, 1-10 μg/2μl, panel C) and GW0742 (PPAR-β/δ agonist,1-10 μg/2μl, panel D) were i.c.v. injected just before pentobarbital administration (20 mg/kg, iv). After injecting pentobarbital, LORR time was measured in mice (n=6, each group) and expressed as mean ± SEM.

Control animals received i.c.v. vehicle (10% PEG, 5% Tween 80 in distilled water); *** $p<0.001$ versus vehicle.

To confirm the involvement of PPAR-α receptor in PEA and GW7647 modification of the hypnotic effect of pentobarbital, we tested the most active dose of PEA and GW7647 (3 μg) on wild-type (+/+) and PPAR-α knockout (-/-) mice (fig. 17). No differences in LORR time induced by pentobarbital was evidenced between wild-type and knockout mice, however the increased LORR time induced by PPAR-α agonists (PEA and GW7647) was evidenced only in wild-type mice (fig 17 A and B), whereas disappeared in knockout mice (fig 17 C and D).

Meanwhile, also other nuclear receptors belonging to the class of PPAR have been reported in CNS, we decided to determine if PPAR-γ and PPAR-δ agonist could increase pentobarbital-induced LORR time, as endogenous PPAR-α agonist PEA does.

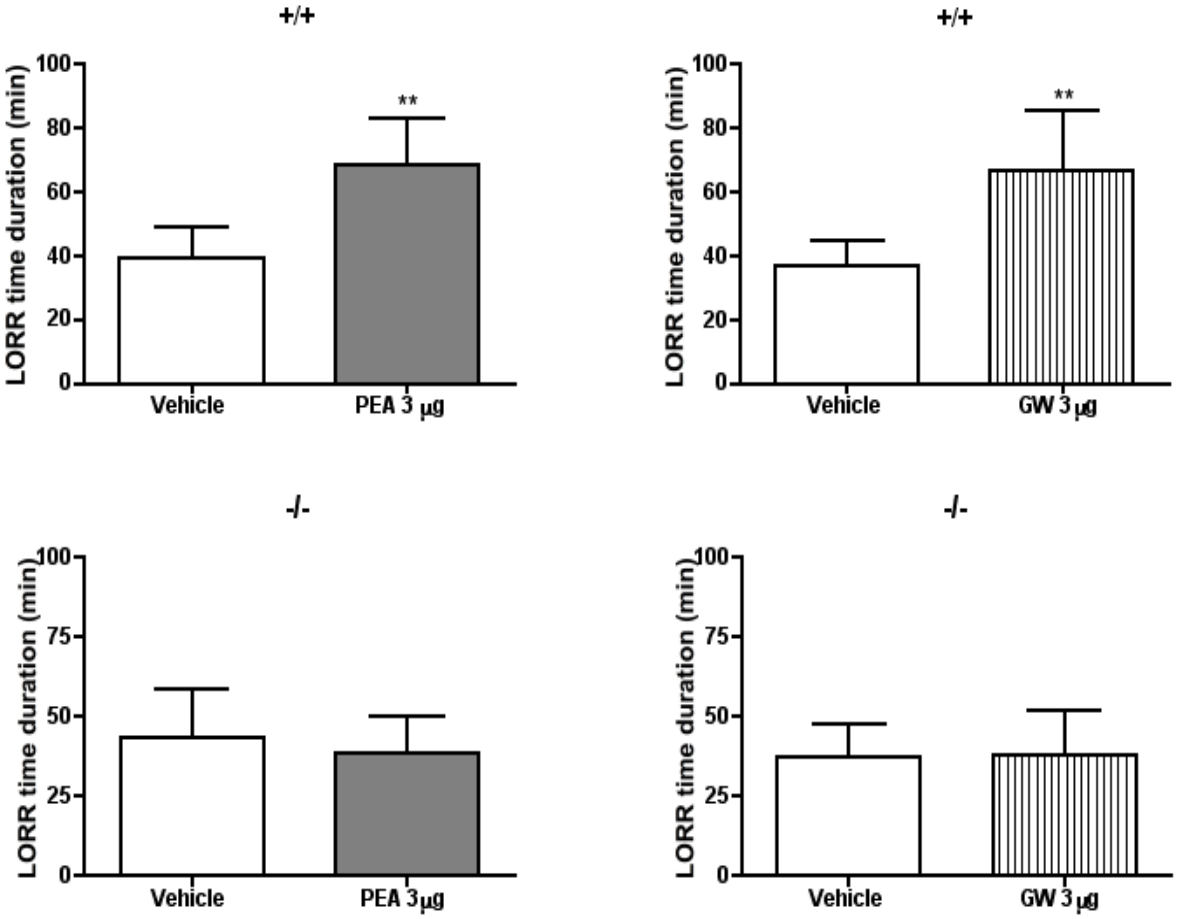


Fig. 17. Effects of PPAR-α agonists (PEA and GW7647) on pentobarbital-induced LORR time in wild-type (+/+) and PPAR-αknockout (-/-) mice. PEA (3 µg/2µl) or GW 7647 (3 µg/2µl) were i.c.v. injected just before pentobarbital administration in wild-type (+/+) (panel A and B, respectively) or PPAR-α (-/-) (panel C and D, respectively) mice. After injecting pentobarbital, LORR time was measured in mice (n=6, each group) and expressed as mean ± SEM. Control animals received i.c.v. vehicle (10% PEG, 5% Tween 80 in distilled water); **p<0.01 versus vehicle.

Involvement of cannabinoid system in PEA-induced effect on pentobarbital-induced LORR.

Several possible scenarios have been proposed to describe the mechanism of action of PEA and one of this is the indirect action on cannabinoid system. To elucidate the involvment of CB1 receptor we used Rimonabant, a CB1 antagonist. Fig. 18 reports the Rimonabant (i.p.; 1 mg/kg, 30 min before pentobarbital injection) effect on PEA increase pentobarbital-induced LORR time. Rimonabant alone did not modify this parameter and and in combination with PEA did revert its effect.

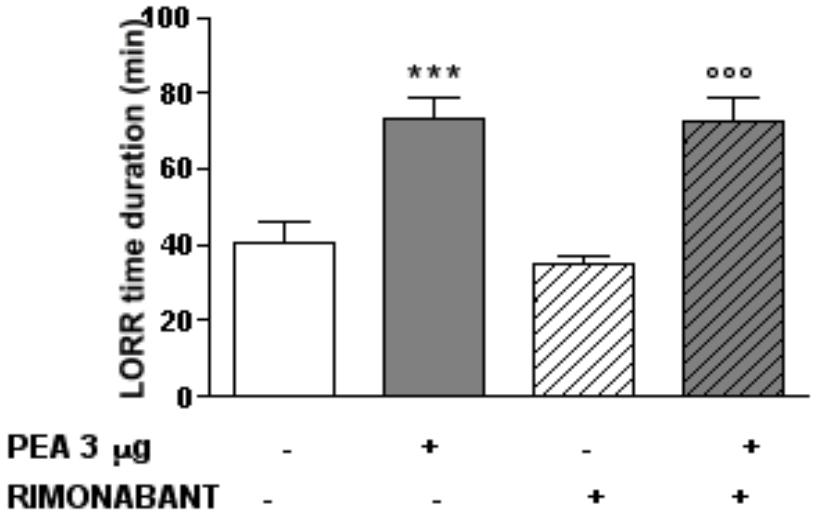


Fig. 18. Effect of CB1 antagonist on PEA-increased LORR time induced by pentobarbital. Rimonabant, a CB1 antagonist, alone (1 mg/kg) or in combination with PEA (3 μg) was i.p. Injected 30 min. before pentobarbital administration. After injecting pentobarbital, LORR time was measured in mice (n=6, each group) and expressed as mean ± SEM. Control animals (open bars) received i.c.v. vehicle just before pentobarbital administration (10% PEG, 5% Tween 80 in distilled water); *** $p<0.001$ and °°° $p<0.001$ versus vehicle.

Effects of PEA on behavioural and EEG LORR induced by pentobarbital.

To exclude that PEA *per se* could induce motor neurological deficits, PEA (1 and 3µg/2µl) was given i.c.v. in mice (n=6-8 miœ for each group). The treatment did not produce any noticeable change in locomotor activity and EEG parameters during an observation time of 150 min.

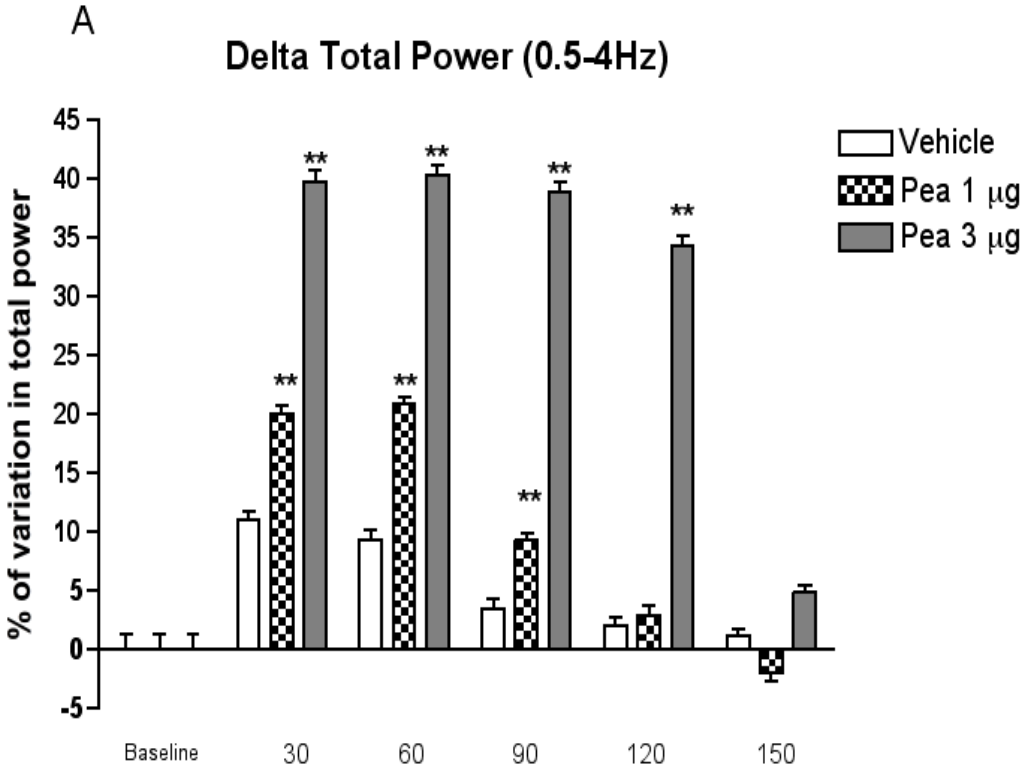
Pentobarbital (40 mg/kg), injected i.p. induced behavioral and EEG signs with a mean latency of 167 ± 24 sec; the LORR state lasted for an average of 55±6 min (n=6), considered as complete immobility of the animal accompanied by a typical slow wave activity on the EEG. FFT analysis of EEG recordings revealed a significant time-dependent modifications in total voltage power (10-20%), as well as in the 0.5-4 Hz band (delta ~10%), a small no significant increase in the 13-18 (beta ~5%) and a significant decrease in the 8-13 (alpha ~10%) Hz frequency bands. No significant changes in the 4-8 Hz (theta) frequency band were observed (fig. 19).

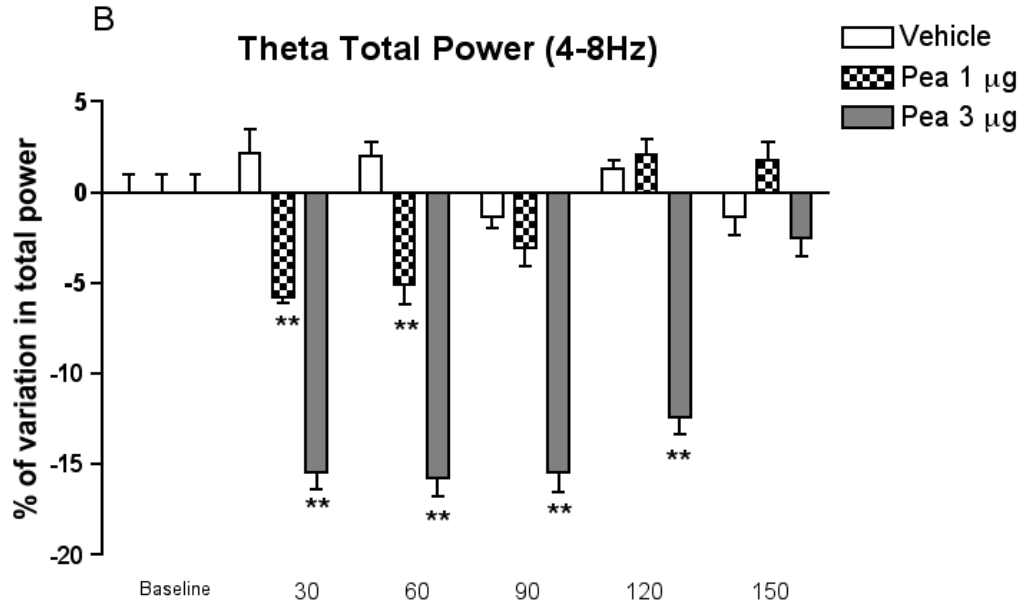
Pretreatment with PEA (i.c.v.; 1 or 3µg/2µl, 30 min before pentobarbital administration) in mice, enhanced the behavioral and EEG effects induced by pentobarbital (40 mg/kg, i.p.).

The LORR state was significantly prolonged to 69.6±6 min after the combined treatment with PEA (1µg/2µl) and 115±10 min after PEA (3µg/2µl). During the combined treatment, EEG recordings were characterized by an evident increase in slow wave activity; FFT analysis

revealed a significant increase in total voltage power in comparison to control animals. It was more pronounced and significantly different (~25% PEA 1µg/2µl; ~35% PEA 3µg/2µl) when compared to vehicle (10-20%) administration.

A statistical comparison between combined treatment and pentobarbital alone revealed a significant dose-dependent increase of delta (0.5-4Hz; Fig 19 A), beta (13-18Hz; Fig. 14 D) band and a significantly lowering of theta and alpha total power (fig. 19 B and C, respectively) for PEA pre-treated animals, at both doses.


A
Delta Total Power (0.5-4Hz)
% of variation in total power
45
40
35
30
25
20
15
10
5
0
-5
Baseline
30
60
90
120
150
Vehicle
Pea 1 µg
Pea 3 µg
**


B
Theta Total Power (4-8Hz)
% of variation in total power
5
0
-5
-10
-15
-20
Baseline
30
60
90
120
150
Vehicle
Pea 1 µg
Pea 3 µg
**

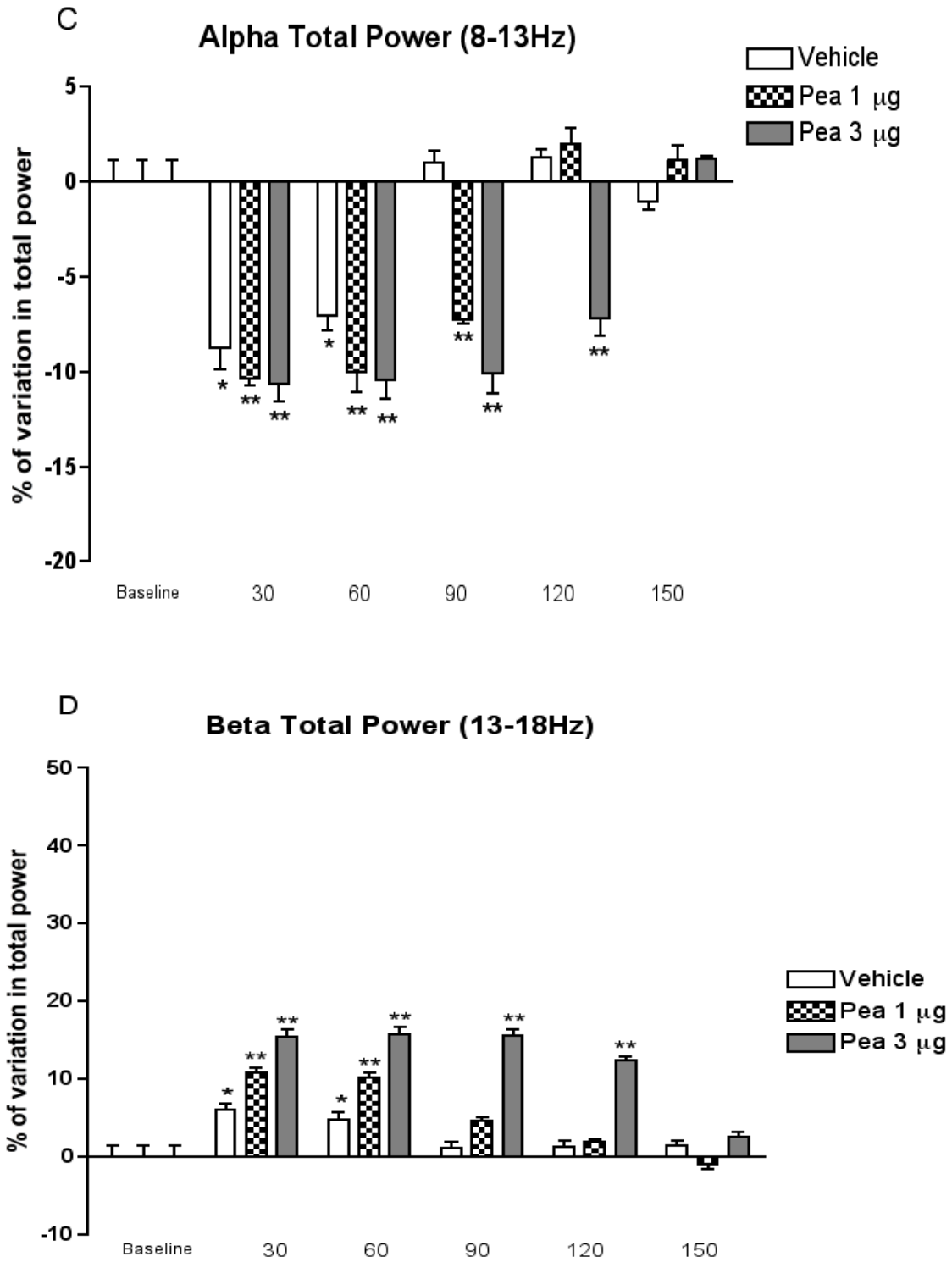


Fig. 19. Elettroencephalografic effect of PEA (1 and 3 μg/2μl) on pentobarbital induced LORR. PEA was i.c.v. injected 30 min. before pentobarbital administration (40 mg/kg, ip.). Control animals received i.c.v. vehicle (10% PEG, 5% Tween 80 in distilled water). Modifications of alpha, beta, delta and theta frequency bands were reportedas % of total variations of total power; * $p<0.05$ and ** $p<0.01$versus vehicle.

Effects of PEA on performance in the rotaroad test.

To evaluate the possible muscle relaxant or sedative activity of PEA, we used a the rotaroad test. PEA showed no reduction in the permanence (time in seconds) of the mice on the apparatus 30 min. and 60 min. after the administration of all doses used (Table 1), suggesting the absence of sedative effects.

TREATMENT	n	30 min.	60 min.
Vehicle	10	56.10 ± 4.70	55.80 ± 5.00
PEA 1 µg	10	55.30 ± 5.41	54.80 ± 4.54
PEA 3 µg	10	56.80 ± 5.36	55.70 ± 5.42
PEA 5 µg	10	57.00 ± 6.30	59.90 ± 4.73

Table 1: Effect of PEA (1-5 µg/ 2µl/ mouse) on rotarod test. The length of time that the animal remained on the rod was recorded 30 and 60 min after administration of PEAand expressed as mean ± SEM. Control animals received i.c.v. vehicle (10% PEG, 5% Tween 80 in distilled water).

Involvement of neurosteroids in PEA-induced effect on pentobarbital-induced LORR.

To explore the role of neurosteroids on the increased LORR time induced by pentobarbital, we used the inhibitors of two key enzymes of neurosteroidogenesis, AMG and FIN.

AMG, an inhibitor of P450scc, blocks the conversion of cholesterol to pregnanolone, the first biosynthetic step of neurosteroid cascade. FIN, is an inhibitor of 5α reductase, a key enzyme for the 5α reduced neurosteroid production, such as ALLO. Both inhibitors caused a significant dose/response reduction of pentobarbital-induced LORR time at the dose of 3-10 μg/2μl i.c.v. (fig. 20). For this reason we chose the dose of 1 μg/2μl, that it was ineffective *per se* on pentobarbital-induced LORR time by itself, in order to verify the involvement of steroidogenic pathway on PEA-induced increase of LORR time. Fig. 21 A and B reports the AMG and FIN effects on PEA increase pentobarbital-induced LORR time, respectively. AMG and FIN alone did not modify this parameter, while they significantly reverted PEA effect ($p<0.01$).

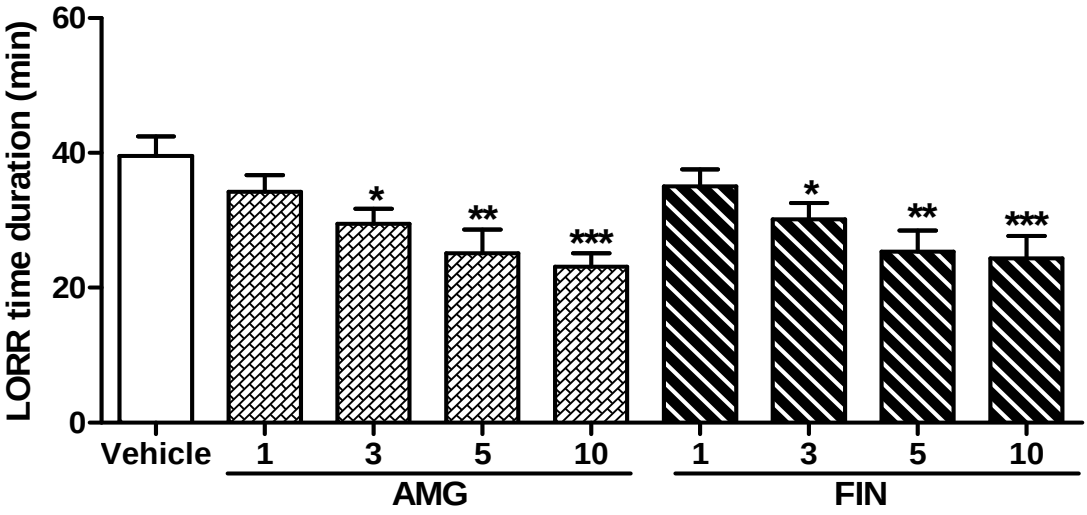


Fig.4

Fig. 20. Effect of AMG (P450scc inhibitor, 1-10 μg/ 2μl/ mouse) and FIN (5-α reductase inhibitor, 1-10 μg/ 2μl/ mouse) on pentobarbital.induced LORR time. AMG and FIN were i.c.v. injected just before pentobarbital administration. After pentobarbital injection, LORR time was measured in mice (n=6, each groups) and expressed as mean ± SEM. Control animals received i.c.v. vehicle (10% PEG, 5% Tween 80 in distilled water) just before pentobarbital administration; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus vehicle.

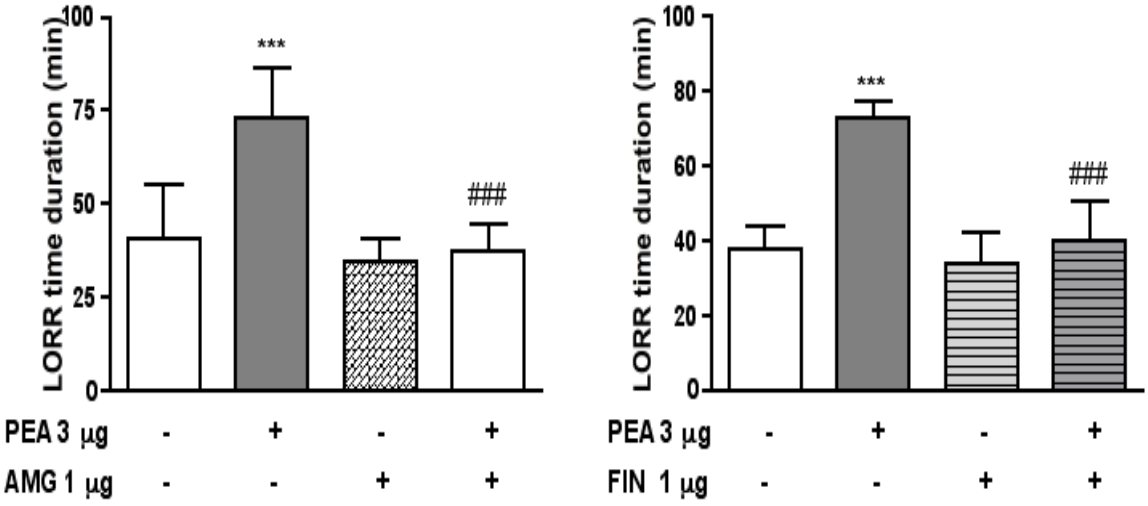


Fig. 21. Effect of neurosteroidogenic enzyme inhibitors on PEA-increased LORR time induced by pentobarbital. Panel A: aminoglutethimide, a P450scc inhibitor, alone (AMG, 1 µg) or in combination with PEA (3 µg) was i.c.v. injected just before pentobarbital administration. Panel B: finasteride, a 5-α reductase inhibitor, alone (FIN, 1 µg) or in combination with PEA (3 µg) was injected just before pentobarbital administration. After injecting pentobarbital, LORR time was measured in mice (n=6, each group) and expressed as mean ± SEM. Control animals (open bars) received i.c.v. vehicle just before pentobarbital administration (10% PEG, 5% Tween 80 in distilled water); *** $p<0.001$ versus vehicle, and ### $p<0.001$ versus PEA.

PEA increases ALLO concentration in the brainstem.

To further investigate if the increase of PEA on pentobarbital-induced LORR involves neurosteroid synthesis, we evaluated the concentration of neurosteroids in the brainstem. Among neurosteroids that might be modulated by PEA, we focused our attention on ALLO, because it is well known to be the most important neurosteroid related to LORR, acting as an allosteric modulator of $GABA_A$ receptor.

We evaluated the ALLO content in this tissue after pentobarbital injection alone or in combination with PEA. PEA (3 μg) significantly increased ALLO concentration ($p<0.01$) both at 15 and 30 minutes after pentobarbital treatment (fig. 22). We chose these point times to compare the ALLO content between vehicle and PEA treated groups. In particular, the second time point (30 minutes) was chosen as last time in which pentobarbital was still active, just before the recovering of righting reflex. Interestingly, a trend increase of ALLO was seen in tissue obtained from animals treated with pentobarbital alone, both at 15 and 30 minutes.

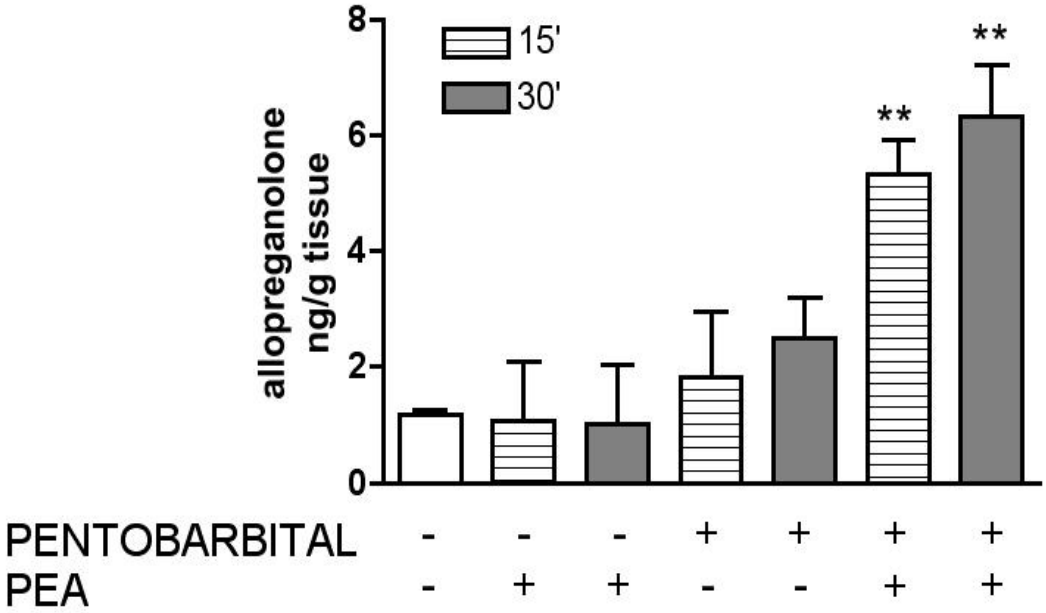


Fig. 22. Brainstem ALLO levels, expressed as ng/g of tissue, were obtained from untreated (naïve, open bar), PEA (3 μg, i.c.v.) and /or pentobarbital treated animals. Brainstems were collected 15 and 30 min after pentobarbital injection or PEA treatment. All data are expressed as mean ± SEM; ** $p<0.01$ versus pentobarbital alone.

Effect of allopregnanolone alone or in combination with PEA on pentobarbital-induced LORR.

The ability of neurosteroids to increase pentobarbital-induced LORR time was determined using exogenous ALLO (1-30 μg, i.c.v.), the principal 5 α–reduced neurosteroid able to modulate $GABA_A$ receptor. At the highest dose used, ALLO significantly prolonged ($p<0.01$) the duration of pentobarbital-induced LORR time (fig. 23 A).

Interestingly, when the inactive doses of ALLO and PEA (1 μg each) were co-injected, an evident synergistic effect was shown, inducing a two-fold increase ($p<0.01$) of pentobarbital-induced LORR duration (fig. 23 B).

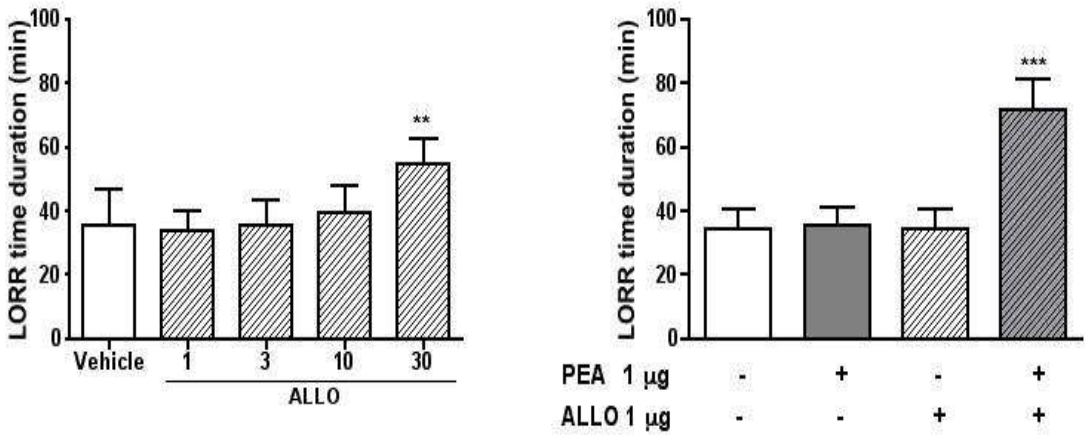


Fig. 23. Effect of ALLO on pentobarbital-induced LORR time: ALLO (1-30 µg/2µl, panel A), positive allosteric modulator of $GABA_A$ receptor, was i.c.v. injected just before pentobarbital administration (20 mg/kg, i.v.). PEA (1 µg/1µl) and ALLO (1 µg/1µl) (panel B) were coadministrated just before pentobarbital injection. LORR time duration was measured in mice (n=6, each group) and expressed as mean ± SEM. Control animals received i.c.v. vehicle (10% PEG, 5% Tween 80 in distilled water) just before pentobarbital administration; ** $p<0.01$ and *** $p<0.001$ versus vehicle.

Effect of Calcium-Activated K^+ Channels (KCa) on PEA increasing pentobarbital-induced LORR time.

To exclude the involvement of KCa channels in PEA increasing pentobarbital-induce LORR time, we used charybdotoxin (1µg/2µl), an inhibitor of large-conductance KCa channels (BKca, KCa1.1) and intermediate-conductance KCa channels (IKCa, KCa3.1) and the selective BKca inhibitor iberiotoxin (1µg/2µl). The administration of two blockers did not modify the effect of PEA, indicating that IKca and BKca channels are not involved (Fig. 24).

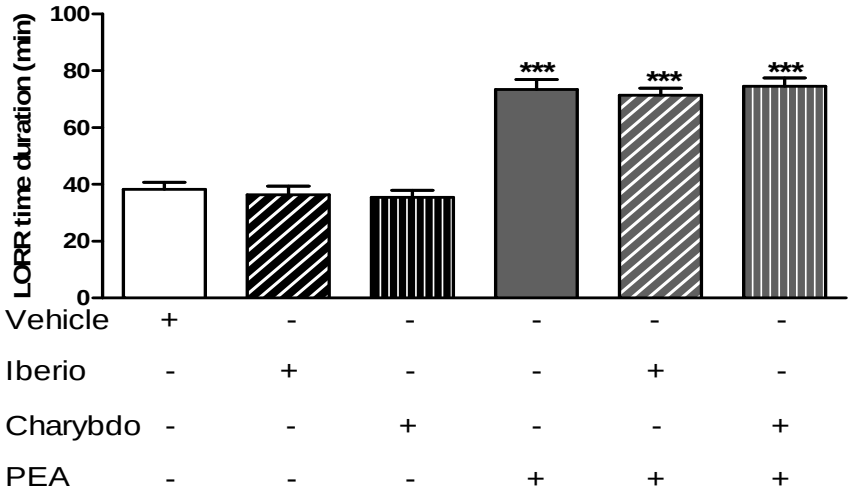


Fig.8

Fig. 24. Effect of Calcium-Activated K^+Channels blockers on PEA increasing pentobarbital-induced LORR time. Charybdotoxin, an inhibitor of BKca and IKca, and the selective BKca inhibitor iberiotoxin, alone (1 μg/ 2 μl/ mouse) or in combination with PEA (3 μg) were i.c.v. injected just before pentobarbital administration. After pentobarbital injection, LORR time was measured in mice (n=6, each groups) and expressed as mean ± SEM. Control animals received i.c.v. vehicle (10% PEG, 5% Tween 80 in distilled water) just before pentobarbital administration; *** $p<0.001$ versus vehicle.

Effect of PEA and GW7647 on StAR and P450scc expression in the brainstem.

To further confirm the involvement of de novo-neurosteroid synthesis in PEA-induced LORR time increase, we evaluated the brainstem-expression of StAR and P450scc, two enzymes that have been implicated in the early step of neurosteroidogenesis. When the animals were treated with pentobarbital and immediately euthanized, no significant difference in basal expression of either enzyme was seen (fig. 25). On the other hand, Western blot analysis revealed an increase of StAR and CYP450scc 30 minutes after co-administration of PEA (3 μg) and pentobarbital (fig. 25 A). Densitometric analysis of all determinations at 15 and 30 minutes was also reported, confirming the augment of both proteins (fig. 25 B and C).

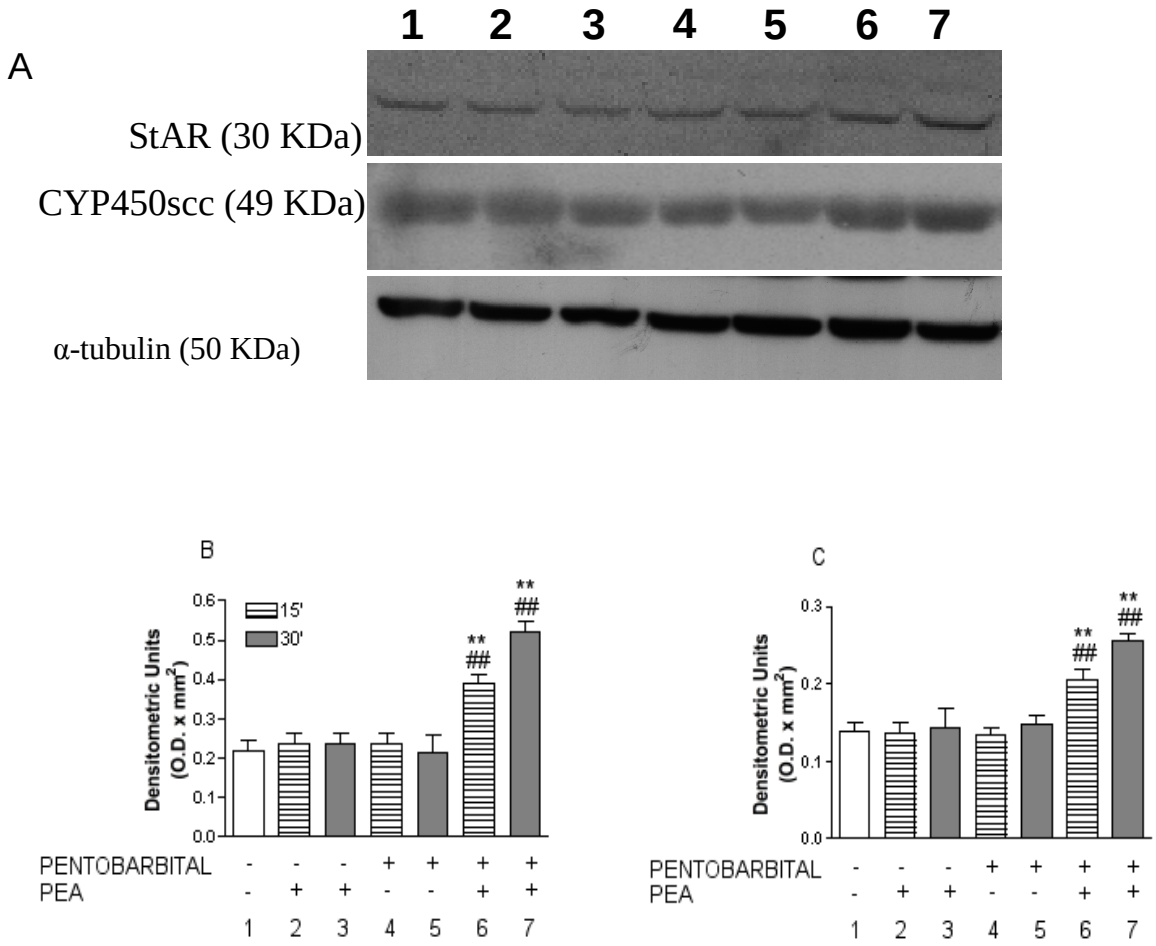


Fig. 25. Expression of StAR and P450scc in the brainstem of animals treated with PEA(3 μg/2μl) and/or pentobarbital. The effect was evaluated 15 and 30 min after pentobarbital or drug injection. A representative immunoblot (panel A) is shown. The densitometric quantification of all determinations (n=6 mice) of StAR and P450scc expression at 15 and 30 min. are also reported (panels B and C): All data are expressed as mean ± SEM. Basal level of enzyme expression was also reported (lane 1). Equal loading was confirmed by alpha-tubulin staining. **p<0.01 versus basal level; ##p<0.01 versus PEA alone.

Discussion.

Palmitoylethanolamide (PEA), a member of the fatty-acid ethanolamide family, acts as an endogenous PPAR-α ligand exerting analgesic and anti-inflammatory effects (Calignano et al., 2001, Lo Verme et al., 2005 and 2006; D'Agostino et al., 2007). Although the presence of PEA (Cadas et al., 1997; Petrosino et al., 2007) and the discovery of PPAR-α receptors in the CNS (Kainu et al., 1994; Moreno et al., 2004) has been reported, their role in these areas remain to be elucidate.

The present study provides evidence that PPAR-α receptor and its putative endogenous ligand, PEA, could regulate neurosteroidogenesis.

After *in vitro* experiments, we report that the incubation of astrocytes with PEA, at higher concentration than physiological levels, enhances the expression of P450scc the first and main steroidogenic enzyme involved in de novo synthesis of neurosteroids, which converts cholesterol to pregnenolone, the precursor of neuroactive hormones. The rate of cholesterol transport from intracellular stores to the inner mitochondrial membrane is another limiting step in pregnenolone formation and involves both PBR and StAR both in peripheral and central tissues (Hauet et al., 2002; Karri et al., 2007).

Differently from steroidogenic enzymes, which have long half-lives and are chronically regulated, StAR protein is not an enzyme, is acutely regulated and its active form is highly labile and must be continuously

synthesized. Several differences were noted between StAR expression and steroidogenesis in astrocytes and other steroid-producing cells. One important difference is that astrocytes in cell culture have significant level of StAR expression in absence of stimulation (Karri et al., 2007). This basal level of StAR can be upregulated by hormone stimulation but this process occurs over a range of hours, and not minutes, such as in various endocrine cells (Stocco, 2001 a). In our experimental conditions, the capability of PEA to modulate cholesterol metabolism not only implies the modulation of P450scc, but also StAR protein activation, increasing both mitochondrial cholesterol availability and its subsequent conversion into pregnenolone. PEA effects on StAR and P450scc are accompanied by a strong increase of ALLO, one of the metabolites of the neuroactive steroid progesterone. Neuroendocrinological studies have demonstrated that a variety of vital neuronal and glial functions are influenced by the autocrine/paracrine actions of locally produced neurosteroids. After brain injury, progesterone and its metabolites can exert protective effects on neurons and glial cells by preventing cerebral edema, necrosis, apoptosis, and inflammation, while enhancing neuroregenerative mechanisms (De Nicola, 1993; He et al., 2004; Djebaili et al., 2004; Shear et al., 2002). In particular, ALLO's neuroprotective and antineurodegenerative effects affect neurogenesis (Brinton and Wang, 2006), and inhibit toxin-induced cell death (Waters et al., 1997). Recently, ALLO was identified as a regenerative factor to promote functional neurogenesis and diminish

Alzheimer's pathology (Wang et al., 2008).

Here, we show that PEA stimulation induces a significant increase in ALLO concentration in C6 and primary astrocyte medium, as quantitatively revealed by gas chromatography-mass spectrometry analysis. The appreciable level of StAR in untreated cells suggest that this protein has a basal physiological activity in astrocytes, according with previous findings of Karri et al. (2007). We also observed that these cells required a long time period (24 hr) to induce a detectable release of ALLO from untreated or PEA-stimulated astrocytes, suggesting that the regulation of neurosteroid synthesis in astrocytes is mediated by StAR, but with a slower time course of action, as it occurs in endocrine cells.

About the molecular mechanisms underlining several PEA effects, they have been related to several cell surface and intracellular receptors. PEA exhibits poor affinity for cannabinoid CB1 or CB2 receptors (Lambert et al., 1999) and exerts *per se* only a negligible effect on vanilloid type-1 receptor even at high concentrations (Zygmunt et al., 1999; Smart et al., 2000), its effects have been proposed to involve indirect activation of these receptors, either through inhibition of AEA hydrolysis, or indirectly increasing 2-AG production (Lo Verme et al., 2005). However, the anti inflammatory effects of PEA involve nuclear PPAR-α which leads to the transcription of target genes upon ligand activation. PEA activates this receptor in cell based assays and its transcriptional activity, causing anti-inflammatory, in both 12-O-tetradecanoylphorbol-13-acetate-induced and

carrageenan-induced oedema, blunted in PPAR-αknockout mice (Lo Verme et al., 2005; D'Agostino et al., 2007).

The role of PPAR-α receptor on PEA induced neurosteroids synthesis is supported by our experimental evidence: the synthetic PPAR-α antagonist, GW6471, reverted the effects of PEA (i.e. StAR activation, P450scc expression and ALLO production) and most importantly, knock-down of PPAR-α in C6 cells completely blunted PEA-induced increase of StAR mature form and P450scc expression.

Our data support a physiological role and pharmacological properties of PEA in CNS, where PEA is found in significant levels in whole mouse or rat brains and could play homeostatic and protective role, also trough neurosteroids, both in physiological and pathological conditions, when its concentration strongly increases. In fact, PEA concentration dramatically increased as a result of diverse neuropathological conditions (Franklin et al., 2003), and a protective role of PEA in neuroinflammation or after transient focal cerebral ischemia was strongly reinforced (Lambert et al., 2002 a; Schomacher et al., 2008).

According with previous data (Watanabe et al., 1999), with *in vivo* experiments we demonstrate that PEA significantly and in a dose-dependent manner increases pentobarbital-induced LORR duration. It is worthy of note that PEA did not cause a sedative or hypnotic effect by itself after i.c.v. injection. Thus, PEA did not cause but was able to modulate molecular mechanisms involved in pentobarbital-induced effect.

The pivotal role of PPAR-α activation in PEA effect was strongly supported by the evidence that it was absent in PPAR-$\alpha^{-/-}$ mice and mimicked by the synthetic PPAR-α agonist GW7647. Moreover, synthetic agonists of PPAR-γ and PPAR-δ, ciglitazone and GW0742 respectively, did not modulate pentobarbital-induced LORR duration.

These results show the key role of PPAR-α for this central effect of PEA, strengthening our previous observation about the involvement of this receptor in anti-inflammatory and analgesic effect of PEA (Calignano et al., 2001, Lo Verme et al., 2005 and 2006; D'Agostino et al., 2007 and 2009).

Our results also indicate that PEA may be involved in the alteration of EEG spectra inducing a higher delta and lower theta range during LORR.

Pentobarbital-induced hypnotic effects involve more pronounced depression of the central nervous system than mere sedation. We show that these effects include shortening of LORR latency, increase of total LORR time and efficiency, and enhancement of EEG power in the delta frequency range. Indeed, the intraperitoneal injection of pentobarbital (40 mg/kg) produced LORR lasting about 55 min, and muscle relaxing effects.

In the EEG spectra, the LORR induced by pentobarbital was associated to the wake EEG power spectra, with an increase in the delta and a reduction in the theta and alpha activity.

Pentobarbital is a rapidly acting barbiturate. Barbiturates are agonists of the $GABA_A$-receptor complex, and the combinations of PEA with this barbiturate exhibit synergistic interactions. In this study PEA, prolonged

twice the duration of the LORR induced by pentobarbital. The effects of PEA on LORR and EEG parameters were not reported previously in mice. From our experiments, it appears that a single administration of PEA did not produce any noticeable change in LORR and EEG activity in these mice. Instead, analysis of the experimental results in which PEA was co-administered with pentobarbital revealed that mice showed an elongation of LORR time in comparison with mice administrated only with pentobarbital.

Also the EEG frequencies in LORR changed in presence of PEA and pentobarbital: higher EEG power in the low-frequency band (0.5–4 Hz), and a lower EEG power in the 4–8 Hz frequency band relative to pentobarbital alone. The magnitude of EEG slow wave activity during LORR (0.5–4 Hz, SWA) is considered an indicator of LORR intensity (Daan et al., 1984; Tobler and Borbely, 1986). The increase of the 4–8 Hz frequency band PEA-induced suggested that PEA is involved in the modulation of pentobarbital-induced LORR. EEG theta activity has been linked to motor activity (Brown and Shryne, 1964; Franken et al., 1998). Thus, the highly significant decrease in theta activity after PEA administration may be related to the impairment of motor activity.

The results of EEG analysis spectra showing an increase of slow-wave activity (EEG power density in the range 0.5-4Hz) and a concomitant reduction in frequencies above 15 Hz suggests that PEA, successfully increased the intensity of LORR by producing a reduction of motor

activity.

The results of this study also showed that during pentobarbital-induced LORR, the power of alpha (8-13 Hz) and beta (13-18 Hz) frequency waves changed; in particular the results indicate that PEA is involved in the higher beta and lower alpha frequency bands in LORR.

PEA administration leads to a different sleep-wake EEG activity in mice, probably reflecting variable effects on LORR-promoting and maintaining neuronal networks. We believe that administration of PEA might modulate the hypnotic effect evoked by pentobarbital modifying molecular mechanisms involved in pentobarbital effect.

It is well known that pentobarbital positively modulates $GABA_A$ receptor and its hypnotic activity can be modulated by various factors, such as stress and several endogenous molecules. Neurosteroids are involved in a variety of brain functions, such as learning and memory, psychiatric mood disorders and behavioural abnormalities. Among neurosteroids, only ALLO and its 3-α,5-β stereoisomer pregnanolone positively and/or allosterically modulate GABA or pentobarbital actions at $GABA_A$ receptors at low nanomolar concentrations (Paul and Purdy, 1992; Puia et al., 2003) increasing both the probability and the frequency of chloride channel opening (Twyman and Mcdonald, 1992; Macdonald and Olsen, 1994). It has also been demonstrated that ALLO has somnogenic properties that are very similar to those of other $GABA_A$ modulators, especially of short-acting benzodiazepines (Lancel et al., 1997; Damianisch et al., 2001), and

it may be considered an efficacious modulator of sleep-wake behaviour. Moreover, ALLO itself displays anxiolytic, sedative, hypnotic, analgesic and anaesthetic properties (Rupprecht et al., 2001).

Here, we report that the increase of pentobarbital-induced LORR duration produced by PEA was linked to an increase of ALLO production.

In our experimental conditions, ALLO increases pentobarbital-induced LORR time only at the highest dose used (30μg). Interestingly, when inactive doses of ALLO and PEA (1 μg, respectively) were co-administrated a significant additive effect was shown, inducing a two-fold increase of pentobarbital-induced LORR time. This evidence suggests an additional role of exogenous ALLO on PEA activity in our experimental and time conditions.

Hypothesizing the role of the *de novo* synthesis of ALLO in PEA effect, we used the inhibitor of 5-α reductase (FIN), reducing the availability of 5-α reduced neurosteroids including ALLO. As expected, the inhibitor reverted the PEA effect on pentobarbital-induced LORR duration. To confirm our observation, we then measured ALLO content in the brainstem evidencing the PEA capability to increase ALLO concentrations in this area already 15-30 min after co-administration of PEA and pentobarbital.

The biosynthesis of steroids in all steroidogenic tissues studied to date begins with the enzymatic conversion of cholesterol to pregnenolone. This reaction is catalyzed by CYP450scc, which is located on the matrix side of the inner mitochondrial membrane.

Here, we reported data indicating that PEA-induced increase in ALLO biosynthesis is related to an increase of CYP450scc expression, evidenced in *ex vivo* by western blot analysis, and *in vivo* using the inhibitor of CYP450scc, AMG.

Although the rate-limiting step of steroidogenesis was previously thought to be the production of pregnenolone, the true rate-limiting step in this process is the delivery of cholesterol to the inner mitochondrial membrane where the CYP450scc is located (Lambeth et al., 1987; Privalle et al., 1987; Miller, 2008).

It was recently discovered that StAR, localized in mitochondria, is a critical protein in the regulation of cholesterol availability in the mitochondria of peripheral and central steroidogenic cells (Stocco, 2001 a; King et al., 2002, Sierra, 2004). StAR is synthesized as a 37-kDa preprotein that is rapidly imported into the mitochondria, and processed to the inactive mature 30-kDa form found in the mitochondrial matrix. The C-terminal region contains a hydrophobic pocket that might be involved in the 'desorption' of cholesterol from the sterol-rich outer membrane to the sterol-poor inner membrane. The N-terminal domain targets StAR in the mitochondria, and its cleavage effectively terminates the delivery of cholesterol to CYP450scc and therefore the synthesis of pregnenolone (Stocco, 2001 b; Bose et al., 2002; Mathieu et al., 2002; Thomson, 2003).

In our experimental conditions both at 15 and 30 minutes after pentobarbital, PEA increased StAR (30-kDa) expression, suggesting an

augmentation in mitochondrial cholesterol import after PEA treatment. Based on these evidence, the increase of StAR and CYP450scc induced by PEA could act in a coordinated manner to induce steroidogenesis and in turn ALLO level augmentation. Further studies may identify other StAR-independent mechanisms, such as START proteins or the peripheral benzodiazepine receptor, in PEA effect.

We have, furthermore, contemplated the possibility that PEA could extend pentobarbital-induced LORR time in mice by increasing endocannabinoid levels in the CNS as suggested by Benn-Shabat (Benn-Shabat et al., 1998), since anandamide causes a cataleptic effect in mice (Sulcova et al., 1998). Hence, we used rimonabant, a CB1 receptor antagonist, which did not modify the effect of PEA, excluding the involvement of cannabinoid system in the central effect of PEA here reported.

Recently, we demonstrated that rapid analgesic effect of PEA is related to an increased potassium efflux (Lo Verme et al., 2006). In light of this observation, we have also explored the possibility that PEA, by increasing potassium efflux, might indirectly cause an improvement of pentobarbital-induced chlorum-dependent hyperpolarization. Both charybdotoxin and iberiotoxin, two different and selective KCa channel blocker failed to modify PEA effect on pentobarbital-induced LORR, suggesting that effect here described was independent of potassium channel increase outward conductance.

Finally, our results provide a framework to better understand the role of this

naturally occurring fatty-acid ethanolamide in the CNS, expanding our previous observation about its role in controlling pain and inflammation. Here we report, for the first time, that pharmacological stimulation of central PPAR-alpha receptor by endogenous or synthetic agonist could positively modulate pentobarbital-induced LORR at least in part by the increase of central ALLO production.

References.

Aldrich, M., 1998. Effect of alcohol on sleep, in: E.S. Lisansky-Gomberg, A.M. Hegedus, R.A. Zucker (Eds.), Alcohol Problems and Aging, National Institute on Alcohol Abuse and Alcoholism Research Monograph No. 33, National Institutes of Health, Bethesda, MD, pp. 281-300.

Aloe, L., Leon, A., Levi-Montalcini, R., 1993. A proposed autacoid mechanism controlling mastocyte behaviour. *Agents and Actions.* 39, C145-C147 (Spec No.).

Andersson, S., Russell, D.W., 1990. Structural and biochemical properties of cloned and expressed human and rat steroid 5 alpha-reductases. *Proc. Natl. Acad. Sci. USA.* 87, 3640-3644.

Andersson, S., Berman, D.M., Jenkins, E.P., Russell, D.W., 1991. Deletion of steroid 5 alpha-reductase 2 gene in male pseudohermaphroditism. *Nature* 354, 159-161.

Astarita, G., Di Giacomo, B., Gaetani, S., Oveisi, F., Compton, T.R., Rivara, S. et al., 2006. Pharmacological characterization of hydrolysis resistant analogs of oleoylethanolamide with potent anorexiant properties. *J Pharmacol Exp Ther.* 318, 563-570.

Atzori, G., Fratta, W., Gessa, G.L., Burov, Y., 2001. Inhibition of neurosteroid synthesis fails to reduce the anaesthetic effect of alcohol and

other anaesthetics in mice. *Soc. Neurosci. Abs.* 27, 1714.

Bachur, N.R., Masek, K., Melmon, K.L., Udenfriend, S., 1965. Fatty acid amides of ethanolamine in mammalian tissues. *Journal of Biological Chemistry.* 240 (3), 1019-1024.

Barbaccia, M.L., Affricano, D., Trabucchi, M., Purdy, R.H., Colombo, G., Agabio, R., Gessa, G.L., 1999. Ethanol markedly increases 'GABAergic' neurosteroids in alcohol-preferring rats. *Eur. J. Pharmacol.* 384, R1-R2.

Baulieu, E.E., Robel, P., Schumacher, M., 1999. Contemporary Endocrinology, Humana Press, Totowa.

Belelli, D., Lambert, J.J., 2005. Neurosteroids: Endogenous regulators of the GABA(A) receptor. *Nat. Rev. Neurosci.* 6, 565-575.

Benani, A., Heurtaux, T., Netter, P., Minn, A., 2004. Activation of peroxisome proliferator-activated receptor alpha in rat spinal cord after peripheral noxious stimulation. *Neurosci Lett.* 369, 59-63.

Benmessahel, Y., Troadec, J.D., Cadepond, F., Guennoun, R., Hales, D.B., Schumacher, M., Groyer, G., 2004. Downregulation of steroidogenic acute regulatory protein (StAR) gene expression by cyclic AMP in cultured Schwann cells. *Glia.* 45, 213-228.

Ben-Shabat, S., Fride, E., Sheskin, T., Tamiri, T., Rhee, M.H., Vogel, Z, Bisogno, T., De Petrocellis, L., Di Marzo, V., Mechoulam, R., 1998. An entourage effect: inactive endogenous fatty acid glycerol esters enhance 2-arachidonoyl-glycerol cannabinoid activity. *Eur J Pharmacol.* 353, 23-31.

Berdyshev, E., Boichot, E., Corbel, M., Germain, N., Lagente, V., 1998. Effects of cannabinoid receptor ligands on LPS-induced pulmonary inflammation in mice. *Life Sciences.* 63 (8), L125-L129.

Berman, D.M., Russell, D.W., 1993. Cell-type-specific expression of rat steroid 5 alpha-reductase isozymes. *Proc. Natl. Acad. Sci. USA.* 90, 9359-9363.

Bitran, D., Foley, M., Audette, D., Leslie, N., Frye, C.A., 2000. Activation of peripheral mitochondrial benzodiazepine receptors in the hippocam- pus stimulates allopregnanolone synthesis and produces anxiolytic- like effects in the rat, *Psychopharmacol.* (Berl.) 151, 64-71.

Borel, V., Gallot, D., Marceau, G., Sapin, V., Blanchon, L., 2008. Placental implications of peroxisome proliferator-activated receptors in gestation and parturition. *PPAR Res.* 2008, 758562.

Bose, H.S., Lingappa, V.R., Miller, W.L., 2002. The steroidogenic acute regulatory protein, StAR, works only at the outer mitochondrial membrane. *Endocr Res.* 28,295-308.

Bouchard, J.F., Lepicier, P., Lamontagne, D., 2003. Contribution of endocannabinoids in the endothelial protection afforded by ischemic preconditioning in the isolated rat heart. *Life Sciences.* 72 (16), 1859-1870.

Bose H.S., Lingappa V.R., Miller W.L. ,2002a. The steroidogenic acute regulatory protein, StAR, works only at the outer mitochondrial membrane. *Endocr Res.* 28: 295-308.

Bose H., Lingappa V.R., Miller W.L., 2002b. Rapid regulation of

steroidogenesis by mitochondrial protein import. *Nature*. 417: 87-91.

Bracey, M.H., Hanson, M.A., Masuda, K.R., Stevens, R.C., Cravatt, B.F., 2002. Structural adaptations in a membrane enzyme that terminates endocannabinoid signaling. *Science*. 298 (5599), 1793-1796.

Brinton R.D,. Wang J.M., 2006. Preclinical analyses of the therapeutic potential of allopregnanolone to promote neurogenesis in vitro and in vivo in transgenic mouse model of Alzheimer's disease. Curr Alzheimer Res. 3: 11-17.

Brot, M.D., Akwa, Y., Purdy, R.H., Koob, G.F., Britton, K.T., 1997. The anxiolytic-like effects of the neurosteroid allopregnanolone: interactions with GABAA receptors. *Eur. J. Pharmacol*. 325, 1-7.

Brown, B.B., Shryne, J.E., 1964. EEG theta activity and fast activity sleep in cats as related behavioral traits. *Neuropsychologia* 2, 311-326.

Bowlby, M.R., 1993. Pregnenolone sulfate potentiation of N-methyl-D-aspartate receptor channels in hippocampal neurons. *Mol. Pharmacol*. 43, 813-819.

Burade, V.S., Jain, M.R., Khan, F.A., Saha, S.G., Subhedar, N., 1996. Involvement of corticosteroid-like neurosteroids in pentobarbital-induced sleep. *Neuroreport*. 8, 139-41.

Burudi E.M.E., Riese S., Stahl P.D., Regnier-Vigouroux A., 1999. Identification and functional characterization of the mannose receptor in astrocytes. *Glia*. 25: 44-55.

Cadas, H., Gaillet, S., Beltramo, M., Venance, L., Piomelli, D., 1996.

Biosynthesis of an endogenous cannabinoid precursor in neurons and its control by calcium and cAMP. *Journal of Neuroscience.* 16 (12), 3934-3942.

Cadas, H., di Tomaso, E., Piomelli, D., 1997. Occurrence and biosynthesis of endogenous cannabinoid precursor, N-arachidonoyl phosphatidylethanolamine, in rat brain. *J Neurosci.* 17, 1226-1242.

Calignano, A.; La Rana, G.; Giuffrida, A.; Piomelli, D., 1998. Control of pain initiation by endogenous cannabinoids. *Nature.* 394, 277-281.

Calignano, A., La Rana, G., Piomelli, D., 2001. Antinociceptive activity of the endogenous fatty acid amide, palmitylethanolamide. *Eur J Pharmacol.* 11, 191-8.

Capasso, R., Izzo, A.A., Fezza, F., Pinto, A., Capasso, F., Mascolo, N., Di Marzo, V., 2001. Inhibitory effect of palmitoylethanolamide on gastrointestinal motility in mice. *British Journal of Pharmacology.* 134 (5), 945-950.

Caruso, D., Scurati, S., Maschi, O., De Angelis, L., Roglio, I., Giatti, S., Garcia-Segura, L.M., Melcangi, R.C., 2007. Evaluation of neuroactive steroid levels by liquid chromatography-tandem mass spectrometry in central and peripheral nervous system: Effect of diabetes. Neurochem.Int.,doi :10.1016/j.neuint.2007.06.004.

Celotti, F., Melcangi, R.C., Martini, L., 1992. The 5 alpha-reductase in the brain: Molecular aspects and relation to brain function. *Front. Neuroendocrinol.* 13, 163-215.

Chung, B.C., Matteson, K.J., Voutilainen, R., Mohandas, T.K., Miller, W.L., 1986. Human cholesterol side-chain cleavage enzyme, P450scc: cDNA cloning, assignment of the gene to chromosome 15, and expression in the placenta. *Proc. Natl. Acad. Sci. USA.* 83, 8962-8966.

Coburn, A., Moore, L., 1943. *American Journal of Diseases of Children.* 65 (744).

Coburn, O.H., Graham, C.E., Haninger, J., 1954. The effect of egg yolk in diets on anaphylactic arthritis (passive Arthus phenomenon) in the guinea pig. *Journal of Experimental Medicine.* 100 (5), 425-435. (November 1).

Concas, A., Mostallino, M.C., Porcu, P., Follesa, P., Barbaccia, M.L., Trabucchi M., Purdy, R.H., Grisenti, P., Biggio, G., 1998. Role of brain allopregnanolone in the plasticity of gamma-aminobutyric acid type A receptor in rat brain during pregnancy and after delivery. *Proc. Natl. Acad. Sci. USA.* 95, 13284-13289.

Compagnone, N.A., Bulfone, A., Rubenstein, J.L., Mellon, S.H., 1995. Expression of the steroidogenic enzyme P450scc in the central and peripheral nervous systems during rodent embryogenesis. *Endocrinology.* 136, 2689-2696.

Compagnone, N.A., Mellon, S.H., 2000. Neurosteroids: Biosynthesis and function of these novel neuromodulators. *Front. Neuroendocrinol.* 21, 1-56.

Conti, S., Costa, B., Colleoni, M., Parolaro, D., Giagnoni, G., 2002.

Antiinflammatory action of endocannabinoid palmitoylethanolamide and the synthetic cannabinoid nabilone in a model of acute inflammation in the rat. *British Journal of Pharmacology.* 135 (1), 181-187.

Costa, B.; Conti, S.; Giagnoni, G.; Colleoni, M., 2002. Therapeutic effect of the endogenous fatty acid amide, palmitoylethanolamide, in rat acute inflammation: inhibition of nitric oxide and cyclooxygenase systems. *Br J Pharmacol.* 137, 413-420.

Corpechot, C., Robel, P., Axelson, M., Sjovall, J.,Baulieu, E.E., 1981. Characterization and measurement of dehydroepiandrosterone sulfate in rat brain. *Proc. Natl. Acad. Sci. USA.* 78, 4704-4707.

Corpechot, C., Synguelakis, M., Talha, S., Axelson, M., Sjovall, J., Vihko, R., Baulieu, E.E., Robel, P., 1983. Pregnenolone and its sulfate ester in the rat brain. *BrainRes.* 270, 119-125.

Cravatt, B.F., Lichtman, A.H., 2002. The enzymatic inactivation of the fatty acid amide class of signaling lipids. *Chemistry and Physics of Lipids*. 121 (1-2), 135-148.

Cravatt, B.F., Giang, D.K., Mayfield, S.P., Boger, D.L., Lerner, R.A., Gilula, N.B., 1996. Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature.* 384 (6604), 83-87.

Cravatt, B.F., Saghatelian, A., Hawkins, E.G., Clement, A.B., Bracey, M.H., Lichtman, A.H., 2004. Functional disassociation of the central and peripheral fatty acid amide signaling systems. *Proceedings of the National Academy of Sciences of the United States of America.* 101 (29), 10821-

10826.

Czlonkowska, A.I., Krzascik, P., Sienkiewicz-Jarosz, H., Siemiatkowski, M., Szyndler, J., Bidzinski, A., Plaznik, A., 2000. The effects of neurosteroids on picrotoxin-, bicuculline- and NMDA-induced seizures, and a hypnotic effect of ethanol. *Pharmacol. Biochem. Behav.* 67, 345-353.

Czlonkowska, A.I., Krzascik, P., Sienkiewicz-Jarosz, H., Siemiatkowski, M., Szyndler, J., Bidzinski, A., Plaznik, A., 2001. Tolerance to the anticonvulsant activity of midazolam and allopregnanolone in a model of picrotoxin seizures. *Eur. J. Pharmacol.* 425, 121-127.

Daan, S., Beersma, D.G., Borbely, A.A., 1984. Timing of human sleep: recovery process gated by a circadian pacemaker. *Am. J. Physiol.* 246, R161-R18

Damianisch, K., Rupprecht, R., Lancel, M., 2001. The influence of subchronic administration of the neurosteroid allopregnanolone on LORR in the rat. *Neuropsychopharmacology.* 25, 576-584.

D'Agostino, G., La Rana, G., Russo, R., Sasso, O., Iacono, A, Esposito, E., Mattace Raso, G., Cuzzocrea, S., Lo Verme, J., Piomelli, D., Meli, R., Calignano, A., 2007. Acute intracerebroventricular administration of palmitoylethanolamide, an endogenous peroxisome proliferator-activated receptor-alpha agonist, modulates carrageenan-induced paw edema in mice. *J Pharmacol Exp Ther.* 322, 1137-43.

D'Agostino, G., La Rana, G., Russo, R., Sasso, O., Iacono, A, Esposito,

E., Mattace Raso, G,. Cuzzocrea, S., Loverme, J., Piomelli, D., Meli, R., Calignano, A., 2009. Central administration of palmitoylethanolamide reduces hyperalgesia in mice via inhibition of NF-kappaB nuclear signalling in dorsal root ganglia. *Eur J Pharmacol.* 24, 54-9.

Dazzi, L.S., Cherchi, E., Sarigu, G., Biggio, S., 2002. The neurosteroid allopregnanolone potentiates the effect of ethanol on cortical dopa- minergic neurons. *Alcohol Clin. Exp. Res.* 26, 103A.

Deboer, T., Ruijgrok, G., Meijer, J.H., 2007. Short light-daek cycles affect LORR in mice. *Eur J Neurosci.* 26, 3518-23.

De Nicola AF., 1993. Steroid hormones and neuronal regeneration. *Adv Neurol.* 59: 199-206.

Djebaili M., Hoffman S.W., Stein D.J., 2004. Allopregnanolone and progesterone decrease cell death and cognitive deficits after a contusion of the rat pre-frontal cortex. *Neuroscience*. 123: 349-359.

De Petrocellis, L., Bisogno, T., Ligresti, A., Bifulco, M., Melck, D., Di Marzo, V., 2002. Effect on cancer cell proliferation of palmitoylethanolamide, a fatty acid amide interacting with both the cannabinoid and vanilloid signalling systems. *Fundamental & Clinical Pharmacology.* 16 (4), 297-302.

De Roo, M., Rodeau, J.L., Schlichter, R., 2003. Dehydroepiandrosterone potentiates native ionotropic ATP receptors containing the P2X2 subunit in rat sensory neurones. *J. Physiol.* 552, 59-71.

De Sarro, G.B., Masuda, Y., Ascioti, C., Audino, M.G., Nistico, G., 1990. Behavioural and EcoG spectrum changes induced by intracerebral infusion of interferons and interleukin 2 in rats are antagonized by naloxone. *Neuropharmacology.* 29, 167-79.

De Sarro, G., Ammendola, D., Nava, F., De Sarro, A., 1995. Effects of some excitatory amino acid antagonists on imipenem-induced seizures in DBA/2 mice. *Brain Res.* 671, 131-40.

De Sarro, G., Palma, E., Costa, N., Marra, R., Gratteri, S., De Sarro, A., Rotiroti, D., 2000. Effects of compounds acting on GABA(B) receptors in the pentylenetetrazole kindling model of epilepsy in mice. *Neuropharmacology.* 39, 2147-61.

Désarnaud, F., Cadas, H., Piomelli, D., 1995. Anandamide amidohydrolase activity in rat brain microsomes. Identification and partial characterization. *Journal of Biological Chemistry.* 270 (11), 6030-6035.

Desvergne, B., Wahli, W., 1999. Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr Rev.* 20 (5), 649-688.

Di Marzo, V., Fontana, A., Cadas, H., et al., 1994. Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature*. 372, 686-691.

Di Marzo, V., Melck, D., Orlando, P., Bisogno, T., Zagoory, O., Bifulco, M., Voge,l Z., De Petrocellis, L., 2001. Palmitoylethanolamide inhibits the expression of fatty acid amide hydrolase and enhances the anti-proliferative effect of anandamide in human breast cancer cells. *Biochem J.*

358, 249-255.

Dreyer. C., Krey, G., Keller, H., Givel, F., Helftenbein, G., Wahli, W., 1992. Control of theperoxisomal beta-oxidation pathway by a novel family of nuclear hormone receptors. *Cell.* 68 (5), 879-887.

Ehlers, C.L., 2000. Alcohol and sleep, in: A. Noronha, M. Eckardt, K. Warren (Eds.), Alcohol Problems and Aging, Review of NIAAA's Neuroscience and Behavioral Research Portfolio. National Institute on Alcohol Abuse and Alcoholism Research Monograph, Vol. 33, National Institutes of Health, Bethesda, MD, pp. 417-433.

Escher, P., Wahli, W., 2000. Peroxisome proliferator-activated receptors: insight into multiple cellular functions. *Mutat Res.* 448 (2), 121-138.

Escriva, H., Safi, R., Hanni, C., Langlois, M.C., Saumitou-Laprade, P., Stehelin, D., et al., 1997. Ligand binding was acquired during evolution of nuclear receptors. *Proc Natl Acad Sci U S A.* 94 (13), 6803-6808.

Facci, L., Dal Toso, R., Romanello, S., Buriani, A., Skaper, S.D., Leon, A., 1995. Mast cells express a peripheral cannabinoid receptor with differential sensitivity to anandamide and palmitoylethanolamide. *Proceedings of the National Academy of Sciences of the United States of America*. 92 (8), 3376-3380.

Fajas, L., Fruchart, J.C., Auwerx, J, 1998. PPARgamma3 mRNA: a distinct PPARgamma mRNA subtype transcribed from an independent promoter. *FEBS Lett.* 438 (1-2), 55-60.

Farquhar-Smith, W.P., Rice, A.S., 2001. Administration of endocannabinoids prevents a referred hyperalgesia associated with inflammation of the urinary bladder. *Anesthesiology.* 94 (3), 507-513.

Farquhar-Smith, W.P., Rice, A.S., 2003. A novel neuroimmune mechanism in cannabinoid-mediated attenuation of nerve growth factor-induced hyperalgesia. *Anesthesiology*. 99 (6), 1391-1401.

Franken, P., Malafosse, A., Tafti, M., 1998. Biochemistry. Genetic variation in EEG activity during sleep in inbred mice. *Am. J. Physiol.* 275, R1127-R1137.

Franklin, A., Parmentier-Batteur, S., Walter, L., Greenberg, D.A., Stella, N., 2003. Palmitoylethanolamide increases after focal cerebral ischemia and potentiates microglial cell motility. *Journal of Neuroscience.* 23 (21), 7767-7775.

Frye, C.A., 2001. The role of neurosteroids and non-genomic effects of progestins and androgens in mediating sexual receptivity of rodents. *Brain Res. Rev.* 37, 201-222.

Fu, J., Gaetani, S., Oveisi, F., Lo Verme, J., Serrano, A., Rodriguez De Fonseca, F. et al., 2003. Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha. *Nature.* 425, 90-93.

Genovese, T., Mazzon, E., Di Paola, R., Cannavò, G., Muià, C., Bramanti, P., Cuzzocrea, S., 2005. Role of endogenous ligands for the peroxisome proliferators activated receptors alpha in the secondary damage

in experimental spinal cord trauma. *Exp Neurol*. 194, 267-78.

Glass, C.K., Ogawa, S., 2006. Combinatorial roles of nuclear receptors in inflammation and immunity. *Nat Rev Immunol*. 6 (1), 44-55.

Gonzalez Deniselle, M.C., Lopez-Costa, J.J., Saavedra, J.P., Pietranera, L., Gonzalez, S.L., Garay, L., Guennoun, R., Schumacher, M., De Nicola, A.F., 2002. Progesterone neuroprotection in the Wobbler mouse, a genetic model of spinal cord motor neuron disease. *Neurobiol. Dis.* 11, 457-468.

Greene, M.E., Blumberg, B., McBride, O.W., Yi, H.F., Kronquist, K., Kwan, K., et al., 1995. Isolation of the human peroxisome proliferator activated receptor gamma cDNA: expression in hematopoietic cells and chromosomal mapping. *Gene Expr*. 4, 281-299.

Guidotti, A., Dong, E., Matsumoto, K., Pinna, G., Rasmusson, A.M., Costa, E., 2001. The socially-isolated mouse: a model to study the putative role of allopregnanolone and 5alpha-dihydroprogesterone in psychiatric disorders. *Brain Res. Brain Res. Rev.* 37, 110-115.

Gulyas, A.I., Cravatt, B.F., Bracey. M.H., et al., 2004. Segregation of 2 endocannabinoid-hydrolyzing enzymes into pre- and postsynaptic compartments in the rat hippocampus, cerebellum and amygdala. *Eur J Neurosci*. 20, 441-458.

Guzman, M., Lo Verme, J., Fu, J., Oveisi, F., Blazquez, C., Piomelli, D., 2004. Oleoylethanolamide stimulates lipolysis by activating the nuclear receptor peroxisome proliferator-activated receptor alpha (PPAR-alpha). *J*

Biol Chem. 279, 27849-27854.

Haley, T.J., Mc Cormick, W.G., 1957. Pharmacological effects produced by intracerebral injection of drugs in the conscious mouse. *Br J Pharmacol.* 12,12-15.

Hansen, H.S., Lauritzen, L., Strand, A.M., Vinggaard, A.M., Frandsen, A., Schousboe, A., 1997. Characterization of glutamate-induced formation of N-acylphosphatidylethanolamine and N-acylethanolamine in cultured neocortical neurons. *J Neurochem*. 69, 753-761.

Hartl F.U., Schmidt B., Wachter E., Weiss H., Neupert W., 1986. Transport into mitochondria and intramitochondrial sorting of the Fe/S protein of ubiquinol-cytochrome c reductase. *Cell.* 47: 939-951.

Hauet T., Liu J., Li H., Gazouli M., Culty M., Papadopoulos V., 2002. PBR, StAR, and PKA: partners in cholesterol transport in steroidogenic cells. *Endocr Res.* 28: 395-401.

He J., Evans C.O., Hoffman S.W., Oyesiku N.M., Stein D.G., 2004. Progesterone and allopregnanolone reduce inflammatory cytokines after traumatic brain injury. *Exp Neurol.* 189: 404-412.

Helyes, Z., Nemeth, J., Than, M., Bolcskei, K., Pinter, E., Szolcsanyi, J., 2003. Inhibitory effect of anandamide on resiniferatoxin-induced sensory neuropeptide release in vivo and neuropathic hyperalgesia in the rat. *Life Sciences.* 73 (18), 2345-2353.

Hiemke, C., Jussofie, A., Juptner, M., 1991. Evidence that 3 alpha-hydroxy- 5 alpha-pregnan-20-one is a physiologically relevant modulator

of GABA-ergic neurotransmission, *Psychoneuroendocrinology.* 16, 517-523.

Hillard, C.J., Wilkison, D.M., Edgemond, W.S., Campbell, W.B., 1995. Characterization of the kinetics and distribution of N-arachidonylethanolamine (anandamide) hydrolysis by rat brain. *Biochimica et Biophysica Acta.* 1257 (3), 249-256.

Hirani, K., Khisti, R.T., Chopde, C.T., 2002. Behavioral action of ethanol in Porsolt's forced swim test: modulation by 3 alpha-hydroxy-5 alpha-pregnan- 20-one. *Neuropharmacology.* 43, 1339-1350.

Hodge, C.W., Nannini, M.A., Olive, M.F., Kelley, S.P., Mehmert, K.K., 2001. Allopregnanolone and pentobarbital infused into the nucleus accumbens substitute for the discriminative stimulus effects of ethanol. *Alcohol Clin. Exp. Res.* 25, 1441-1447.

Hosie, A.M., Wilkins, M.E., da Silva, H.M., Smart, T.G., 2006. Endogenous neurosteroids regulate GABAA receptors through two discrete transmembrane sites. *Nature.* 444, 486-489.

Jacobsson, S.O., Fowler, C.J., 2001. Characterization of palmitoylethanolamide transport in mouse Neuro-2a neuroblastoma and rat RBL-2H3 basophilic leukaemia cells: comparison with anandamide. *British Journal of Pharmacology.* 132 (8), 1743-1754.

Jaggar, S.I., Hasnie, F.S., Sellaturay, S., Rice, A.S., 1998. The anti-hyperalgesic actions of the cannabinoid anandamide and the putative CB2 receptor agonist palmitoylethanolamide in visceral and somatic

inflammatory pain. *Pain.* 76 (1-2), 189–199.

Janak, P.H., Redfern, J.E.M., Samson, H.H., 1998. The reinforcing effects of ethanol are altered by the endogenous neurosteroid, allopregnanolone. *Alcohol Clin. Exp. Res.* 22, 1106-1112.

Jenkins, E.P., Andersson, S., Imperato-McGinley, J., Wilson, J.D., Russell, D.W., 1992. Genetic and pharmacological evidence for more than one human steroid 5 alpha-reductase. *J. Clin. Invest.* 89, 293-300.

Jez, J.M., Flynn, T.G., Penning, T.M., 1997. A new nomenclature for the aldo-keto reductase superfamily. *Biochem. Pharmacol.* 54, 639-647.

Jez, J.M, Penning, T.M., 2001. The aldo-keto reductase (AKR) superfamily: An update. *Chem. Biol. Interact.* 130-132.

Jung-Testas, I., Hu, Z.Y., Baulieu, E.E., Robel, P., 1989. Neurosteroids: Biosynthesis of pregnenolone and progesterone in primary cultures of rat glial cells. *Endocrinology.* 125, 2083-2091.

Jung-Testas, I., Baulieu, E.E., 1998. Steroid hormone receptors and steroid action in rat glial cells of the central and peripheral nervous system. J *Steroid Biochem Mol Biol.* 65, 243-251.

Kainu, T., Wikstrom, A.C., Gustafsson, J.A., Pelto-Huikko, M., 1994. Localization of the peroxisome proliferator-activated receptor in the brain. *Neuroreport.* 5, 2481-2485.

Karry S., Dertien J.S., Stocco D.M., Syapin P.J., 2007. Steroidogenic acute regulatory protein expression and pregnenolone synthesis in rat astrocyte cultures. *J Neuroendocrinol.* 19: 860-869.

Kassiou, M., Meikle, S.R., Banati, R.B., 2005. Ligands for peripheral benzodiazepine binding sites in glial cells. *Brain Res. Rev.* 48, 207-210.

Kathuria, S., Gaetani, S., Fegley, D., Valino, F., Duranti, A., Tontini, A., Mor, M., Tarzia, G., La Rana, G., Calignano, A., Giustino, A., Tattoli, M., Palmery, M., Cuomo, V., Piomelli, D., 2003. Modulation of anxiety through blockade of anandamide hydrolysis. *Nature Medicine*. 9 (1), 76-81.

Keller, J.M., Collet, P., Bianchi, A., Huin, C., Bouillaud-Kremarik, P., Becuwe, P., et al., 2000. Implications of peroxisome proliferator-activated receptors (PPARS) in development, cell life status and disease. *Int J Dev Biol*. 44 (5), 429-442.

Keller, A.F., Breton, J.D., Schlicnter, R., Poisbeau, P., 2004. Production of 5alpha-reduced neurosteroids is developmentally regulated and shapes GABA(A) miniature IPSCs in lamina II of the spinal cord. *J Neurosci.* 24, 907-915.

Khanna, M., Qin, K.N., Cheng, K.C., 1995. Distribution of 3 alpha-hydroxysteroid dehydrogenase in rat brain and molecular cloning of multiple cDNAs encoding structurally related proteins in humans. *J. Steroid Biochem. Mol. Biol.* 53, 41-46.

Kilgore, K.S., Billin, A.N., 2008. PPARbeta/deltal igands as modulators of the inflammatory response. *Curr Opin Investig Drugs*. 9 (5), 463-469.

King, S.R., Manna, P.R., Ishii, T., Syapin, P.J., Ginsberg, S.D., Wilson, K., Walsh, L.P., Parker, K.L., Stocco,D.M., Smith, R.G., Lamb, D.J., 2002.

An essential component in steroid synthesis, the steroidogenic acute regulatory protein, is expressed in discrete regions of the brain. *J Neurosci.* 22, 10613-10620.

Klaunig, J.E., Babich, M.A., Baetcke, K.P., Cook, J.C., Corton, J.C., David, R.M., et al., 2003. PPARalpha agonist-induced rodent tumors: modes of action and human relevance. *Crit Rev Toxicol.* 33 (6), 655-780.

Kliewer, S.A., Forman, B.M., Blumberg, B., Ong, E.S., Borgmeyer, U., Mangelsdorf, D.J., et al., 1994. Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proc Natl Acad Sci U SA.* 91 (15), 7355-7359.

Kliewer, S.A., Sundseth, S.S., Jones, S.A., Brown, P.J., Wisely, G.B., Koble, C.S. et al., 1997. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. *Proc Natl Acad Sci USA.* 94, 4318-4323.

Korneyev, A., Costa, E., 1996. Allopregnanolone (THP) mediates anesthetic effects of progesterone in rat brain. *Horm. Behav.* 30, 37-43.

Kozak, K.R., Crews, B.C., Morrow, J.D., Wang, L.H., Ma, Y.H., Weinander, R. et al., 2002. 15-Lipoxygenase metabolism of 2-arachidonylglycerol. Generation of a peroxisome proliferator-activated receptor alpha agonist. *J Biol Chem.* 277, 23278-23286.

Krieger, N.R., Scott, R.G., 1984. 3alpha-hydroxysteroid oxidoreductase in rat brain. *J. Neurochem.* 42, 887-890.

Krieger, N.R., Scott, R.G., 1989) Nonneuronal localization for steroid converting enzyme : 3 alpha-hydroxysteroid oxidoreductase in olfactory tubercle of rat brain. *J. Neurochem.* 52, 1866-1870.

Lacor, P., Gandolfo, P., Tonon, M.C., Brault, E., Dalibert, I., Schumacher, M., Benavides, J., Ferzaz, B., 1999. Regulation of the expression of peripheral benzodiazepine receptors and their endogenous ligands during rat sciatic nerve degeneration and regeneration : A role for PBR in neurosteroidogenesis. *Brain Res.* 815, 70-80.

Lambert D.M., DiPaolo F.G., Sonveaux P., Kanyonyo M., Govaerts S.J., Hermans E., Bueb J., Delzenne N.M,. Tschirhart E.J., 1999 a. Analogues and homologues of N-palmitoylethanolamide, a putative endogenous CB(2) cannabinoid, as potential ligands for the cannabinoid receptors. *Biochim Biophys Acta.* 1440: 266-274.

Lambert, D.M., Di Marzo, V., 1999 b. The palmitoylethanolamide and oleamide enigmas: are these two fatty acid amides cannabimimetic? *Current Medicinal Chemistry.* 6 (8), 757 -773.

Lambert, D.M., Vandevoorde, S., Diependaele, G., Govaerts, S.J., Robert, A.R., 2001. Anticonvulsant activity of N-palmitoylethanolamide, a putative endocannabinoid, in mice. *Epilepsia*. 42 (3), 321-327.

Lambert, D., Vandevoorde, S., Jonsson, K., Fowler, J., 2002. The palmitoylethanolamide family: a new class of anti-nflammatory agents? *Current Medicinal Chemistry.* 9 (6), 663-674.

Lambert, J.J., Belelli, D., Peden, D.R., Vardy, A.W., Peters, J.A., 2003.

Neurosteroid modulation of GABAA receptors. *Prog. Neurobiol.* 71, 67-80.

Lambeth, J.D., Xu, X.X., Glover, M., 1987. Cholesterol sulfate inhibits adrenal mitochondrial cholesterol side chain cleavage at a site distinct from cytochrome P-450scc. Evidence for an intramitochondrial cholesterol translocator. *J Biol Chem.* 262, 9181-9188.

Labombarda, F., Guennoun, R., Gonzalez, S., Roig, P., Lima, A., Schumacher, M, De Nicola, A.F., 2000. Immunocytochemical evidence for a progesterone receptor in neurons and glial cells of the rat spinal cord. *Neurosci. Lett.* 288, 29-32.

Lancel, M., Faulhaber, J., Schiffelholz, T., Romeo, E., Di Michel, F., Holsboer, F., Rupprecht, R., 1997. Allopregnanolone affects LORR in a benzodiazepine-like fashion. *J Pharmacol Exp Ther.* 282, 1213-1218.

Lavaque, E., Sierra, A., Azcoitia, I., Garcia-Segura, L.M., 2006. Steroidogenic acute regulatory protein in the brain. *Neuroscience.* 138, 741-747.

Le Goascogne, C., Robel, P., Gouezou, M., Sananes, N., Baulieu, E.E., Waterman, M., 1987. Neurosteroids: Cytochrome P-450scc in rat brain. *Science.* 237, 1212-215.

Lemberger, T., Desvergne, B., Wahli, W., 1996. Peroxisome proliferator-activated receptors: a nuclear receptor signaling pathway in lipid physiology. *Annu Rev Cell Dev Biol.* 12, 335-63.

Lichtman, A.H., Hawkins, E.G., Griffin, G., Cravatt, B.F., 2002. Pharmacological activity of fatty acid amides is regulated, but not

mediated, by fatty acid amide hydrolase in vivo. *Journal of Pharmacology and Experimental Therapeutics.* 302 (1), 73-79.

Long, D., Martin, A., 1956. Factors in arachis oil depressing sensitivity to tuberculin in BCG infected guinea pigs. *Lancet.* 270 (6921), 464 -466.

Long, D., Miles, A., 1950. *Lancet.* 492.

Lo Verme, J., Fu J., Astarita, G., La Rana, G., Russo, R., Calignano, A., Piomelli, D., 2005. The nuclear receptor peroxisome proliferator-activated receptor-alpha mediates the anti-inflammatory actions of palmitoylethanolamide. *Mol Pharmacol.* 67, 15-9.

Lo Verme, J., Russo, R., La Rana, G., Fu, J., Farthing, J., Mattace-Raso, G., Meli, R., Hohmann, A., Calignano,A., Piomelli D., 2006. Rapid broad-spectrum analgesia through activation of peroxisome proliferator-activated receptor-alpha. *J Pharmacol Exp Ther.* 319, 1051-61.

Lumbroso, S., Sandillon, F., Georget, V., Lobaccaro, J.M., Brinkmann, A.O., Privat, A., Sultan, C., 1996. Immunohistochemical localization and immunoblotting of androgen receptor in spinal neurons of male and female rats. *Eur. J. Endocrinol.* 134, 626-632.

Macdonald, R.L., Olsen, R.W., 1994. GABAA receptor channels. *Annu Rev Neurosci.* 17, 569-602.

Majewska, M.D., 1992. Neurosteroids: Endogenous bimodal modulators of the GABAA receptor. Mechanism of action and physiological significance. *Prog. Neurobiol.* 38, 379-395.

Mandard, S., Muller, M., Kersten, S., 2004. Peroxisome proliferator-activated receptor alpha target genes. *Cell Mol Life Sci.* 61 (4), 393-416.

Marriot D.R., Hirst W.D., Ljungberg M.C., 1995. Astrocytes. In: Neural Cell Culture, a Practical Approach. Cohen J, Wilkin GP, eds.New York: Oxford University Press. P. 83-96.

Mathieu, A.P., Fleury, A., Ducharme, L., Lavigne, P., LeHoux, J.G., 2002. Insights into steroidogenic acute regulatory protein (StAR)-dependent cholesterol transfer in mitochondria: evidence from molecular modeling and structure-based thermodynamics supporting the existence of partially unfolded states of StAR. *J Mol Endocrinol.* 29, 327–345.

Matsumoto, K., Satoh, T., Bing, L.H., Ohta, H., Watanabe, H., 1991. Effects of forced shaking stress at low temperature on pentobarbital-induced LORRing in mice. *Gen. Pharmacol.* 22, 729-733.

Matsumoto, A., 1997. Hormonally induced neuronal plasticity in the adult motoneurons. *Brain Res. Bull.* 44, 539-547.

Matsumoto, K., Uzunova, V., Pinna, G., Taki, K., Uzunov, D.P., Watanabe, H., Mienville, J.M., Guidotti, A., Costa, E., 1999. Permissive role of brain allopregnanolone content in the regulation of pentobarbital-induced righting reflex loss. *Neuropharmacology.* 38, 955-963.

Matthews, D.B., Morrow, A.L., Tokunaga, S., McDaniel, J.R., 2002. Acute ethanol administration and acute allopregnanolone administration impair spatial memory in the Morris water task. *Alcohol. Clin. Exp. Res.* 11, 1747-1751.

Mazzari, S., Canella, R., Petrelli, L., Marcolongo, G., Leon, A., 1996. N-(2-hydroxyethyl)hexadecanamide is orally active in reducing edema formation and inflammatory hyperalgesia by down-modulating mast cell activation. *European Journal of Pharmacology.* 300 (3), 227-236.

Maurice, T., Gregoire, C., Espallergues, J., 2006. Neuro(-active)steroids actions at the neuromodulatory sigma1 (sigma1) receptor: Biochemical and physiological evidences, consequences in neuroprotection. *Pharmacol. Biochem. Behav.* 84, 581-597.

Melcangi, R.C., Celotti, F., Ballabio, M., Poletti, A., Castano, P., Martini, L., 1988. Testosterone 5 alpha-reductase activity in the rat brain is highly concentrated in white matter structures and in purified myelin sheaths of axons. *J. Steroid Biochem.* 31, 173-179.

Melcangi, R.C., Celotti, F., Ballabio, M., Poletti, A., Martini, L., 1990. Testosterone metabolism in peripheral nerves: Presence of the 5alpha-reductase-3alpha-hydroxysteroid-dehydrogenase enzymatic system in the sciatic nerve of adult and aged rats. *J. Steroid Biochem.* 35, 145-148.

Melcangi, R.C., Celotti, F., Castano, P., Martini, L., 1993. Differential localization of the 5 alpha-reductase and the 3 alpha-hydroxysteroid dehydrogenase in neuronal and glial cultures. *Endocrinology.* 132, 1252-1259.

Melcangi, R.C., Poletti, A., Cavarretta, I., Celotti, F., Colciago, A., Magnaghi, V., Motta, M., Negri-Cesi, P,. Martini, L., 1998. The 5alpha-reductase in the central nervous system: Expression and modes of control.

J. Steroid Biochem. Mol. Biol. 65, 295-299.

Melis, M., Pillola, G., Luchicchi, A., Muntoni, A.L., Yasar, S., Goldberg, S.R., Pistis, M,. 2008. Endogenous fatty acid ethanolamides suppress nicotine-induced activation of mesolimbic dopamine neurons through nuclear receptors. *J Neurosci.* 28, 13985-13994.

Mendelson, W.B., Martin, J.V., Perlis, M., Wagner, R., Majewska, M.D., Paul, S.M., 1987. Sleep induction by an adrenal steroid in the rat. *Psychopharmacology.* 93, 226-229.

Mensah-Nyagan, A.G., Do-Rego, J.L., Beaujean, D., Luu-The, V., Pelletier, G., Vaudry, H., 1999. Neurosteroids: Expression of steroidogenic enzymes and regulation of steroid biosynthesis in the central nervous system. *Pharmacol. Rev.* 51, 63-81.

Michalik, L., Desvergne, B., Dreyer, C., Gavillet, M., Laurini, R.N., Wahli, W., 2002. PPAR expression and function during vertebrate development. *Int J Dev Biol.* 46 (1), 105-114.

Miller, W.L., 2008. Steroidogenic enzymes. *Endocr Dev.* 13, 1-18.

Mok, W.M., Bukusoglu, C., Krieger, N.R., 1995. 3 alpha-hydroxy-5 alpha- pregnan-20-one is the only active anesthetic steroid in anesthetized mouse brain. *Steroids.* 58, 112-114.

Monnet, F.P., Mahe, V., Robel, P., Baulieu, E.E., 1995. Neurosteroids, via sigma receptors, modulate the [3H]norepinephrine release evoked by N-methyl-D-aspartate in the rat hippocampus. *Proc. Natl. Acad. Sci. USA.* 92, 3774-3778.

Morato, G.S., Ferreira, V.M., Ferrara, P., Farges, R.C., 2001. Effects of central and systemic injections of peripheral benzodiazepine receptor ligands on the anxiolytic actions of ethanol in rats. *Addict. Biol.* 6, 129-136.

Moreno, S., Farioli-Vecchioli, S., Ceru, M.P. 2004. Immunolocalization of peroxisome proliferator-activated receptors and retinoid X receptors in the adult rat CNS. *Neurosci.* 123, 131-145.

Morohashi, K., Sogawa, K., Omura, T., Fujii-Kuriyama, Y., 1987. Gene structure of human cytochrome P-450(SCC), cholesterol desmolase. *J. Biochem.* (Tokyo). 101, 879-887.

Morrow, A.L., Suzdak, P.D., Paul, S.M., 1987. Steroid hormone metabolites potentiate GABA receptor-mediated chloride ion flux with nanomo- lar potency. *Eur. J. Pharmacol.* 142, 483-485.

Morrow, A.L., Pace, J.R., Purdy, R.H., Paul, S.M., 1990. Characterization of steroid interactions with gamma-aminobutyric acid receptor-gated chloride ion channels: evidence for multiple steroid recognition sites. *Mol. Pharmacol.* 37, 263-270.

Morrow, A.L., Devaud, L.L., Purdy, R.H., Paul, S.M., 1995. Neuroactive steroid modulators of the stress response, in: G. Chrousos, R. McCarty, K. Pacak, G. Cizza, E. Sternberg, P. Gold, R. Kvetnansky (Eds.), Stress: Basic Mechanisms and Clinical Implications, Vol. 771, Annals New York Academy of Science, New York, pp. 257-272.

Morrow, A.L., VanDoren, M.J., Penland, S.N., Matthews, D.B., 2001a. The role of GABAergic neuroactive steroids in ethanol action, tolerance

and dependence. *Brain Res. Brain Res. Rev.* 37, 98-109.

Morrow, A.L., NVanDoren, M.J., Fleming, R., Penland, S., 2001b. Ethanol and neurosteroid interactions in the brain, in: G. Biggio, R.H. Purdy (Eds.), Neurosteroids and Brain Function, Vol. 46, Academic Press, San Diego, pp. 349-377.

Muller-Preuss, P., Rupprecht, R., Lancel, M., 2002. The effects of the neuroactive steroid 3alpha,5alpha-THDOC on sleep in the rat, *Neuroreport.* 13, 487-490.

Natarajan, V., Schmid, P.C., Reddy, P.V., Schmid, H.H., 1984. Catabolism of N-acylethanolamine phospholipids by dog brain preparations. *Journal of Neurochemistry.* 42 (6), 1613-1619.

Nelson, L.E., Guo, T.Z., Lu, J., Saper, C.B., Franks, N.P., Maze, M., 2002. The sedative component of anesthesia is mediated by GABA(A) receptors in an endogenous sleep pathway. *Nat. Neurosci.* 5, 979-984.

Normington, K., Russell, D.W., 1992. Tissue distribution and kinetic characteristics of rat steroid 5 alpha-reductase isozymes. Evidence for distinct physiological functions. *J. Biol. Chem.* 267, 19548-19554.

Okamoto, Y., Morishita, J., Tsuboi, K., Tonai, T., Ueda, N., 2004. Molecular characterization of a phospholipase D generating anandamide and its congeners. *Journal of Biological Chemistry.* 279 (7), 5298-5305.

Oonk, R.B., Parker, K.L., Gibson, J.L., Richards, J.S., 1990. Rat cholesterol side-chain cleavage cytochrome P-450 (P-450scc) gene. Structure and regulation by cAMP in vitro. *J. Biol. Chem.* 265, 22392-

22401.

Papadopoulos, V., Lecanu, L., Brown, R.C., Han, Z., Yao, Z.X., 2006a. Peripheral-type benzodiazepine receptor in neurosteroid biosynthesis, neuropathology and neurological disorders. *Neuroscience.* 138, 749-756.

Papadopoulos, V., Baraldi, M., Guilarte, T.R., Knudsen, T.B., Lacapere, J.J., Lindemann, P., Norenberg, M.D., Nutt, D., Weizman, A., Zhang, M.R., Gavish, M., 2006b. Translocator protein (18kDa): New nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. *Trends Pharmacol. Sci.* 27, 402-409.

Patel, S., Carrier, E.J., Ho, W.S., Rademacher, D.J., Cunningham, S., Reddy, D.S., Falck, J.R., Cravatt, B.F., Hillard, C.J., 2004. The post-mortal accumulation of brain N-arachidonylethanolamine (anandamide) is dependent upon fatty acid amide hydrolase activity. *Journal of Lipid Research.* 1 December, Electronic publication.

Pathirathna, S., Brimelow, B.C., Jagodic, M.M., Krishnan, K., Jiang, X., Zorumski, C.F., Mennerick, S., Covey, D.F., Todorovic, S.M., Jevtovic-Todorovic, V., 2005. New evidence that both T-type calcium channels and GABAA channels are responsible for the potent peripheral analgesic effects of 5alpha-reduced neuroactive steroids. *Pain.* 114, 429-443.

Patte-Mensah, C., Kappes, V., Freund-Mercier, M.J., Tsutsui, K., Mensah-Nyagan, A.G., 2003. Cellular distribution and bioactivity of the key steroidogenic enzyme, cytochrome P450side chain cleavage, in sensory

neural pathways. *J. Neurochem.* 86, 1233-1246.

Patte-Mensah, C., Penning, T.M., Mensah-Nyagan, A.G., 2004. Anatomical and cellular localization of neuroactive 5 alpha/3 alpha-reduced steroid-synthesizing enzymes in the spinal cord. *J. Comp. Neurol.* 477, 286-299.

Patte-Mensah, C., Kibaly, C., Mensah-Nyagan, A.G., 2005. Substance P inhibits progesterone conversion to neuroactive metabolites in spinal sensory circuit: A potential component of nociception. *Proc. Natl. Acad. Sci. USA.* 102, 9044-9049.

Patte-Mensah, C., Kibaly, C., Boudard, D., Schaeffer, V., Begle, A., Saredi, S., Meyer, L., Mensah-Nyagan, A.G., 2006. Neurogenic pain and steroid synthesis in the spinal cord. *J. Mol. Neurosci.* 28, 17-31.

Paul, S.M., Purdy,RH., 1992. Neuroactive steroids. *FASEB J.* 6, 2311-2322. Paxinos, G., Franklin, K.B.J., 2001. The mouse brain in stereotaxic coordinates, second ed. Academic Press, San Diego.

Pawlowski, J.E., Huizinga, M., Penning, T.M., 1991. Cloning and sequencing of the cDNA for rat liver 3 alpha-hydroxysteroid/dihydrodiol dehydrogenase. *J. Biol. Chem.* 266, 8820-8825.

Penning, T.M., Jin, Y., Heredia, V.V., Lewis, M., 2003. Structure-function relationships in 3alpha-hydroxysteroid dehydrogenases: A comparison of the rat and humaniso-forms. *J. Steroid Biochem. Mol. Biol.* 85, 247-255.

Peraza, M.A., Burdick, A.D., Marin, H.E., Gonzalez, F.J., Peters, J.M.,

2006. The toxicology of ligands for peroxisome proliferator-activated receptors (PPAR). *Toxicol Sci.* 90 (2), 269-295.

Poletti, A., Celotti, F., Rumio, C., Rabuffetti, M., Martini, L. (1997) Identification of type 1 5alpha-reductase in myelin membranes of male and female rat brain. *Mol. Cell. Endocrinol.* 129, 181-190.

Pozzi, P., Bendotti, C., Simeoni, S., Piccioni, F., Guerini, V., Marron, T.U., Martini, L., Poletti, A. 2003. Androgen 5-alpha-reductase type 2 is highly expressed and active in rat spinal cord motor neurones. *J. Neuroendocrinol.* 15, 882-887.

Pelletier, G., Luu-The, V., Labrie, F., 1994. Immunocytochemical localization of 5 alpha-reductase in rat brain. *Mol. Cell. Neurosci.* 5, 394-399.

Petrosino, S., Palazzo, E., de Novellis, V., Bisogno, T., Rossi, F., Maione, S., Di Marzo, V., 2007. Changes in spinal and supraspinal endocannabinoid levels in neuropathic rats. *Neuropharmacology.* 52, 415-422.

Pinna, G., Uzunova, V., Matsumoto, K., Puia, G., Mienville, J.M., Costa, E., Guidotti, A., 2000. Brain allopregnanolone regulates the potency of the GABAA receptor agonist muscimol. *Neuropharmacology.* 39, 440-448.

Poletti, A., Coscarella, A., Negri-Cesi, P., Colciago, A., Celotti, F., Martini, L., 1998a. 5 alpha-reductase isozymes in the central nervous system. *Steroids.* 63, 246-251.

Poletti, A., Negri-Cesi, P., Rabuffetti, M., Colciago, A., Celotti, F., Martini, L., 1998b. Transient expression of the 5 alpha-reductase type 2 isozyme in the rat brain in late fetal and early postnatal life. *Endocrinology.* 139, 2171-2178.

Privalle, C.T., Mc Namara, B.C., Dhariwal, M.S., Jefcoate, C.R., 1987. ACTH control of cholesterol side-chain cleavage at adrenal mitochondrial cytochrome P-450scc. Regulation of intramitochondrial cholesterol transfer. *Mol Cell Endocrinol.* 53, 87-101.

Puia, G., Mienville, J.M., Matsumoto, K., Takahata, H., Watanabe, H., Costa, E., Guidotti, A., 2003. On the putative physiological role of allopregnanolone on GABA(A) receptor function. *Neuropharmacology.* 44, 49-55.

Reddy, D.S., 2003. Is there a physiological role for the neurosteroid THDOC in stress-sensitive conditions? *Trends Pharmacol. Sci.* 24, 103-106.

Rockwell, C.E., Snider, N.T., Thompson, J.T, Vanden Heuvel J.P., Kaminski Kuehl, F.A., Jacob, T.A., Ganley, O.H., Ormond, R.E., Meisinger, M.A.P., 1957. The identification of N-(2-hydroxyethyl)-palmitamide as a naturally occurring anti-inflammatory agent. *Journal of the American Chemical Society.* 79, 5577-5578.

Rodriguez, E.M., Désarnaud, F., García, O.P., 2006. Diurnal variation of arachidonoylethanolamine, palmitoylethanolamide and oleoylethanolamide in the brain of the rat. *Life Sci.* 76, 30-37.

Rodríguez de Fonseca, F., Navarro, M., Go´ mez, R., Escuredo, L., Nava, F., Fu, J., Murillo-Rodríguez, E., Giuffrida, A., LoVerme, J., Gaetani, S., Kathuria, S., Gall, C., Piomelli, D., 2001. An anorexic lipid mediator regulated by feeding. *Nature.* 414 (6860), 209-212.

Roehrs, T., Papineau, K., Rosenthal, L., Roth, T., 1999. Ethanol as a hypnotic in insomniacs: self administration and effects on sleep and mood, *Neuropsychopharmacology.* 20, 279-286.

Roehrs, T., Roth, T., 2001. Sleep, sleepiness, and alcohol use. *Alcohol Res. Health.* 25, 101-109.

Romeo, E., Brancati, A., De Lorenzo, A., Fucci, P., Furnari, C., Pompili, E., Sasso, G.F., Spalletta, G., Troisi, A., Pasini, A., 1996. Marked decrease of plasma neuroactive steroids during alcohol withdrawal. *Clin. Pharmacol.* 247, 309-322.

Ross, R.A., Brockie, H.C., Pertwee, R.G., 2000. Inhibition of nitric oxide production in RAW264.7 macrophages by cannabinoids and palmitoylethanolamide. *European Journal of Pharmacology.* 401 (2), 121-130.

Rupprecht, R., di Michele, F., Hermann, B., Ströhle, A., Lancel, M., Romeo, E., Holsboer F., 2001. Neuroactive steroids: molecular mechanisms of action and implications for neuropsychopharmacology. *Brain Res Rev.* 37, 59-67.

Saitoh, H., Hirato, K., Yanaihara, T., Nakayama, T. 1982. A study of 5 alpha-reductase in human fetal brain. *Endocrinol. Jpn.* 29, 461-467.

Schmid, P.C., Zuzarte-Augustin, M.L., Schmid, H.H., 1985. Properties of rat liver N-acylethanolamine amidohydrolase. *Journal of Biological Chemistry.* 260 (26), 14145-14149.

Schmid, H.H., Schmid, P.C., Natarajan, V., 1990. N-acylated glycerophospholipids and their derivatives. *Progress in Lipid Research.* 29 (1), 1-43.

Schmidt, A., Endo, N., Rutledge, S.J., Vogel, R., Shinar, D., Rodan, G.A., 1992. Identification of a new member of the steroid hormone receptor superfamily that is activated by a peroxisome proliferator and fatty acids. *Mol Endocrinol.* 6 (10), 1634-1641.

Schumacher, M., Weill-Engerer, S., Liere, P., Robert, F., Franklin, R.J., Garcia-Segura, L.M., Lambert, J.J., Mayo, W., Melcangi, R.C., Parducz, A., Suter, U., Carelli, C., Baulieu, E.E., Akwa, Y., 2003. Steroid hormones and neurosteroids in normal and pathological aging of the nervous system. *Prog. Neurobiol.* 71, 3-29.

Schomacher M., Müller H.D., Sommer C., Schwab S., Schäbitz W.R., 2008. Endocannabinoids mediate neuroprotection after transient focal cerebral ischemia. *Brain Res.* 1240: 213-220.

Schumacher, M., Guennoun, R., Robert, F., Carelli, C., Gago, N., Ghoumari, A., Gonzalez Deniselle, M.C., Gonzalez, S.L., Ibanez, C., Labombarda, F., Coirini, H., Baulieu, E.E., De Nicola, A.F., 2004. Local synthesis and dual actions of progesterone in the nervous system: Neuroprotection and myelination. *Growth Horm. IGF Res.* 14, S18-33.

Shear D.A., Galani R., Hoffman S.W., Stein D.G., 2002. Progesterone protects against necrotic damage and behavioral abnormalities caused by traumatic brain injury. *Exp Neurol.* 178: 59-67.

Sheerin, A.H., Zhang, X., Saucier, D.M., Corcoran, M.E., 2004. Selective antiepileptic effects of N-palmitoylethanolamide, a putative endocannabinoid. *Epilepsia.* 45 (10), 1184-1188.

Sher, T., Yi, H.F., McBride, O.W., Gonzalez, F.J., 1993. cDNA cloning, chromosomal mapping, and functional characterization of the human peroxisome proliferator activated receptor. *Biochemistry.* 32 (21), 5598-5604.

Showalter, V.M., Compton, D.R., Martin, B.R., Abood, M.E., 1996. Evaluation of binding in a transfected cell line expressing a peripheral cannabinoid receptor (CB2): identification of cannabinoid receptor subtype selective ligands. *Journal of Pharmacology and Experimental Therapeutics*. 278 (3), 989-999.

Sierra, A., Lavaque, E., Perez-Martin, M., Azcoitia, I., Hales, D. B., Garcia-Segura, L.M., 2003. Steroidogenic acute regulatory protein in the rat brain: Cellular distribution, developmental regulation and overexpression after injury. *Eur. J. Neurosci.* 18, 1458-1467.

Sierra, A., 2004. Neurosteroids: the StAR protein in the brain. *J Neuroendocrinol.* 16, 787-793.

Skaper, S.D., Buriani, A., Dal Toso, R., Petrelli, L., Romanello, S., Facci, L., Leon, A., 1996. The aliamide palmitoylethanolamide and

cannabinoids, but not anandamide, are protective in a delayed postglutamate paradigm of excitotoxic death in cerebellar granule neurons. *Proceedings of the National Academy of Sciences of the United States of America*. 93 (9), 3984-3989.

Smart D., Gunthorpe M.J., Jerman J.C., Nasir S., Gray J., Muir A.I., Chambers J.K., Randall A.D., Davis J.B., 2000. The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). *Br J Pharmacol.* 129: 227-230.

Stella, N., Piomelli, D., 2001. Receptor-dependent formation of endogenous cannabinoids in cortical neurons. *Eur J Pharmacol*. 425, 189-196.

Stocco, D.M., 2001 a. StAR protein and the regulation of steroid hormone biosynthesis. *Annu Rev Physiol*. 63, 193-213.

Stocco, D.M., 2001 b. Tracking the role of a star in the sky of the new millennium. *Mol Endocrinol.* 15, 1245-1254.

Stoffel-Wagner, B., 2003. Neurosteroid biosynthesis in the human brain and its clinical implications. *Ann. N. Y. Acad. Sci.* 1007, 64-78.

Sugiura, T., Kondo, S., Kishimoto, S., Miyashita, T., Nakane, S., Kodaka, T., Suhara, Y., Takayama, H., Waku, K., 2000. Evidence that 2-arachidonoylglycerol but not N -palmitoylethanolamine or anandamide is the physiological ligand for the cannabinoid CB2 receptor. Comparison of the agonistic activities of various cannabinoid receptor ligands in HL-60 cells. *Journal of Biological Chemistry.* 275 (1), 605-612.

Sulcova, E., Mechoulam, R., Fride, E., 1998. Biphasic effects of anandamide. *Pharmacol Biochem Behav.* 59, 347-352.

Sun, Y.X., Tsuboi, K., Okamoto, Y., Tonai, T., Murakami, M., Kudo, I., Ueda, N., 2004. Biosynthesis of anandamide and N-palmitoylethanolamine by sequential actions of phospholipase A2 and lysophospholipase D. *Biochemical Journal*. 380 (Part 3), 749-756.

Sun, Y.X., Tsuboi, K., Zhao, L.Y., Okamoto, Y., Lambert, D.M., Ueda, N., 2005. Involvement of N-acylethanolamine-hydrolyzing acid amidase in the degradation of anandamide and other N-acylethanolamines in macrophages. *Biochim Biophys Acta*. 1736, 211-220.

Sun, Y., Alexander, S.P., Kendall, D.A., Bennett, A.J., 2006. Cannabinoids and PPARalpha signalling. *Biochem Soc Trans.* 34, 1095-1097.

Tan, N.S., Michalik, L., Desvergne, B., Wahli, W., 2005. Multiple expression control mechanisms of peroxisome proliferator-activated receptors and their target genes. *J Steroid Biochem Mol Biol.* 93 (2-5), 99-105.

Thigpen, A.E., Cala, K.M., Russell, D.W., 1993. Characterization of Chinese hamster ovary cell lines express- ing human steroid 5 alpha-reductase isozymes. *J. Biol. Chem.* 268, 17404-17412.

Thomas, A.J., Nockels, R.P., Pan, H.Q., Shaffrey, C.I., Chopp, M., 1999. Progesterone is neuroprotective after acute experimental spinal cord trauma in rats. *Spine.* 24, 2134-2138.

Thomson, M., 2003. Does cholesterol use the mitochondrial contact site as aconduit to the steroidogenic pathway? *Bioessays.* 25: 252–258.

Tien, E.S., Hannon, D.B., Thompson J.T., Vanden Heuvel, J.P., 2006. Examination of ligand-dependent coactivator recruitment by peroxisome proliferator-activated receptor-alpha (PPARalpha). *PPAR Res.* 2006, 69612.

Tobler, I., Borbely, A.A., 1986. Sleep EEG in the rat as a function of prior waking. *Electroencephalogr. Clin. Neurophysiol.* 64, 74-76.

Torres, J.M., Ortega, E., 2003. Differential regulation of steroid alpha-reductase isozymes expression by androgens in the adult rat brain. *FASEB J.* 17, 1428-1433.

Tsuboi, K., Sun, Y.X., Okamoto, Y., Araki, N., Tonai, T., Ueda, N., 2005. Molecular characterization of N-acylethanolamine-hydrolyzing acid amidase, a novel member of the choloylglycine hydrolase family with structural and functional similarity to acid ceramidase. *J Biol Chem*. 280, 11082-11092.

Twyman, R.E., Mcdonald, R.L., 1992. Neurosteroid regulation of GABAA receptor single-channel kinetic properties of mouse spinal cord neurons in culture. *J Physiol.* 456, 215-45.

Ueda, N., Kurahashi, Y., Yamamoto, S., Tokunaga, T., 1995. Partial purification and characterization of the porcine brain enzyme hydrolyzing and synthesizing anandamide. *Journal of Biological Chemistry.* 270 (40), 23823-23827.

Ueda, N., Yamanaka, K., Yamamoto, S., 2001. Purification and

characterization of an acid amidase selective for N-palmitoylethanolamine, a putative endogenous anti-inflammatory substance. *Journal of Biological Chemistry.* 276 (38), 35552-35557.

Ueda, N., Tsubo, K., Lambert, D.M., 2005. A second N-acylethanolamine hydrolase in mammalian tissues. *Neuropharmacology*. 48, 1079-1085.

Vallée M., Rivera J.D., Koob G.F., Purdy R.H., Fitzgerald R.L., 2000. Quantification of neurosteroids in rat plasma and brain following swim stress and allopregnanolone administration using negative chemical ionization gas chromatography/mass spectrometry. *Anal Biochem.* 287: 153-166.

VanDoren, M.J., Matthews, D.B., Janis, G.C., Grobin, A.C., Devaud, L.L., Morrow, A.L., 2000. Neuroactive steroid 3a-hydroxy-5a-pregnan- 20-one modulates electrophysiological and behavioral actions of ethanol. *J. Neurosci.* 20, 1982-1989.

Vanover, K.E., Suruki, M., Robledo, S., Huber, M., Wieland, S., Lan, N.C., Gee, K.W., Wood, P.L., Carter, R.B., 1999. Positive allosteric modulators of the GABAA receptor: differential interaction of benzodiazepines and neuroactive steroids with ethanol. *Psychopharmacology.* 141, 77-82.

Walter, L., Franklin, A., Witting, A., Möller, T., Stella, N., 2002. Astrocytes in culture produce anandamide and other acylethanolamides. *J Biol Chem*. 277, 20869-20876.

Walter, L., Stella, L., 2003. Endothelin-1 increases 2-arachidonyl glycerol (2-AG) production in astrocytes. *Glia*. 44, 85-90.

Wang J.M., Liu L., Irwin R.W., Chen S,. Brinton R.D., 2008. Regenerative potential of allopregnanolone. *Brain Res Rev.* 57: 398-409.

Watanabe, K., Matsunga, T., Nakamura, S., Kimura, T., Ho, I.K., Yoshimura, H., Yamamoto, I., 1999. Pharmacological effects in mice of anandamide and its related fatty acid ethanolamides, and enhancement of cataleptogenic effect of anandamide by phenylmethylsulfonyl fluoride. *Biol Pharm Bull.* 22, 366-370.

Waters S.L., Miller G.W., Aleo M.D., Schnellmann R.G., 1997. Neurosteroid inhibition of cell death. *Am J Physiol.* 273: F869-876.

Wilson, J D., Griffin, J.E., Russell, D.W., 1993. Steroid 5 alpha-reductase 2 deficiency. *Endocr. Rev.* 14, 577-593.

Wu, F.S., Gibbs, T.T., Farb, D.H., 1991. Pregnenolone sulfate: A positive allosteric modulator at the N-methyl-D- aspartate receptor. *Mol. Pharmacol.* 40, 333-336.

Zoete, V., Grosdidier, A., Michielin, O., 2007. Peroxisome proliferator-activated receptor structures: ligand specificity, molecular switch and interactions with regulators. *Biochim Biophys Acta.* 1771, 915-925.

Zhu, Y., Bond, J., Thomas, P., 2003a. Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. *Proc. Natl. Acad. Sci.*

USA. 100, 2237-2242.

Zhu, Y., Rice, C.D., Pang, Y., Pace, M., Thomas, P., 2003b. Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. *Proc. Natl. Acad. Sci. USA.* 100, 2231-2236.

Zygmunt P.M., Petersson J., Andersson D.A., Chuang H., Sørgård M., Di Marzo V., Julius D., Högestätt E.D., 1999. Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature.* 400: 452-457.