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## SSRIs act as selective brain steroidogenic stimulants (SBSSs) at low doses that are inactive on 5-HT reuptake

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### Summary

Brain principal glutamatergic neurons synthesize 3 $\alpha$ -hydroxy-5 $\alpha$ -pregnan-20-one (Allo), a neurosteroid that potently, positively, and allosterically modulates GABA action at GABA<sub>A</sub> receptors. Cerebrospinal fluid (CSF) Allo levels are decreased in patients with posttraumatic stress disorder (PTSD) and major depression. This decrease is corrected by fluoxetine in doses that improve depressive symptoms. Depression-like behavioral dysfunctions (aggression, fear, and anxiety) associated with a decrease of corticolimbic Allo content can be induced in mice by social isolation. In socially isolated mice, fluoxetine and analogs stereospecifically normalize the decrease of Allo biosynthesis and improve behavioral dysfunctions by a mechanism independent from 5-HT reuptake inhibition. Thus, fluoxetine and related congeners facilitate GABA<sub>A</sub> receptor neurotransmission and effectively ameliorate emotional and anxiety disorders and depression by acting as selective brain steroidogenic stimulants (SBSSs).

### Keywords

allopregnanolone; aggressive behavior; contextual fear conditioning; depression; PTSD; GABA<sub>A</sub> receptors; selective brain steroidogenic stimulants (SBSSs)

### Introduction

Emotional disorders such as impulsivity, irritability, aggression, and anxiety spectrum disorders, including generalized anxiety, panic, and posttraumatic stress disorder (PTSD) are frequently associated with major depression [1,2].

Although the brain structures responsible for these complex psycho-pathologies are not yet precisely defined, there is growing evidence that these clinical manifestations are associated with functional alterations of monoamine (5-HT, NE, DA) neurotransmitters expressed by specific cortico-limbic circuit neurons [2-5]. For example, the frontal cortex and hippocampus mediate cognitive deficits, feelings of worthlessness, hopelessness, guilt, and suicidality while the corpus striatum, nucleus accumbens, and amygdala are important in processing aversive or reward responses to emotional stimuli, thereby mediating the onset of anhedonia, anxiety, and the reduced behavioral motivation frequently shown in patients with major depression [2].

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Reduced cortical GABA levels in depressed patients, determined by positron magnetic resonance [6], and the beneficial effects following the administration of benzodiazepines (BZs) that positively and allosterically modulate GABA action at GABA<sub>A</sub> receptors [7], suggest that in addition to monoamines, a perturbation of the GABAergic signaling could also play a fundamental role in the pathogenesis of the emotional and anxiety disorders associated with depression.

Here, we will review results from human and rodent studies suggesting that emotional disorders, anxiety disorders, and depression may reflect a cortico-limbic perturbation of GABAergic neurotransmission that may be the result of a reduction of the GABA<sub>A</sub> receptor-active neurosteroid, 3 $\alpha$ -hydroxy-5 $\alpha$ -pregnan-20-one (allopregnanolone, abbreviated as Allo).

### Neurons expressing neurosteroid biosynthesis

Allo is a potent (nM affinity) positive endogenous allosteric modulator of GABA action that acts at the majority of synaptic and extrasynaptic GABA<sub>A</sub> receptor subtypes [8\*\*,9\*\*-13]. Hence, Allo fails to exhibit the receptor subunit selectivity typical of BZs. Importantly, Allo is particularly active (nmol concentrations) at extrasynaptic GABA<sub>A</sub> receptors expressing subunits  $\alpha_4\beta_x\delta$  or  $\alpha_5\beta_x\delta$  where classical BZs fail to act or act with low affinity [12].

Allo is the most abundant brain neurosteroid acting at GABA<sub>A</sub> receptors [14\*\*,15\*\*]. In both human and rodent brains, Allo is synthesized from progesterone by the sequential action of two reducing enzymes: 5 $\alpha$ -reductase (5 $\alpha$ -R) type I, which transforms progesterone into 5 $\alpha$ -DHP, and 3 $\alpha$ -hydroxysteroid dehydrogenase (3 $\alpha$ -HSD), which transforms 5 $\alpha$ -DHP into Allo and vice-versa [15\*\*-17\*\*] (Fig. 1). These two enzymes co-localize and are highly expressed in cortical, hippocampal, and amygdala glutamatergic pyramidal neurons and in olfactory bulb glutamatergic mitral neurons. However, these enzymes are not expressed in glial cells or GABAergic interneurons [17\*\*].

Taken together, these considerations suggest that Allo synthesized by glutamatergic neurons of the olfactory bulb, frontal cortex, hippocampus, and amygdala modulates GABA action at synaptic or extrasynaptic GABA<sub>A</sub> receptors. These receptors are located on dendritic shafts or cell bodies of the above-mentioned glutamatergic neurons by an autocrine mechanism or more likely by this neurosteroid reaching GABA<sub>A</sub> receptor intracellular sites through lateral membrane diffusion (Fig. 1) [17\*\*,18].

### Allo is decreased in cerebrospinal fluid (CSF) of depressed and PTSD patients

Based on the observation made in our laboratory, that fluoxetine and paroxetine increase the content of Allo in neurons of various rat brain areas (olfactory bulb > frontal cortex > hippocampus > striatum > cerebellum) [19\*\*], we hypothesized that by normalizing brain Allo levels in depressed and in PTSD patients, administration of selective serotonin reuptake inhibitors (SSRIs) may alleviate both the anxiety and dysphoria symptomatology of these psychiatric disorders [20].

As proof of concept, we measured Allo levels in the cerebrospinal fluid (CSF) of patients with psychiatric disorders [21\*\*,22\*], based on the assumption that the amount of Allo in the CSF is a reliable index of brain Allo levels. We found that the concentration of Allo in the CSF of non-psychiatric subjects (~40 fmol/ml) was approximately 2-fold higher than that measured in the CSF of depressed patients [21\*\*].

To support the hypothesis that this decrease in the CSF Allo levels of depressed patients reflects a decrease of brain Allo content, we compared the expression of 5 $\alpha$ -R type I mRNA in samples (N=12) of the prefrontal-cortical area (BA9) from depressed patients that were age- and sex-matched with non-psychiatric subjects. In depressed patients, the level of 5 $\alpha$ -R type I mRNA

was dramatically decreased (about 50%) compared to that of non-psychiatric subjects. However, 5 $\alpha$ -R type I mRNA expression failed to change in the cerebellum of the same patients (Agis-Balboa, personal communication).

In a recent human study, we also reported that in PTSD patients, Allo level downregulation in the CSF was in keeping with an increase of PTSD re-experiencing and comorbid depressive symptoms [22\*]. Also, Allo levels were decreased in all PTSD patients but were lowest in those patients with PTSD and comorbid depression [22\*].

In 15 depressed patients affected by major depression, treatment with fluoxetine and fluvoxamine (8-10 weeks, doses of 0.8-4.8 for fluoxetine and 1.7-9.1  $\mu$ mol/kg for fluvoxamine) normalized the CSF Allo content [21\*\*]. Moreover, a statistically significant correlation existed between symptomatic improvement (Hamilton Rating Scale for Depression Score) and the increase of CSF Allo elicited by fluoxetine or fluvoxamine. Similar results were reported when Allo or 5 $\alpha$ -tetrahydrodeoxycorticosterone levels were measured in the plasma of depressed patients treated with SSRIs [23].

Taken together, these data suggest that among the molecular mechanisms underlying major depression and PTSD symptomatology must be included a deficit of GABAergic neurotransmission likely caused by a downregulation of brain Allo biosynthesis.

### **Downregulation of neurosteroid biosynthesis in cortico-limbic circuits mediates aggression, anxiety, and fear induced in mice by social isolation**

To examine whether a downregulation of GABA action at GABA<sub>A</sub> receptors is related to the emotional or anxiety spectrum disorders observed in depressed or PTSD patients, we and others [24,25\*,26\*,27,28\*,29\*-32] studied rodents (mice and rats) exposed to a protracted period (3-4 weeks) of social isolation stress. It is known that in mice this condition causes i) aggression [24,27,28\*,30, reviewed in 32], ii) an enhanced contextual fear response to stressful stimuli [26\*, reviewed in 32], and iii) a decreased response to barbiturates and BZs and other GABA-mimetic drugs [24,30,33\*\*]. In these socially isolated mice, the behavioral abnormalities were associated with a marked decrease of brain Allo content caused by a decrease of 5 $\alpha$ -R type I mRNA and protein expression [25\*,26\*,27,28\*,29\*-32]. In socially isolated mice, the intensity of aggression and the enhancement of fear are inversely related to the extent of Allo content downregulation measured in the olfactory bulb, frontal cortex, hippocampus, and amygdala [26\*,28\*]. Moreover, when Allo was administered subcutaneously to socially isolated mice, it attenuated their aggression and fear responses to stressful stimuli (i.e. mild electric foot shock) in a dose-dependent manner [26\*,28\*,32]. These doses of Allo failed to alter gross behavioral patterns or trends of locomotor activity in group-housed mice (used as control).

To provide further evidence that the decrease of brain Allo content in socially isolated mice is responsible for the altered behavioral responses, we induced decreased brain Allo content by administering the potent 5 $\alpha$ -R type I inhibitor, 17 $\beta$ -(N,N-diisopropylcarbamoyl)-androst-3,5diene-3-carboxylic acid (SKF 105,111) to group-housed mice [26\*,32,33\*\*]. The subcutaneous administration of SKF 105,111 (1-80  $\mu$ mol/kg) induced a fast occurring (30-60 min delay) and marked (~80%) reduction of brain Allo content that lasted for at least 6 hrs [11,16,26\*,32].

The SKF 105,111-induced decrease of brain Allo content by an extent comparable to the Allo content decrease induced by social isolation can be related to the shorter duration of pentobarbital-induced sedation, increased aggressiveness, and enhanced expression of contextual fear after exposure to a conditioning stimulus [25\*,26\*,27,28\*,29\*-32,33\*\*].

Measurements of Allo and 5 $\alpha$ -R type I mRNA and protein levels show that the neurosteroid biosynthesis reduction in socially isolated mice does not occur uniformly in every brain areas but is greater in cortico-limbic circuits, which are known to regulate the levels of emotions and anxiety, and particularly in neurons that express the highest levels of 5 $\alpha$ -R type I [26\*,27, 28\*,29\*,30,32].

It has been suggested that the neuronal networks that underlie the expression of aggression and fear conditioning responses include excitatory glutamatergic projections from the medial frontal (prelimbic and infralimbic) cortex (mFC) and hippocampus (CA1) (Fig. 2) to the basolateral nucleus of the amygdala [34-38]. In the basolateral amygdala (BLA), cortical and hippocampal projections establish excitatory synapses with GABAergic interneurons and also pyramidal-like glutamatergic output neurons. These project either directly to the neurons of the central amygdala (CeA) or to the *intercalated* (ITC) inhibitory GABAergic neurons located on the capsule surrounding the central amygdaloid nucleus. The CeA spiny output neurons (presumably GABAergic) project to the brainstem and hypothalamus, thereby modulating *inter alia* the intensity of emotional responses to environmental stimuli (Fig. 2). In socially isolated mice, the expression of 5 $\alpha$ -R type I and Allo is downregulated selectively in layer V/VI glutamatergic pyramidal neurons of the mFC and in glutamatergic pyramidal-like neurons of the BLA (Fig. 2) [29\*]. Hence, a selective decrease of Allo in cortical layer V/VI pyramidal neurons or BLA glutamatergic output neurons may reduce the inhibitory potency of GABA at GABA<sub>A</sub> receptors located on dendrites or cell bodies of these principal neurons. In functional terms, this may represent the molecular mechanisms that underlie the decreased plasticity of the cortico-limbic pathways converging on the ITC and CeA spiny neurons in socially isolated mice, ultimately resulting in an altered output from the CeA neurons projecting into the hypothalamic and brainstem nuclei.

Therefore, by altering the function of cortico-amygdaloid circuits, the reduction of 5 $\alpha$ -R type I expression and consequently that of the Allo levels in glutamatergic neurons of FC and BLA may be involved in the increased aggressive behavior and in the enhancement of the contextual fear responses and anxiety-like behaviors observed in socially isolated mice.

### **Effects of fluoxetine and norfluoxetine on neurosteroid biosynthesis are unrelated to their efficacy as 5-HT reuptake inhibitors**

To address the question of whether the mechanisms whereby SSRIs increase brain and CSF Allo levels and improve clinical symptoms are dependent on changes in 5-HT neurotransmission, we tested whether fluoxetine, norfluoxetine, and other specific SSRIs stereoselectively upregulate brain neurosteroid content, reduce aggression, or prevent the enhancement of fear expression at doses capable of inhibiting 5-HT reuptake in socially isolated mice.

In these studies, we showed that intraperitoneal doses of fluoxetine (1.4-2.9  $\mu$ mol/kg) correct the brain Allo level decrease and reduce the behavioral deficits associated with prolonged social isolation. Further, fluoxetine continues to do so in socially isolated mice in which brain 5-HT synthesis was inhibited by pretreatment with p-chlorophenylalanine (1.2 mmol/kg i.p. at 72, 48, and 24 hr before measurement) that reduces brain 5-HT content by 80% [25\*].

Since fluoxetine is an S and R racemic mixture that is metabolized into S or R norfluoxetine [40], we used S- or R-fluoxetine and S- or R-norfluoxetine in a dose-response study to evaluate their stereospecificity in modifying brain Allo content and related behavioral responses. We also tested whether neurosteroidogenic doses differ from the doses of these compounds that inhibit 5-HT reuptake.

We found [27,28\*,41\*\*] that fluoxetine and norfluoxetine in sub $\mu$ molar doses and in a stereospecific manner (S-isomers > R-isomers) reverse the decrease of brain Allo levels and at the same doses, correct the behavioral deficits expressed by socially isolated mice. Importantly, these actions of S-fluoxetine and S-norfluoxetine cannot be related to their intrinsic SSRI activity because to normalize pentobarbital-induced sedation, to reduce aggression, and to upregulate brain Allo levels in socially isolated male mice, the EC<sub>50</sub>s are at least 10-to-50 times lower than the EC<sub>50</sub> required to inhibit 5-HT reuptake (Fig. 3). More importantly, the 5-HT reuptake inhibition is not stereospecific (Table 1).

For the first time, these studies provide evidence suggesting that fluoxetine upregulates endogenous brain stores of Allo and regulates GABAergic tone and related behaviors by a mechanism that may be independent from modifications of 5-HT reuptake mechanisms.

### Selective and potent action of S-fluoxetine, S-norfluoxetine, and other SSRIs on neurosteroid biosynthesis

The mechanisms by which fluoxetine and norfluoxetine [27,28\*,41\*\*] and other SSRIs (i.e., paroxetine, fluvoxamine, sertraline) [19\*\*-21\*\*,42,43] cause a rapid (minutes) increase of brain Allo levels in rodents remain unclear.

A possible hypothesis is that fluoxetine or norfluoxetine correct the brain Allo level decrease in socially isolated mice via a direct action on 5 $\alpha$ -R type I. However, studies *in vitro* using recombinant rat 5 $\alpha$ -R type I or 3 $\alpha$ -HSD showed that fluoxetine, paroxetine, or sertraline in concentrations as high as 50  $\mu$ M failed to activate 5 $\alpha$ -R type I. In contrast, these drugs directly activated 3 $\alpha$ -HSD, decreasing the K<sub>m</sub> of this enzyme for 5 $\alpha$ -DHP by 100-fold and thereby favoring the reduction of 5 $\alpha$ -DHP into Allo [44].

When the results of these *in vitro* studies [44] are compared to those of our *in vivo* studies [27,28\*,41\*\*], it becomes evident that in mice the doses of fluoxetine and norfluoxetine that cause a rapid increase in brain Allo levels do not exceed brain concentrations in the low nanomolar range, whereas the fluoxetine concentrations that directly activate 3 $\alpha$ -HSD *in vitro* are in the  $\mu$ molar range. Moreover, the high potency and stereospecificity of fluoxetine and norfluoxetine in decreasing aggressive behavior and normalizing brain Allo content during social isolation (see Table 1, and Fig. 3) support the notion that these compounds facilitate the action of 5 $\alpha$ -R type I or 3 $\alpha$ -HSD by an unidentified indirect mechanism, which is very likely perturbed by protracted social isolation.

Thus, these drugs, which originally were termed “SSRI” antidepressants, may be beneficial in psychiatric disorders because in doses that are inactive on 5-HT reuptake mechanisms, they increase the bioavailability of neuroactive GABAergic steroids [27]. Based on these considerations, we now propose that the term “SSRIs” should be changed to the more appropriate term “selective brain steroidogenic stimulants” (SBSSs), which more accurately defines the pharmacological mechanisms expressed by fluoxetine and its congeners [27].

## 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- to 50-fold lower than those required to induce SSRI activity. However, the precise mechanisms of action by which S-fluoxetine and S-norfluoxetine increase neurosteroids remain to be investigated.

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, may represent a new class of pharmacological tools important for the management of anxiety, related mood disorders, dysphoria, fear, and impulsive aggression.

Based on these data, new drugs devoid of SSRI activity but that are potent neurosteroidogenic agents should be developed for the treatment of psychiatric disorders that result from the downregulation of neurosteroid expression, including major depression, and in the prevention of PTSD.

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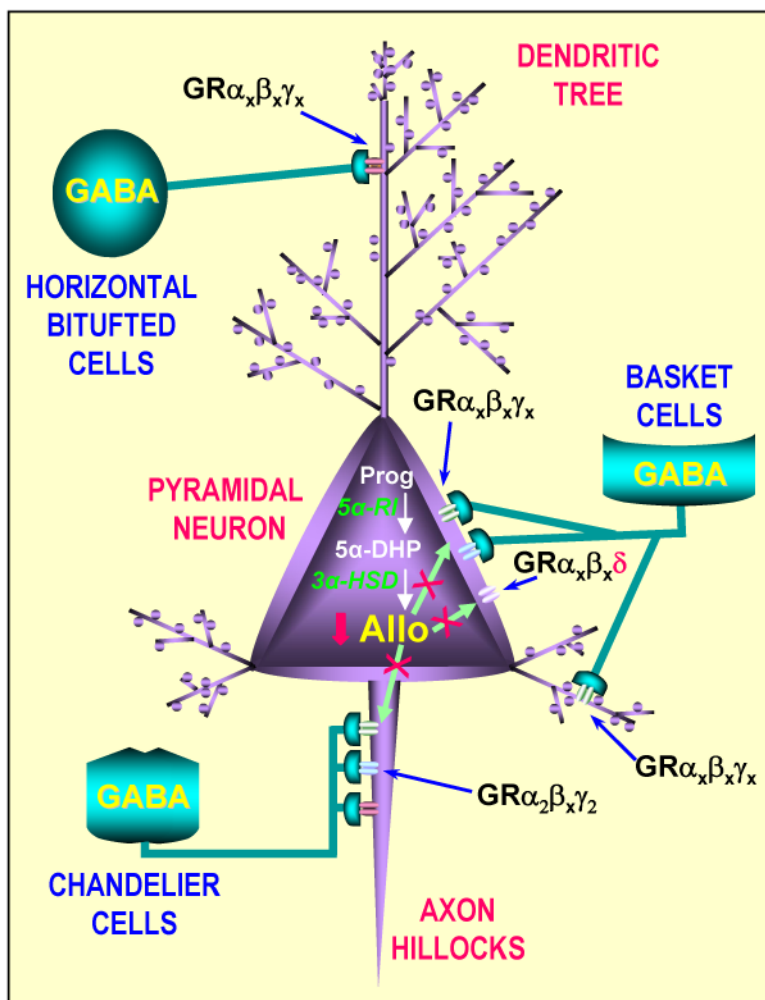
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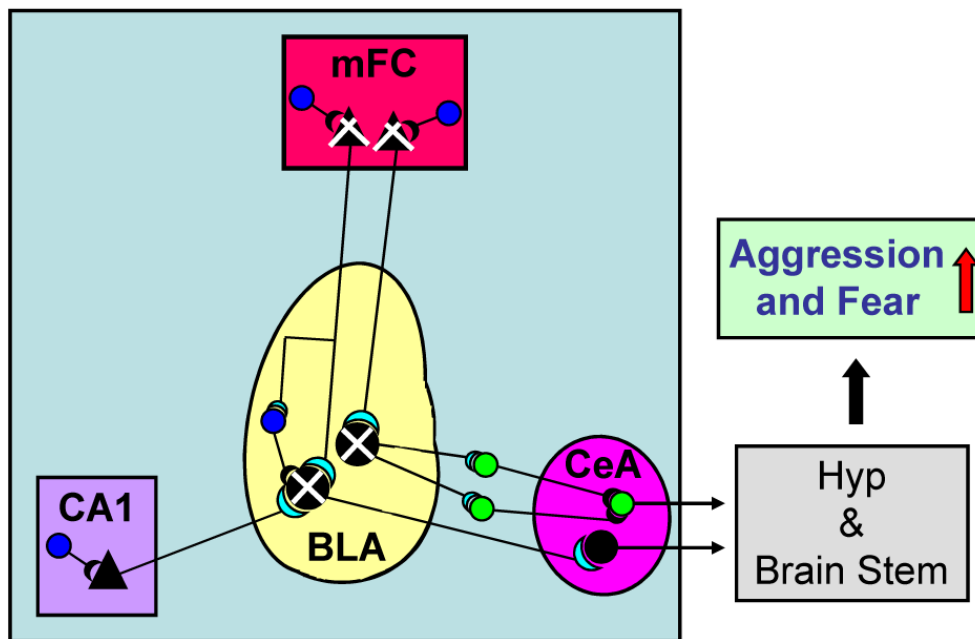
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## Abbreviations

<b>3<math>\alpha</math>-HSD</b>	3 $\alpha$ -hydroxysteroid dehydrogenase
<b>5<math>\alpha</math>-RI</b>	5 $\alpha$ -reductase type I
<b>Allo</b>	allopregnanolone
<b>BLA</b>	baso-lateral nuclei of the amygdala
<b>mFC</b>	medial prefrontal cortex
<b>SBSSs</b>	selective brain steroidogenic stimulants
<b>CSF</b>	cerebrospinal fluid
<b>PTSD</b>	posttraumatic stress disorder
<b>SSRI</b>	selective serotonin reuptake inhibitors



**Fig. 1.** Simplified cortical circuitry that depicts the action of Allo on GABA<sub>A</sub> receptors (GR) expressed on the cell body, dendrites, or axon hillocks of a pyramidal neuron. Allo is synthesized in pyramidal neurons by the action of 5 $\alpha$ -R-type I and 3 $\alpha$ -HSD. Allo diffuses (indicated by  $\rightarrow$ ) to cell membranes and facilitates the action of GABA at synaptic and extrasynaptic GABA<sub>A</sub> receptors.  $\downarrow$  denotes Allo biosynthesis downregulation in pyramidal neurons of socially isolated mice.  $\times$  denotes a decrease of Allo levels reaching synaptic or extrasynaptic GABA<sub>A</sub> receptors located on pyramidal neurons in socially isolated mice. GR $\alpha_x\beta_x\delta$ , extrasynaptic GABA<sub>A</sub> receptors that express  $\delta$  subunits.

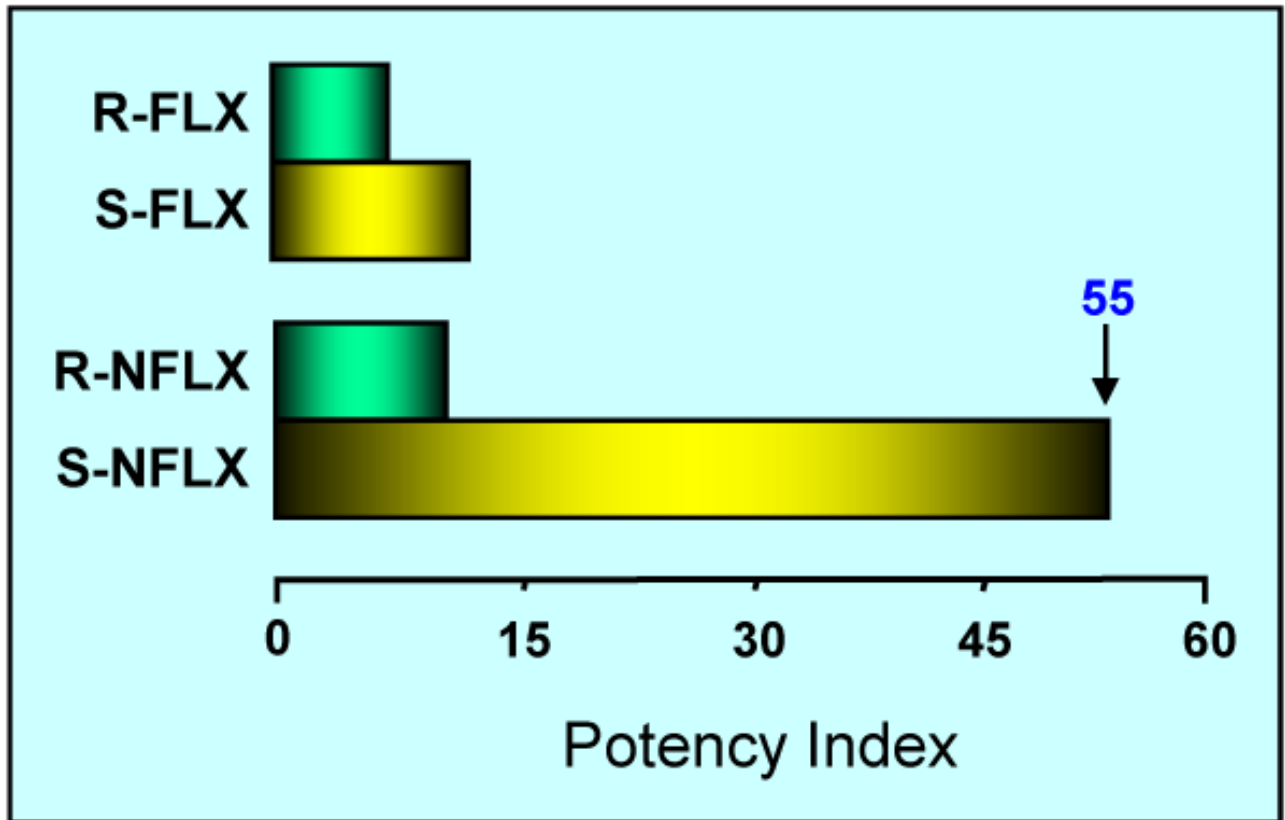


**Fig. 2.** Schematic representation of the main intrinsic connections of the basolateral (BLA) and central (CeA) nuclei of the amygdala and extrinsic projections from the medial frontal cortex (mFC) (layer V/VI) and hippocampus (CA1) pyramidal neurons to the BLA.

In socially isolated mice, the decrease in Allo biosynthesis in layer V/VI pyramidal glutamatergic neurons of the mFC and in pyramidal-like glutamatergic neurons of the BLA (indicated by ⊗△) downregulates the inhibitory potency of GABAergic interneurons (indicated by ●○) impinging on these pyramidal neurons. This Allo content decrease results in an increased excitatory output from BLA to the intercalated (ITC) neurons or to neurons of the CeA nucleus, which project to the hypothalamus (Hyp) and brain stem, enhancing fear and aggression (indicated by ↑).

- Pyramidal-like glutamatergic neurons expressing Allo
- ▲△ Pyramidal glutamatergic neurons expressing Allo
- Inhibitory GABAergic interneurons
- Intercalated (ITC) GABAergic neuron
- ⊗△ Decreased Allo biosynthesis in mFC pyramidal and BLA pyramidal-like glutamatergic neurons

Modified from Sah and Westbrook [45].



**Fig. 3.** The stereospecific potency of S-norfluoxetine required to stimulate Allo biosynthesis is 55 times higher than 5-HT reuptake inhibition. Data on the x-axis (potency index) represent the ratios between the  $EC_{50}$  doses that inhibit 5-HT reuptake and the  $EC_{50}$  doses that stimulate Allo biosynthesis. Each value is the mean of four to six socially isolated mice (data from Table 1).

**Table 1**

**Fluoxetine and norfluoxetine stereoisomers induce normalization of pentobarbital (PTB) right reflex loss (RRL), reduce the duration of attacks against an intruder (Aggression), activate neurosteroidogenesis (Allo) at doses that fail to affect 5-HT reuptake**

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

Drugs were administered 30 min before behavioral tests and [<sup>14</sup>C]5-HT reuptake measurement. Data represent the mean ± SEM of four to six mice socially isolated for 4 weeks before testing.

\*  $P < 0.01$  when S-fluoxetine is compared with R-fluoxetine.

\*\*  $P < 0.001$  when S-norfluoxetine is compared with R-norfluoxetine and S-fluoxetine. The EC<sub>50</sub> were calculated from dose-response curves analyzed by the "quantal dose-response: probits test" [46] equipped with a statistical package. Statistical comparisons among the different IC<sub>50</sub> values were performed by using the COHORT package. For details see Pinna et al., [27,28,41]