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REVIEW

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Fluoxetine and norfluoxetine stereospecifically and selectively increase brain neurosteroid content at doses that are inactive on 5-HT reuptake

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Abstract It has recently become more clearly understood that in human brain pathophysiology, neurosteroids play a role in anxiety disorders, premenstrual syndrome, postpartum depression, posttraumatic stress disorder, and depression. In the treatment of major depression, recent clinical studies indicate that the pharmacological profiles of fluoxetine and fluvoxamine are correlated with the ability of these drugs to increase the brain and cerebrospinal fluid content of allopregnanolone (Allo), a potent positive allosteric modulator of gamma-aminobutyric acid (GABA) action at $GABA_A$ receptors. Thus, the neurosteroid-induced positive allosteric modulation of GABA action at $GABA_A$ receptors is facilitated by fluoxetine or its congeners (i.e., paroxetine, fluvoxamine, sertraline), which may not block 5-HT reuptake at the doses currently prescribed in the clinic. However, these doses are effective in the treatment of premenstrual dysphoria, anxiety, and depression. In socially isolated mice, we tested the hypothesis that fluoxetine, norfluoxetine, and other specific serotonin reuptake inhibitor (SSRI) congeners stereoselectively upregulate neurosteroid content at doses insufficient to inhibit 5-HT reuptake; although they potentiate pentobarbital-induced sedation and exert antiaggressive action. Very importantly, the inhibition of 5-HT reuptake lacks stereospecificity and requires fluoxetine and norfluoxetine doses that are 50-fold greater than those required to increase brain Allo content, potentiate the action of pentobarbital, or antagonize isolation-induced aggression. Based on these findings, it could be inferred that the increase of brain Allo content elicited by fluoxetine and norfluoxetine, rather than the selective inhibition of 5-HT reuptake, may be operative in the fluoxetine-induced remission of the behavioral abnormalities associated with mood disorders. Therefore, the term "SSRI" may be misleading in defining the pharmacological profile of flouxetine and its congeners. To this extent, the term "selective brain steroidogenic stimulants" (SBSSs) could be proposed.

Keywords Allopregnanolone · Testosterone · 5α-reductase type I · SSRIs · Aggressive behavior · $GABA_A$ receptors · Social isolation · Premenstrual dysphoria · Anxiety · Depression

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Introduction

Neurosteroids are synthesized in the central nervous system (CNS), where they turn over in a manner that is unrelated to peripheral source renovation rates (Baulieu, 1981; Baulieu et al. 2001; Cheney et al. 1995; Guidotti et al. 2001). The two most studied neurosteroids are the progesterone derivative 3α-hydroxysteroid-5α-pregnan-20-one (allopregnanolone, Allo) and 5α-dihydroprogesterone (5α-DHP). In the brain, they are synthesized from progesterone by the sequential action of two enzymes: 5α-reductase type I, which transforms progesterone into 5α-DHP; and 3α-hydroxysteroid oxidoreductase (3α-HSD), which reduces 5α-DHP into Allo in a reversible manner (Dong et al. 2001).

The exact CNS cellular location of these enzymes has not been defined, but it is known that their distribution and the content of 5α-DHP and Allo in various brain regions is not uniform (Pinna et al. 2000; Dong et al. 2001). For example, in rodents, the olfactory bulb contains concentrations of 5α-DHP and Allo that are three- to five-fold higher than those expressed in the frontal cortex or in other brain areas. 5α-DHP mediates a genomic action and interacts with high affinity with the intracellular progesterone receptors (Rupprecht and Holsboer 1999). On the other hand, Allo acts as a potent (nM affinity) positive allosteric modulator of the action of gamma-aminobutryic acid (GABA) at $GABA_A$ receptors, where it potentiates the intensity of GABA-gated Cl^- currents (Puia et al. 1990, 1991, 2003; Lambert et al. 2003).

When administered systemically, Allo expresses anticonvulsant, anxiolytic, and, at the highest doses, sedative-hypnotic actions that are similar to those elicited by other positive allosteric modulators of GABA action at $GABA_A$ receptors [i.e., barbiturates, benzodiazepines (BZs)] (Guidotti et al. 2001; Majewska 1992): These pharmacological actions define Allo as a neuroactive steroid. In fact, the term "neuroactive steroid" refers to the actions of Allo when it is not synthesized in brain, but reaches the brain and acts on $GABA_A$ receptors, positively and allosterically modifying neuronal activity.

Link between $GABA_A$ receptor function and 3α-pregnane steroids

A clear link between neurosteroids (steroids that are synthesized in the brain) and $GABA_A$ receptor function upregulation was first detected in the early '90s in studies with patch-clamp technology (Mienville and Vicini 1989; Puia et al. 1990, 1993; Vicini et al. 1991). In these studies, the actions of the two progesterone metabolites, 3α-5α-Allo and 3α,5β-Allo (which are collectively termed Allo), as well as the neuroactive steroid 3α,21-dihydroxy-5α-pregnan-20-one [tetrahydrodeoxycorticosterone (TH-DOC)], were investigated on various recombinant $GABA_A$ receptor subtypes transfected in 293 kidney cell lines (Puia et al. 1990, 1993). GABAergic signal transduction facilitation elicited by Allo was also tested in postsynaptic $GABA_A$ receptors located on inhibitory synapses expressed in hippocampal and cortical pyramidal neurons (Vicini et al. 1991). These studies were successively confirmed in other laboratories and established that Allo and its 3α-pregnane steroid congeners positively and allosterically modulate the action of GABA at various native or recombinant $GABA_A$ receptor subtypes when applied in the low nM concentration range (reviewed in Lambert et al. 1987, 2003; Paul and Purdy 1992). Allo and its congeners bind to specific high-affinity sites expressed on $GABA_A$ receptors, which differ from those that mediate the BZ-positive allosteric modulation of GABA action. In fact, the upregulation of GABA-active BZ recognition sites expressed at $GABA_A$ receptors requires the presence of two α subunits (α1, α2, α3, or α5), two β subunits (β1, β2, or β3), and one γ2 subunit to form the hetero-pentameric $GABA_A$ receptor complex (Costa and Guidotti 1996). In contrast, Allo is equipotent in facilitating GABA's action on $GABA_A$ receptor subtypes that either are homo-oligomeric (expressing only β subunits of $GABA_A$ receptors), or heteromeric (expressing α and β, or α, β, and γ subunits) (Puia et al. 1990).

Recent studies report that $GABA_A$ receptors, including α4, α6, and δ subunits in combination with γ and β subunits, may have a higher affinity for neurosteroids than other recombinant $GABA_A$ receptor subtypes that express different subunit combinations (Belelli et al. 2002; Belelli and Lambert 2005). However, the affinity of neurosteroids for these $GABA_A$ receptor subtypes remains in the low nM range. Thus, neurosteroid allosteric positive modulators of the action of GABA at $GABA_A$ receptors show a pharmacological profile much less differentiated than that of the $GABA_A$ receptor modulation by BZs (Puia et al. 1990, 1991; Vicini et al. 1991; Costa and Guidotti 1996). In fact, BZs virtually fail to modulate GABA action at $GABA_A$ receptors containing α4 or α6 subunits. Zolpidem for instance, modulates only $GABA_A$ receptors that include α1 subunits (Rowlett et al. 2005). The spectrum of Allo pharmacological actions is broader than that of BZs and resembles those of barbiturates, even though the mechanisms of $GABA_A$ receptor modulation by neurosteroids differ from those of barbiturates (Puia et al. 1990).

Since Allo and its congeners fail to positively modulate glycine, *N*-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), or voltage sensitive Na^+ or Ca^{2+} channels even in high concentrations (Lambert et al. 2003), it is currently believed that the pharmacological actions of neurosteroids (e.g., anxiolytic, anticonvulsant, sedative, hypnotic) are mediated by a positive allosteric modulation of GABA action at virtually every $GABA_A$ receptor subtype.

Physiological relevance of neurosteroids

Behavioral and electrophysiological evidence substantiates the hypothesis that brain Allo is a physiological modulator of GABA with permissive action at $GABA_A$ receptors (Pinna et al. 2000; Puia et al. 1990, 1993, 2003; Dong et al. 2001). Since brain Allo and its congeners are expressed in physiologically relevant concentrations (nM) and are equipotent in facilitating GABA actions at several $GABA_A$ receptor subtypes, these data suggest that if widely distributed in the brain, defective or excessive production and/or release of neurosteroids can modulate GABA-mediated neuronal inhibition. However, the uneven capacity of various brain structures to synthesize Allo (olfactory bulb> frontal cortex>hippocampus>cerebellum) (Cheney et al. 1995; Pinna et al. 2000; Dong et al. 2001) is probably an important factor that contributes to the diverse efficacy of Allo on GABAergic signal transduction in different brain regions. Recent studies have recognized that certain neurons (i.e., mitral cells in the olfactory bulb, pyramidal neurons in the neocortex and hippocampus) express high levels of the enzymes [5α-reductase type I (5α-RI) and 3α-hydroxysteroid dehydrogenase (3α-HSD)] that catalyze the synthesis of Allo from progesterone (Agis-Balboa et al. 2005). Furthermore, it should be noted that the Allo concentrations (nM) expressed in the cortex and hippocampus are sufficient to amplify the GABA gating of Cl^- channels in cortical pyramidal neuron somata (Puia et al. 2003).

It is assumed that Allo released in the vicinity of GABAergic synapses via region- and neuron-specific local biosynthesis and/or release mechanisms may protect $GABA_A$ receptors from modulatory oscillations in circulating steroid levels as they occur, for example during estrus cycles (Paul and Purdy 1992), premenstrual dysphoric disorders (Girdler et al. 2001), and depressive states (Uzunova et al. 1998); and in rodents during pregnancy (Concas et al. 1998), stress (for a review see

Barbaccia et al. 2001), or protracted social isolation (Matsumoto et al. 1999; Pinna et al. 2003a; for a review see Matsumoto et al. 2005). In addition, other studies have shown that administration of Allo exerts antidepressive (Khisti et al. 2000; Khisti and Chopde 2000), antiaggressive (Pinna et al. 2003a; Rupprecht 2003; Matsumoto et al. 2005), anxiolytic (Bitran et al. 1993, 2000; Le Mellédo and Baker 2002; Jain et al. 2005; Reddy et al. 2005), and anticonvulsant effects (Belelli et al. 1989). Taken together, these findings support the hypothesis that $GABA_A$ receptor signal transduction may be physiologically influenced by brain region- and neuron-specific Allo synthesis and secretion rates (Cheney et al. 1995; Dong et al. 2001; Pinna et al. 2000; Puia et al. 2003; Agis-Balboa et al. 2005).

The clinical pharmacological profile of fluoxetine and other SSRIs may depend on the ability of these drugs to increase brain neurosteroid content

Following the pioneering observation that fluoxetine and paroxetine treatment unevenly increases the content of Allo in various brain structures (olfactory bulb>frontal cortex> cerebellum) of adrenalectomized and castrated rodents, we suggested that an increase of brain Allo content should be considered among the mechanisms that mediate some of the pharmacological actions of fluoxetine and its congeners (Uzunov et al. 1996; Guidotti and Costa 1998). These drugs are termed "specific serotonin reuptake inhibitors" (SSRIs) (Wong et al. 1993). Traditionally, it has been proposed that 5-HT reuptake inhibition is the molecular mechanism underlying the anxiolytic, antipanic, antidysphoric, and antidepressant action of SSRIs (Coppen and Doogan 1988; Bunney and Davis 1965; Schildkraut 1965; reviewed in Hirschfeld 2000, Blier and de Montigny 1994; Mongeau et al. 1997; Burke 2004; Delgado 2004; Leon 2004; Owens 2004; Shelton 2004). However, recent investigations have provided evidence that there are a number of major issues (in addition to the SSRI activity of these drugs) that must be addressed in the interpretation of the broad range of mechanisms mediating the pharmacological actions of fluoxetine and congeners (Nestler et al. 2002; Castrén 2005). Castrén, in a recent review (2005), attempted to explain the mechanism of action of antidepressants, suggesting the "network hypothesis", which proposes instead of the "monoamine hypothesis", that problems in the neuronal communication of specific brain networks might underlie mood disorders in depressive patients. Following this view, antidepressants [e.g., tryciclic antidepressant (TCA) and SSRI] may be beneficial in the treatment of depression not by changing monoamine concentrations at the synapses but by stimulating brain-derived neurotrophic factor (BDNF) expression and neurogenesis, thereby improving synaptic information processing in the affected neural networks (Castrén 2005). For example, why does tianeptine, which downregulates serotonergic neurotransmission and fails to influence noradrenergic transmission (Mennini et al. 1987; Kuroda et al. 1994; McEwen and Olié 2005), elicit significant antidepressant activity? One must also explain why antidepressants, such as fluoxetine and its congeners, act rapidly and are more effective than typical TCA in alleviating the symptoms of premenstrual dysphoria (Steiner et al. 1995; reviewed in Romano et al. 1999; Steiner and Pearlstein 2000; Pearlstein 2002; Guidotti and Costa 1998; van Broekhoven and Verkes 2003), panic disorders (Westenberg 1996; Brambilla et al. 2005), impulsive aggressive behavior in humans (Coccaro and Kavoussi 1997), and the aggressive behavior of socially isolated rodents (Guidotti et al. 2001; Pinna et al. 2003a; Matsumoto et al. 2005). Although the above conditions have been associated with a deficiency in 5-HT and noradrenaline at functionally important receptor sites in the brain (Bunney and Davis 1965; Coppen 1967; Schildkraut 1965; Wong and Licinio 2004), pathogenetically they are also related to an alteration of GABAergic neurotransmission and to the downregulation of neurosteroid biosynthesis (Paul and Purdy 1992; Pearlstein 2002; Guidotti et al. 2001; Pinna et al. 2003a; Costa and Guidotti 1996).

One could argue that a blockade of 5-HT reuptake may neither be necessary nor sufficient to explain the selective ability of fluoxetine and its congeners to counteract premenstrual dysphoria, anxiety, panic, and mood disorders in depressed patients and in impulsive aggression. It is important to note that the fluoxetine doses that alleviate premenstrual dysphoria, aggression, and panic are 0.7 to 1.4 μmol/kg (Romano et al. 1999; Perlstein 2002). From pharmacokinetic data in humans (Altamura et al. 1994) and rats (Potts and Parli 1992), one can hypothesize that a subject treated with a fluoxetine dose of 1 μmol/kg (e.g., 20 mg in a 70 kg subject) expresses brain concentrations of fluoxetine in the submicromolar range. We have shown that in mice, these concentrations of fluoxetine are at least tenfold lower than those necessary to inhibit the reuptake of 5-HT by 50% (i.e., 10–15 μM), but are sufficient to induce an increase of brain neurosteroid levels (Table 3; Fig. 3). Thus, if the results of our experiments in rodents could be applied to humans, one could hypothesize that most of the rapid anxiolytic, antidysphoric, antiaggressive, and antiseizure actions (Pasini et al. 1996; Gigli et al. 1996; Ugale et al. 2004; Favale et al. 1995) of fluoxetine can be explained by the ability of SSRIs to increase brain neurosteroid content. In addition, these actions of fluoxetine most likely cannot be related to 5-HT reuptake inhibition. Importantly, tianeptine, which also inhibits aggression in mice (Mocaer et al. 1988), increases the brain levels of neurosteroids (Pinna et al. 2003b); whereas imipraminine, in doses that completely block 5-HT reuptake, fails to increase brain Allo levels and also fails to block aggression in mice (Pinna et al. 2003a).

The concept that an increase of Allo bioavailability may mediate the anxiolytic and contribute to the antidepressant actions of fluoxetine and its congeners is currently supported by the observation that Allo levels in cerebrospinal fluid (CSF) are ~40 fmol/ml in nonpsychiatric subjects and are lower, approximately 15 fmol/ml, in patients with unipolar major depression (Uzunova et al. 1998). However, in these patients, 8 to 10 weeks of treatment with fluoxetine

and fluvoxamine (doses ranging from 0.8 to 4.8 μmoles/kg for fluoxetine, and 1.7 to 9.1 μmoles/kg for fluvoxamine) normalized the low CSF Allo content (Uzunova et al. 1998). The highest therapeutic dose of fluoxetine used in this study (i.e., 4.8 μmoles/kg) may be too low to produce brain levels of the drug capable of inhibiting reuptake of 5-HT by more than 50%. Therefore, one can expect that even at this dose, fluoxetine may fail to elicit a significant inhibition of 5-HT reuptake in these patients. Interestingly, the largest increase of CSF Allo content after fluoxetine or fluvoxamine treatment occurs in patients who show the greatest improvement in Hamilton depression rating scale (HAM-D) scores (Uzunova et al. 1998).

Pharmacological strategies for the treatment of neurosteroid downregulation

Considering that signs of anxiety, panic, dysphoria, and mood disorders may be related to a reduction in $GABA_A$ receptor signal transduction efficacy due to the low availability of neurosteroids, it was initially suggested that "neurosteroid replacement" may be a strategy for the treatment of these disorders (Gee et al. 1988). However, the systemic use of natural or synthetic pregnane steroids may not be suitable for therapeutic intervention, because these hormones can be metabolized into derivatives that elicit undesirable side effects (Phillipps 1975). Among other effects, they elicit a dose-dependent sedative action (Reddy and Woodward 2004), and after protracted treatment, they may induce cross-tolerance to BZs (Reddy and Rogawski 2000). Withdrawal from protracted progesterone or Allo treatment has been shown to increase seizure susceptibility (Smith et al. 1998).

BZs, such as diazepam, triazolam, or alprazolam, used frequently in the clinic as coadjutants in the treatment of anxiety disorders associated with depression, are potent full-positive allosteric modulators of the action of GABA at various $GABA_A$ receptors, including $GABA_A$ receptors expressing $\alpha 1$ subunits (Costa and Guidotti 1996). These BZs have a broad spectrum of pharmacological actions very similar to that of pregnane steroids. In fact, they have been extensively used to treat anxiety, panic, dysphoria, and impulsive aggression. However, similar to neurosteroids, the appearance of sedation and amnesia, and the development of tolerance and dependence have negatively impacted the clinical use of these BZs (Costa et al. 2001).

Today, we can circumvent these unwanted effects by using positive allosteric modulators of the action of GABA acting at selected $GABA_A$ receptor subtypes. One such selective positive allosteric modulator of GABAergic signal transduction is imidazenil, an imidazobenzodiazepine devoid of sedative actions because it fails to act at $GABA_A$ receptors including $\alpha 1$ subunits. However, it is a full-positive allosteric modulator of GABA action at $GABA_A$ receptors containing $\alpha 5$ subunits (Costa et al. 2002; Guidotti et al. 2005). This drug, unlike classic BZs or neurosteroids, elicits anxiolytic and anticonvulsant actions, but fails to produce sedation or amnesia, and is devoid of tolerance or dependence liability.

In the treatment of psychiatric disorders associated with a downregulation of neurosteroid expression, an alternative to BZs is the use of drugs that stimulate neurosteroid biosynthesis. As discussed, the pharmacological spectrum of Allo and its congeners as modulators of GABA at $GABA_A$ receptors is broader than that of BZs, and the efficacy of Allo is expressed in every subtype of $GABA_A$ receptors so far investigated for doses in the low nM range. Among drugs that stimulate neurosteroid biosynthesis, one may consider agonists of "mitochondrial BZ receptors", such as the indoleacetamide derivatives (FGIN 1-27 and its congeners) (Romeo et al. 1992; Auta et al. 1993), and drugs acting on key enzymes (i.e., 5α-RI, and 3α-HSD) involved in Allo biosynthesis, such as fluoxetine and its congeners (Matsumoto et al. 1999; Guidotti et al. 2001; Pinna et al. 2003a, 2004a). Importantly, due to the brain region- and neuron-specific expression of mitochondrial BZ receptors or 5α-RI and 3α-HSD, the impact of these drugs on neurosteroid biosynthesis is not expressed globally throughout the brain, but is region-specific. Hence, steroidogenic drugs such as fluoxetine and congeners are devoid of those unwanted side effects caused by direct systemic administration of neuroactive steroids.

Since some of the pharmacological actions of fluoxetine and other SSRIs may depend on the ability of these drugs to increase brain neurosteroids, we anticipate that the present understanding of the mechanism(s) whereby fluoxetine and its congeners increase neurosteroid bioavailability could not only help explain the role of the GABAergic dysfunctions operative in depressed patients, but could also become a target for the development of new antidysphoric, antiaggressive, anxiolytic, and antidepressant medications.

Animal models to study the behavioral responses elicited by fluoxetine and its congeners associated with the strong neurosteroidogenic action of these drugs

Some endophenotypic features of depression, including signs of anxiety, dysphoria, increased psychomotor activity, and increased aggression, can be investigated using animal models to study anxiolytic and antidepressant drugs. Here as an example, we will describe experiments involving socially isolated mice to investigate whether fluoxetine and its congeners exert pharmacological effects via their stimulation of brain neurosteroidogenesis.

Aggression against an intruder is a characteristic behavioral abnormality that can be elicited in male mice by social isolation (Valzelli 1969, 1981). Various strategies are available to quantify this territorially related aggressive behavior induced by social isolation. One such model is the resident–intruder test, which reveals a time-related aggressiveness caused by social isolation in the resident male mouse against a same-sex intruder after isolation lasting 4 weeks (Ojima et al. 1995; Pinna et al. 2003a). Interest-

ingly, the increased intensity of aggressive behavior is inversely related to the extent of Allo content downregulation in the olfactory bulb (Fig. 1), frontal cortex, and hippocampus, but not in the cerebellum (Pinna et al. 2003a). Moreover, when Allo is given subcutaneously to socially isolated male mice, it attenuates the aggression against an intruder mouse in a dose-dependent manner (Fig. 2). These doses of Allo fail to alter gross behavior or locomotor activity in group-housed male mice (Matsumoto et al. 1999).

In male mice socially isolated for 4 weeks, the brain expression of 5α-RI mRNA (Table 1) and protein (Dong et al. 2001) was approximately 50% lower than that of group-housed mice (Table 1), whereas the expression of 3α-HSD mRNA was unchanged (Dong et al. 2001). Since 5α-RI is the rate-limiting step enzyme for neurosteroid biosynthesis (Dong et al. 2001), the decrease in Allo measured in the brains of socially isolated mice occurs in the absence of changes in progesterone brain levels (Table 1). This finding suggests a downregulation of brain Allo biosynthesis rather than an upregulation of its rate of degradation.

In contrast to male mice, socially isolated female mice fail to exhibit aggressive behavior against an intruder of the same sex, and neither male nor female mice express a downregulation of brain Allo content or a decrease of brain 5α-RI mRNA content (Table 1). However, similar to the males, female mice receiving daily testosterone propionate while socially isolated for 3 weeks develop aggression and exhibit a downregulation of Allo content in the olfactory bulb (Table 1) and frontal cortex (Pinna et al. 2004b, 2005). Female mice treated with testosterone also develop an approximately 50% decrease of 5α-RI mRNA expression (Table 1). The extent of this brain Allo content decrease is inversely related to the intensity of aggressive behavior and was blocked by administering small doses of Allo, which have no major behavioral effects when administered to group-housed male mice (Pinna et al. 2005). Submicromolar doses of norfluoxetine, which are capable of increasing brain Allo content in socially isolated male mice, inhibit aggression in female mice treated with testosterone propionate, likely because the decreased brain Allo content is upregulated (Pinna et al. 2005).

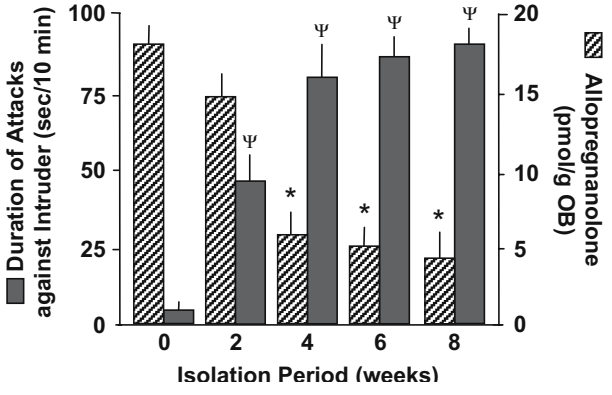

Fig. 1 Time-course of aggressiveness development and olfactory bulb (OB) Allo content decrease during social isolation in male mice. Aggression of a resident mouse against an intruder was measured as the duration of attacks in 10 min. Allo was determined in the OB of the same mice killed immediately after termination of the resident–intruder test. Each value is the mean ±SEM of eight animals. $P<0.01$ OB Allo content at a given social isolation time period compared to social isolation period 0 week (*); $P<0.01$ duration of attacks at a given social isolation time period compared with a social isolation period of 0 week (Ψ). For details see Pinna et al. (2003a)

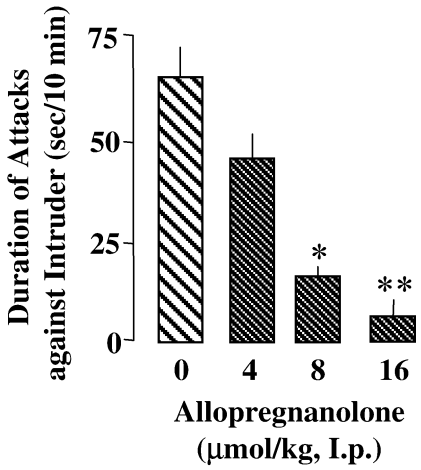

Fig. 2 Dose-dependent inhibition of aggressive behavior elicited by Allo in socially isolated male mice. Each value is the mean ±SEM of six animals. Allo was given 30 min before the resident-intruder test. $P<0.05$ (*); $P<0.01$ (**) Allo-treated mice compared to vehicle-treated mice

Collectively, these findings support the hypothesis that the decrease of brain Allo content is related to the expression of aggressive behavior. These findings also suggest that socially isolated mice may provide a useful model for the study of new potent pharmacological interventions to correct psychiatric and neurological disorders, specifically, those disorders that are associated with the abnormal production or the availability of neurosteroids that positively and allosterically modulate the action of GABA at $GABA_A$ receptors.

Studies in socially isolated mice

Consistent with the finding that the brain concentrations of endogenous Allo regulates the efficacy of $GABA_A$ receptor ligands, we found that socially isolated male mice exhibiting brain Allo content downregulation also display a lower susceptibility to $GABA_A$ receptor agonists and antagonists. For example, they exhibit behavioral impairments and reduced responsiveness to diazepam in tests that detect anxiety levels, such as the elevated-plus maze and the open field test (Pinna et al. 2004c). Doses of norfluoxetine that normalize brain Allo levels reduce the social isolation-induced anxiety-like behavior (Table 2). Socially isolated male mice also express a reduced response to the administration of $GABA_A$ receptor agonists (i.e., pentobarbital) (Table 1), but an increased responsiveness to $GABA_A$ receptor antagonists (i.e., picrotoxin) (Ojima et al. 1995; Matsumoto et al. 1996). In socially isolated male mice, the duration of the loss of righting reflex induced by pentobar-

Table 1 Righting reflex loss (RRL) after pentobarbital (PTB), duration of attacks against an intruder (Aggression), 5α-RI mRNA expression, progesterone (P), Allo, and testosterone (T) levels in the brain of socially isolated (SI) or group housed (GH) male and female mice

Mice	PTB-RRL (min)	Aggression (duration s/10 min)	5α-RI mRNA attomol/μg RNA	P (pmol/g)	Allo (pmol/g)	T (pmol/g)
Male						
GH Sham	78±9.8	none	365±21	19±5.9	16±3.6	31±4.8
SI Sham	40±7.5*	68±11	177±15*	18±6.4	7.1±1.2*	35±4.5
Female						
GH Sham	83±13	none	415±41	65±15	14±2.3	12±4.1
SI Sham	74±5.8	none	437±35	58±13	16±3.1	10±5.0
SI+Spayed+TP	45±8.7**	75±8.3**	280±21**	n.d.	6.9±1.3**	44±18**

Allo, progesterone and testosterone levels were determined in the olfactory bulbs and 5 α-RI mRNA in the frontal cortex of mice socially isolated or group housed for 4 weeks. Decapitation occurred immediately following behavioral tests. Testosterone propionate (TP), (0.5 mg/kg s.c.) was administered daily for 3 weeks. Each value is the mean ±SEM of six to eight animals (Pinna et al. 2005)
n.d. not determined
*$P<0.01$ compared with GH male mice; **$P<0.01$ compared with respective female mouse control group [one-way analysis of variance (ANOVA) followed by Dunnett's test]

bital administration was reduced by approximately 50% (Matsumoto et al. 1996). Instead, picrotoxin-induced seizure susceptibility was increased in male mice following protracted social isolation (Matsumoto et al. 2003). In these mice, the systemic administration of Allo returned the picrotoxin-induced seizure threshold to control values; however, these doses of Allo per se failed to change the picrotoxin-induced seizure threshold in group housed mice (Matsumoto et al. 2003).

Studies in mice treated with SKF105,111, a specific 5α-RI inhibitor

To provide further evidence that the decrease of brain Allo content in socially isolated mice is responsible for the altered responsiveness of $GABA_A$ receptors to specific agonists and antagonists, we induced a decrease of brain Allo content by administering the potent 5α-RI inhibitor 17β-(*N*,*N*-diisopropylcarbamoyl)-androstan-3,5-diene-3-carboxylic acid (SKF 105,111; SKF) to group-housed male mice (Matsumoto et al. 1999; Pinna et al. 2000). The systemic administration of SKF induced a fast-occurring (30 to 60 min) and marked (~80%) reduction of brain Allo content that lasted for at least 6 h (Pinna et al. 2000; Puia et al. 2003). Similar to social isolation, SKF pretreatment shortened the duration of the pentobarbital-induced sedative action (Matsumoto et al. 1999). This pretreatment also reduced the duration of the sedative action of the $GABA_A$ receptor agonist muscimol (Pinna et al. 2000). Furthermore, in SKF-treated mice, the convulsive threshold of picrotoxin was lowered, and this change was reversed by Allo administration (Matsumoto et al. 2003).

Table 2 *S*-norfluoxetine reduces the anxiety-like behavior of socially isolated male mice

Mice	Anxiety-like behavior	
	Elevated plus-maze Open/Closed arm time	Locomotor activity Margin/Center time
GH+VH	0.68±0.05	2.6±0.8
SI+VH	0.49±0.04	5.5±1.1
SI+*S*-NFLX	0.61±0.04*	3.1±0.9*

Elevated plus-maze experiments were performed in mice socially isolated for 4 weeks. Locomotor activity was determined using the open-field monitoring system assisted by VERSAMAT software. Anxiety-like behavior was defined by the ratio of the time spent in the margin versus the time spent in the center of the open field (Pinna et al. 1997). Tests were performed 30 min after the injection of 1.8 μmol/kg *S*-norfluoxetine or vehicle. Each value is the mean ± SEM of six animals
S-NFLX=*S*-norfluoxetine
*$P<0.01$ compared to SI+VH group [One way analysis of variance (ANOVA) followed by Dunnett's test]

In electrophysiological studies conducted with brain slices from mice with a reduced (80%) Allo concentration induced by the administration of SKF, a downregulation of the susceptibility to exogenously applied GABA or muscimol to evoke Cl^- currents was shown, as well as a reduction in the expression of spontaneous inhibitory postsynaptic currents recorded from neocortical pyramidal neurons (Puia et al. 2003). Furthermore, a direct application of Allo to brain slice analogous to those described above reversed the GABA dose-response efficacy to a profile similar to that expressed by nontreated slices (Puia et al. 2003). These results support the concept that endogenous neurosteroids contribute to the maintenance of the physiological modulation of GABAergic signal transduction.

The actions of fluoxetine and norfluoxetine on brain Allo content are stereoisomeric and unrelated to their efficacy as 5-HT reuptake inhibitors

The finding that social isolation is associated with a downregulation of brain Allo content and that Allo pretreatment not only abolishes aggressiveness but corrects the altered

responses of $GABA_A$ receptors to specific agonists and antagonists led to the hypothesis that drugs that upregulate brain Allo content may normalize the behavioral abnormalities expressed in mice with a downregulation of brain Allo content.

We have previously reported that fluoxetine and paroxetine increase the olfactory bulb and frontal cortex Allo content dose-dependently without changing brain progesterone and pregnenolone levels (Uzunov et al. 1996; Matsumoto et al. 1999). Since fluoxetine is an *S* and *R* racemic mixture that is metabolized into *S*- or *R*-norfluoxetine (Potts and Parli 1992), we tested the *S*- or *R*-fluoxetine and *S*- or *R*-norfluoxetine activities to evaluate the stereospecificity of the ability of either drug to modify brain Allo content. Additionally, we investigated whether fluoxetine and norfluoxetine doses that change brain Allo content differ from those that inhibit 5-HT reuptake. Table 3 shows that fluoxetine and norfluoxetine in submicromolar doses and in a stereospecific manner reverse the decrease of Allo brain levels caused by social isolation. The *S*-stereoisomers of fluoxetine or norfluoxetine are several-fold more potent than their respective *R*-stereoisomers (Table 3). We also report (Table 3) that *S*-norfluoxetine is approximately five times more potent than *S*-fluoxetine. Both drugs have a rapid latency period in which they increase brain Allo content (reaching a plateau in 30 min) that lasts for several hours. Importantly, the effective concentrations 50% (EC_{50}s) of *S*-fluoxetine and *S*-norfluoxetine that normalize the brain Allo content are 10- (*S*-fluoxetine) and 50-fold (*S*-norfluoxetine) lower than their respective EC_{50}s needed to inhibit 5-HT reuptake (Table 3 and Fig. 3).

Pharmacokinetic studies of *S*- and *R*-norfluoxetine (Potts and Parli 1992) suggest that the higher potency of *S*-norfluoxetine in the increase of brain Allo is not related to differences in the metabolic rates of these two stereoisomers. In fact, both *S*- and *R*-norfluoxetine have a slow clearance rate. It has been reported that the brain clearing rates of the two stereoisomers are virtually identical (Potts and Parli 1992). Since *S*-fluoxetine elimination is as slow as that of *R*-fluoxetine, the data support a high pharmacological specificity of the *S*-isomers. Further, *S*-norfluoxetine has a higher potency than *S*-fluoxetine (Fig. 3) in increasing neurosteroid levels when given in doses that fail to change 5-HT reuptake (Pinna et al. 2003a, 2004a). In fact, the threshold dose of *S*-norfluoxetine that inhibits 5-HT reuptake is more than 50 times greater than the threshold dose that increases Allo brain levels (Fig. 3). More importantly, 5-HT reuptake action fails to be stereospecific (Table 3).

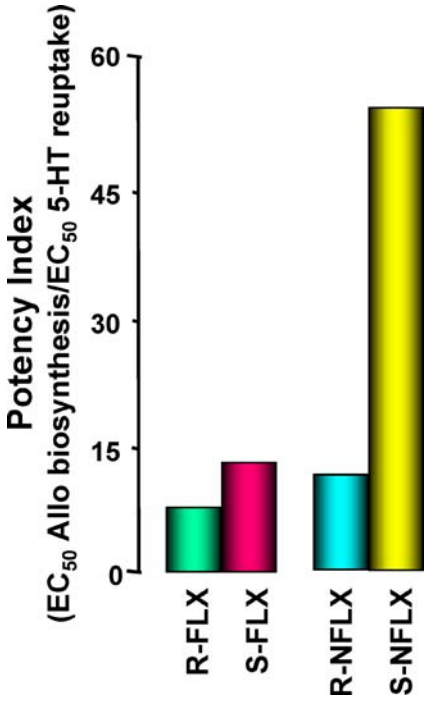

Fig. 3 The stereospecific potency of *S*-norfluoxetine needed to stimulate Allo biosynthesis is 55 times higher than 5-HT reuptake inhibition. Data on the ordinate (potency index) represent the ratios between the EC_{50} doses that stimulate Allo biosynthesis and the EC_{50} doses that inhibit 5-HT reuptake. Each value is the mean of four to six socially isolated mice (data from Table 3)

The possibility that fluoxetine and its congeners could abolish the behavioral deficits associated with protracted

Table 3 Fluoxetine and norfluoxetine stereisomer normalization of pentobarbital-induced righting reflex loss (PTB-LRR), inhibition of duration of attacks against an intruder (Aggression), activation of steroidogenesis (Allo biosynthesis) is not linked to their ability to inhibit 5-HT reuptake

Mice	PTB-RRL (EC_{50}, μmol/kg)	Aggression (EC_{50}, μmol/kg)	Allo biosynthesis (EC_{50}, μmol/kg)	5-HT Reuptake (EC_{50}, μmol/kg)
S-Fluoxetine	0.70±0.2*	0.71±0.03*	0.80±0.07*	10.5±2.4
R-Fluoxetine	>1.80	1.30±0.02	>1.80	13.7±3.2
S-Norfluoxetine	0.25±0.1**	0.20±0.08**	0.15±0.03**	8.3±3.1
R-Norfluoxetine	1.70±0.3	1.53±0.20	>0.9	10.1±3.8

Drugs were administered 30 min before behavioral tests, measurement of Allo, and [^{14}C]5-HT reuptake measurements. Data represent the mean ±SEM of four to six mice socially isolated for 4 weeks before testing. The EC_{50}s were calculated from dose-response curves analyzed by the "quantal dose-response: probits test" as described by Tallarida and Murray (1987) equipped with a statistical package. Statistical comparisons among the different inhibitory concentration 50% (IC_{50}) values were performed by using the COHORT package. For details see Pinna et al. (2003a, 2004a)

*P<0.01 in *S*-fluoxetine compared to *R*-fluoxetine; **P<0.001 in *S*-norfluoxetine compared to *R*-norfluoxetine and *S*-fluoxetine

social isolation by increasing brain Allo content and not by a mechanism involving 5-HT reuptake inhibition was tested by measuring aggressiveness and sedation changes induced by pentobarbital in socially isolated male mice. Fluoxetine dose-dependently (0.1 to 2 μmoles/kg) and stereospecifically (*S*-fluoxetine>*R*-fluoxetine) normalized the duration of pentobarbital-induced sedation and reduced aggressiveness at the same doses that also normalize the downregulation of brain Allo content in socially isolated male mice (Table 3).

The NOR derivative of fluoxetine (norfluoxetine), is about 3–4 times more potent than fluoxetine, both in normalizing pentobarbital-induced sedation and in inhibiting the aggressive behavior induced by social isolation in male mice (Table 3). In addition, with norfluoxetine the normalization of pentobarbital-induced sedation and the antagonism of aggressive behavior are stereospecific (*S*-norfluoxetine>*R*-norfluoxetine) and are paralleled by their ability to increase brain Allo levels (Table 3). The potency of *S*-norfluoxetine in normalizing the duration of pentobarbital-induced sedation and inhibiting aggression is seven-fold higher than that of the *R*-isomer (Table 3).

The action of *S*-fluoxetine and *S*-norfluoxetine on pentobarbital-induced sedation, the inhibition of aggression, or the normalization of brain Allo content cannot be related to their intrinsic SSRI activity because the EC_{50}s of *S*-fluoxetine and *S*-norfluoxetine to normalize pentobarbital-induced sedation, to reduce aggression, and to upregulate brain Allo levels in socially isolated male mice is at least 10–50 times lower than the EC_{50} required to inhibit 5-HT reuptake (Table 3). Moreover, SSRI activity of *S*- or *R*-fluoxetine and of *S*- or *R*-norfluoxetine lacks stereospecificity (Table 3). Indirect evidence that SSRI activity is not part of the mechanisms of fluoxetine and norfluoxetine antagonism on pentobarbital-induced sedation and on aggression was provided by experiments with *P*-chlorophenylalanine (*P*-CPA) and imipramine. *P*-CPA, in doses that reduced brain 5-HT levels by >80%, failed to prevent fluoxetine's action on pentobarbital-induced sedation or on the increase of brain Allo content in socially isolated mice (Matsumoto et al. 1999). Imipramine, in doses that blocked 5-HT reuptake, failed to decrease aggression, to normalize the reduced pentobarbital action, or to change brain Allo level downregulation in socially isolated male mice (Pinna et al. 2003a, 2004a).

Based on published pharmacokinetic studies (Potts and Parli 1992), it can be estimated that the content of fluoxetine or norfluoxetine present in the brain 30 min after the systemic administration of sub-μmol/kg doses of either compound is in the low nM range. Thus, it is likely that at these concentrations, *S*-fluoxetine and *S*-norfluoxetine both fail to inhibit 5-HT reuptake (Pinna et al. 2004a). We conclude that the 5-HT reuptake inhibition elicited by both compounds is not operative in decreasing social isolation-induced aggression, in the potentiation of pentobarbital-induced sedation, or in the normalization of the downregulated brain Allo levels.

Since *S*-norfluoxetine is steroidogenic at the low nM doses used to inhibit aggressive behavior or to potentiate pentobarbital-induced sedation in mice, it could be considered a drug of choice in the treatment of psychiatric or neurological disorders that result from the downregulation of brain neurosteroid expression due to either defective Allo production or to an increase of Allo metabolic rate. Other rodent experiments are in keeping with these results. For example, the effects of fluoxetine on brain Allo level can be mimicked by paroxetine (Uzunov et al. 1996). Fluoxetine, sertraline, and venlafaxine significantly reverse the decline of Allo content in selected cerebrocortical regions elicited by bilateral bulbectomy in rats (Uzunova et al. 2004). Taken together, these data lead to the suggestion that a decrease of Allo is associated with depression, and should be treated with SSRIs that are active on neurosteroidogenesis (Guidotti and Costa 1998; Pisu and Serra 2004; Uzunova et al. 2004).

The mechanism by which fluoxetine and norfluoxetine or other antidepressants (i.e., paroxetine, fluvoxamine, sertaline) cause a rapid (within minutes) increase of brain Allo levels in socially isolated mice or in bulbectomized rats remains elusive. In male mice socially isolated for 4 weeks, 5α-RI expression in the olfactory bulb, cortex, or hippocampus is decreased by at least 50%, whereas the expression of 3α-HSD is not changed (Dong et al. 2001). Since progesterone expression failed to change in the brains of socially isolated mice (Table 1), one may assume that the decrease of brain Allo content is related to the decrease of 5α-RI, which is the rate-limiting step enzyme in Allo biosynthesis from progesterone (Dong et al., 2001).

Based on these data, the hypothesis is that fluoxetine and norfluoxetine correct the deficiency of brain Allo downregulation in socially isolated male mice via a direct action on 5α-RI. However, studies in vitro using recombinant rat 5α-RI showed that fluoxetine, paroxetine, and sertraline in concentrations as high as 50 μM failed to activate 5α-RI. In contrast, these drugs directly activate 3α-HSD by decreasing its K_m for 5α-DHP by 100-fold, and thereby, favoring the reduction of 5α-DHP into Allo (Griffin and Mellon 1999).

When the results of Griffin and Mellon (1999) are compared to our in vivo studies (Pinna et al. 2003a, 2004a), it becomes apparent that the doses of fluoxetine and norfluoxetine that cause a rapid increase in brain Allo levels in mice reach brain concentrations in the low nM range, whereas the fluoxetine concentrations that directly activate 3α-HSD in vitro are in the μM range. The high potency and stereospecificity of fluoxetine and norfluoxetine in decreasing aggressive behavior and normalizing Allo brain content during social isolation (see Table 3 and Fig. 3) may be indirect and involve mechanisms that are not presently understood. An in-depth investigation of these observations requires further investigation at the molecular enzymatic level.

BDNF has drawn considerable interest as mediator of the antidepressant action of fluoxetine and congeners. BDNF synthesis and secretion, which are decreased in the brains of depressed patients and in socially isolated mice (Dong et al. 2004; Westenbroek et al. 2004), can be upregulated by the administration of antidepressant drugs (reviewed in Nestler

et al. 2002; Castrén 2005). Perhaps BDNF, by favoring 5α-RI and/or 3α-HSD expression and/or function, may increase Allo biosynthesis. By such a mechanism, BDNF may decrease aggressive behavior and normalize the response of $GABA_A$ receptor agonists to GABA in socially isolated mice. Such a role for BDNF is under investigation in our experimental paradigms.

Conclusions

The pharmacology of the *S*- stereoisomers of fluoxetine and norfluoxetine appears to be prototypic for molecules that possess specific neurosteroidogenic activity. The doses of *S*-fluoxetine and *S*-norfluoxetine required to normalize brain Allo content downregulation, pentobarbital action, aggressiveness, and anxiety in socially isolated mice are between 10- and 50-fold lower than those required to induce SSRI activity. The precise mechanism of action by which *S*-fluoxetine and *S*-norfluoxetine increase neurosteroids remains to be investigated. Such an investigation may provide important pharmacological evidence suggesting that the ability of *S*-fluoxetine and *S*-norfluoxetine to normalize pentobarbital-induced sedation in socially isolated mice is indirectly mediated by their action at $GABA_A$ receptors and involves their ability to increase brain Allo content. However, a role for 5-HT reuptake inhibition can be excluded based on pharmacodynamic considerations.

The conclusions that can be drawn from the results obtained in this partial mouse model of depression are consistent with clinical studies showing that fluoxetine and other drugs, which are still termed "SSRI" antidepressants, are beneficial in psychiatric disorders because they increase the availability or potency of neuroactive GABAergic steroids (for a review see Dubrovsky 2005; Barbaccia 2004; Pisu and Serra 2004; van Broekhoven and Verkes 2003), and not because of their intrinsic ability to facilitate 5-HT reuptake (Pinna et al. 2003a, 2004a; Khisti and Chopde, 2000; Matsumoto et al. 1999; Guidotti and Costa 1998; Uzunova et al. 1998).

Derivatives of *S*-fluoxetine and *S*-norfluoxetine, acting with high potency and specificity on brain neurosteroid expression at doses devoid of significant action on brain 5-HT reuptake mechanisms, represent a new class of pharmacological tools for the management of anxiety, related mood disorders, dysphoria, and impulsive aggression. These derivatives can be used for these indications as prototypic treatments based on their ability to increase brain steroid biosynthesis. This pharmacology targets an interesting set of behavioral abnormalities and very likely operates via a positive modification of brain neurosteroid bioavailability.

The present study underscores the modulatory role of neurosteroidogenesis by drugs that inhibit 5-HT reuptake in higher doses, and suggests that the term "SSRI" may be misleading in defining the pharmacological profile of fluoxetine and its congeners. To this extent, the term "selective brain steroidogenic stimulants" (SBSSs) could be proposed. The molecular mechanisms subserving the fluoxetine-induced facilitation on neurosteroidogenesis remain to be investigated and defined in terms of structural chemical-related specificity in activating brain steroidogenesis, which is a novel and intriguing branch of psychopharmacology.

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