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Short Communication

# Fluoxetine directly counteracts the adverse effects of chronic stress on T cell immunity by compensatory and specific mechanisms

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ABSTRACT

Chronic stress and depression are widely known to down-regulate the immune system, and several antidepressants can reverse this impairment, with or without effects in normal subjects. Although the central nervous system is undoubtedly involved in these events, some psychotropic drugs can also exert direct effects on lymphoid cells. We have recently shown that the antidepressant fluoxetine enhances T cell proliferation and $T_H1$ cytokine production *in vivo*, without changes on CD4/CD8 subsets. *In vitro*, a direct action of fluoxetine upon T lymphocyte reactivity by complex mechanisms was also described. In another work, we also found that chronic stress reduces T cell mediated immunity, namely a decrease of T cell response to mitogens, $T_H1$ cytokine production and $CD4^+$—but not $CD8^+$—T lymphocytes. Here we investigated the effects of fluoxetine on chronic stress-driven immune system depression. We found that fluoxetine restored T cell proliferation and interleukin-2, interferon-γ and tumor necrosis factor-α production by compensatory mechanisms. In addition, CD4/CD8 ratio was also normalized by antidepressant administration, but this seems to be a non-compensatory effect associated specifically to stress. No changes were observed in other lymphoid cells, i.e. natural killer cells and B lymphocytes. Finally, we observed that fluoxetine is able to reverse T cell reactivity impairment *in vitro* by a direct action at clinically relevant doses. These results highlight the relevance of pharmacological treatment of stress and depression, and may help to begin elucidating the complex events triggered—directly and/or indirectly—by antidepressants in non-neuronal cell types.



## 1. Introduction

Nowadays, neuroimmunomodulation is a growing field supported by a considerable body of evidence pointing to the interaction between psychological factors and the immune system (Serafeim and Gordon, 2001). Prolonged exposure to stressful situations has been associated with the onset and outcome of specific neuropathologies (Willner and Mitchell, 2002), and it is widely known that chronic stress and its related psychiatric diseases such as major depression, post-traumatic stress disorder and anxiety can modulate the immune response (Glaser and Kiecolt-Glaser, 2005; Calcagni and Elenkov, 2006). Additionally, certain psychotropic drugs, e.g. antidepressants, anxiolytics and antipsychotics, reverse the adverse effects triggered by such psychopathologies (Willner and Mitchell, 2002; Edgar et al., 2002). There is also evidence that in non-pathological conditions, antidepressants have effects per se in several cell types. Moreover, increasing evidences indicate that some of these drugs can also act directly on immune cells regulating their functioning (Gordon and Barnes, 2003).

In a recent work, we found that chronic restraint stress for 3 weeks specifically impairs the T helper component of immunity. This was evidenced by a reduction of T cell response to mitogens, a decreased production of the $T_H1$ cytokines interferon-gamma (IFN-γ) and necrosis factor-alpha (TNF-α), and a reduction in the number of $CD4^+$ T cell subpopulation without changes in $CD8^+$ T cells (Frick et al., 2008a). Instead, no changes were found in other immune system components, i.e. natural killer (NK) cells and B lymphocytes. These results emphasize the particular importance of T helper immunity down-regulation induced by prolonged stress. Moreover, given that chronic restraint stress model is proposed as a model for the study of some aspects of depressive-like symptoms in mice similar to those occurring in humans, the reversion by antidepressant treatment of the adverse effects observed further strengthens the importance of previous results (Willner and Mitchell, 2002).

On the other hand, we found that the administration during 4 weeks of the antidepressant fluoxetine, a selective serotonin reuptake inhibitor used for the treatment of major depression in humans, enhances T cell mediated immunity, namely an increase of T cell proliferation and $T_H1$ cytokines IFN-γ and TNF-α production, without changes on CD4/CD8 subsets (Frick et al., 2008b). We also found that, besides its effects on serotonergic neurons in the

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central nervous system and their subsequent action in the periphery, fluoxetine is also able to act directly on T lymphocytes modulating their proliferation in a dual manner depending on cellular activation by two mechanisms: one dependent on serotonin and the other independent of serotonin. Experiments performed using athymic mice—devoid of T lymphocytes—suggested that fluoxetine acts selectively on T cells, but not on other lymphocyte subtypes. These results demonstrate that this antidepressant exerts an immunomodulatory effect in non-pathological conditions in part due to its direct effects on T cells.

However, differential events were observed in these treatments. It seems that chronic stress impairs T helper cell mediated immunity by reducing the number of T $CD4^+$ cells. Instead, fluoxetine seems to alter T cell reactivity without any change in the total number of lymphocyte subsets. Hence, differential events could be triggered by stress and antidepressants. In addition, a direct effect of fluoxetine on normal T cells was demonstrated. Therefore, it is possible that this antidepressant also has a direct regulation of T lymphocytes from stressed animals.

Given that chronic stress has been widely related to the development and progression of major depression, and that fluoxetine is the antidepressant of first choice prescribed for the treatment of depressive patients, here we combined repeated stress exposure and daily fluoxetine administration in order to test whether T cell mediated immunity depression can be counteracted by compensatory and/or not compensatory mechanisms. In addition, we explored more deeply other components of the immune system, i.e. NK activity, B cell response and interleukin-2 (IL-2) production. Finally, we analyzed whether fluoxetine direct effects are also involved in the reversion of T cell mediated immunity impairment induced by chronic stress.

## 2. Materials and methods

### 2.1. Animals

Inbred female BALB/c mice ($n = 42$ mice per treatment) were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (NIH, USA). For the chronic restraint stress model, animals were immobilized daily for 6 h in well-ventilated polypropylene tubes (2.8 cm diameter × 11.5 cm length) without access to food and water (Alfonso et al., 2006; Frick et al., 2008a). Fluoxetine (Gador, Capital Federal, Buenos Aires, Argentina) was administered in the drinking water at a dose of 15 mg/kg/day (Frick et al., 2008b). In the combination of both treatments, animals were restrained and concomitantly given the same dose of fluoxetine (Alfonso et al., 2006). These protocols were performed for 3 weeks and then mice were killed. Animals from control groups were left undisturbed.

### 2.2. Proliferation assays

Lymphocytes from spleens and lymph nodes were prepared in supplemented RPMI 1640 (Invitrogen, Carlsbad, CA, USA), as previously described (Frick et al., 2008a, b). Proliferation was determined by culturing $2 \times 10^5$ cells/ml per well in microplates in the presence of 0.5 μg/ml (suboptimal), 1 μg/ml (optimal) or 2 μg/ml (inhibitory) Concanavalin A (Con A, Sigma–Aldrich, St. Louis, MO, USA); 2.5 μg/ml pokeweed mitogen (PWM, Sigma–Aldrich) or 15 μg/ml lipopolysaccharide (LPS, Sigma–Aldrich). Cells were cultured at 37 °C in a 5% $CO_2$ atmosphere for 72 h. Mitogenic activity was measured by adding 0.75 μCi [$^3$H]-thymidine (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) per well. Thymidine incorporation was measured by scintillation counting after retention over GF/C glass–fiber filters (Whatman, Brentford, Middlesex, UK) of the acid-insoluble macromolecular fraction. Proliferation indexes were calculated as the relation of stimulated—basal/basal proliferation (mean baseline proliferation = 1785 dpms).

### 2.3. Natural killer activity assay

NK cells activity was evaluated according to the JAM method (Frick et al., 2008a). Briefly, YAC-1 (ATCC, Manassas, VA, USA) were labeled with 5 μCi [$^3$H]-thymidine 3 h prior the cytotoxicity assays were carried out. [$^3$H]-Thymidine is then incorporated into the DNA of proliferating YAC-1 cells (Wunderlich et al., 1997). Cell suspensions from spleens were obtained as described above. Different ratios of target and effector cells (1:100 to 1:1) were co-incubated for 3.5 h at 37 °C in a 5% $CO_2$ atmosphere. YAC-1 cells are lysed by NK cells present in the culture and DNA fragments are released to the medium. Chromosomic DNA from intact target cells is retained in filters when harvested, and its radioactivity is inversely related to cytolytic activity of effector cells. Lytic units were calculated by linear regression analysis as the number of lymphocytes that yield a 30% of total lysis.

### 2.4. Lymphocyte subsets determination by flow cytometry

Lymph node $CD4^+$ T helper and $CD8^+$ T-cytotoxic lymphocytes were determined by flow cytometry (Frick et al., 2008a, b). Briefly, cells were stained with fluorescein-conjugated anti-mouse CD4 (CD4-FITC) and phycoerythrin-conjugated anti-mouse CD8 (CD8-PE) monoclonal antibodies (eBioscience, San Diego, CA, USA). Lymphocyte subsets were identified by FACS analysis using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Isotype controls (Sigma–Aldrich) were used to determine non-specific staining.

### 2.5. Real-time reverse transcription polymerase chain reaction

Quantification of gene expression was performed as previously described (Alfonso et al., 2006; Frick et al., 2008a, b). Total RNA was isolated from lymph nodes using Trizol Reagent (Invitrogen). Poly$A^+$ mRNA was isolated from total RNA using the PolyA-Tract mRNA isolation System (Promega, Madison, WI, USA). cDNA was synthesized by retrotranscription using SuperScript II Reverse Transcriptase enzyme (Invitrogen). Real-time RT-PCRs were carried out in a GeneAmp 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). cDNA amounts were determined using SYBR Green PCR Core Reagents kit (Applied Biosystems). Oligonucleotide sequences used were: IL-2 forward 5′-CCTGAGCA GGATGGAGAATTACA-3′, IL-2 reverse 5′-TCCAGAACATGCCGCAG AG-3′, TNF-α forward 5′-GCACCACCATCAAGGACTCAA-3′, TNF-α reverse 5′-TTGCAGAACTCAGGAATGGACA-3′, IFN-γ forward 5′-T GCTGATGGGAGGAGATGTCTAC-3′, IFN-γ reverse 5′-ACCTGACACATT CGAGTGCTGT-3′, β-actin forward 5′-CAACTTGATGTATGAAGGCTTTG GT-3′, β-actin reverse 5′-ACTTTTATTGGTCTCAAGTCAGTGTACAG-3′. For data normalization, values were referred to β-actin.

### 2.6. Statistical analysis

Statistical significances were determined using unpaired two-tailed Student *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's contrast.

## 3. Results

### 3.1. Effects of fluoxetine and chronic stress on immunity

We evaluated the status of the immune response in normal and stressed animals, treated or not with fluoxetine. As we have shown,

chronic restraint stress for 3 weeks reduced T lymphocyte proliferation to mitogenic Con A stimulation, whereas the administration of 15 mg/kg/day fluoxetine during the same period of time increased it (Table 1). The combination of both treatments resulted in [$^3$H]-thymidine incorporation values similar to those from control animals (Table 1). Moreover, the same effects were observed for suboptimal, optimal and inhibitory mitogen concentrations (0.5, 1 and 2 μg/ml Con A, Fig. 1A). Instead these treatments did not modify B cell proliferation induced by LPS or PWM as well as NK cells cytolytic activity against YAC-1 tumor cells (Table 1). Fluoxetine was previously shown to enhance the production of the $T_H1$ cytokines TNF-α and IFN-γ, which was decreased by chronic restraint stress. When both treatments were combined, we observed a normalization of cytokine expression (Table 1). In addition, a similar behavior was observed for the cytokine IL-2, a key regulator of T cell mediated immunity (Table 1). Chronic stress was shown to reduce $CD4^+$ T cells without altering $CD8^+$ T cells, but fluoxetine did not modify CD4/CD8 ratio. Interestingly, fluoxetine administration on stressed mice restored T cells subpopulations (Table 1).

### *3.2. Direct action of fluoxetine on normal and "stressed" T lymphocytes*

We tested whether a direct effect of fluoxetine on T cells is involved in the reversion of lymphoproliferative impairment of these cells observed after antidepressant treatment of stressed mice. Thus, we assayed Con A-induced proliferation of T lymphocytes obtained from normal and chronically stressed animals, in the presence of fluoxetine at a broad range of concentrations. As we have shown, fluoxetine alone produces a direct modulation of T cell reactivity *in vitro* in a dual manner depending on the degree of cellular activation triggered by suboptimal or optimal mitogen concentrations (Fig. 1B). When T cells from normal or stressed mice were stimulated with submitogenic or mitogenic doses of Con A in the presence of fluoxetine, we found that at low concentrations of fluoxetine T cell proliferation remained reduced in lymphocytes from stressed mice, but at therapeutic doses, it was capable of reverse decreased T cell proliferation of stressed animals restoring normal levels (Fig. 1C and D), after subtraction of fluoxetine effects alone (Fig. 1E and F). Finally, when high doses of the antidepressant were tested a general inhibition of T cell reactivity was found (Fig. 1C and D).

## 4. Discussion

We recently found that chronic restraint stress for 3 weeks, in addition to the common behavioral alterations observed in animal models of depression, also reduced T cell proliferation in response to optimal Con A, reduced IFN-γ and TNF-α production and decreased $CD4^+$ T cell counts (Frick et al., 2008a). No changes were observed in $CD8^+$ T lymphocytes, B cell response to LPS and NK activity. These results highlight the importance of the impairment of T cell mediated immunity in response to stress and its relationship with disease, i.e. neoplastic processes. On the other hand, fluoxetine alone enhances T cell reactivity and cytokine production, without effects on $CD4^+$ and $CD8^+$ subpopulations (Frick et al., 2008b). Here we found that fluoxetine ameliorates the adverse effects of chronic stress at the immune system level. The present results contribute to the general knowledge about the beneficial effects of antidepressant treatment in the reversion of stress- or depression-associated impairments of the immune response. In this work, we demonstrated that fluoxetine administration in stressed mice results in a restoration to normal levels of T cell proliferation, cytokine expression and $CD4^+$ T lymphocyte subsets. The lack of effect of fluoxetine on NK cell and B lymphocytes is consistent with our previous studies using athymic nude mice, devoid specifically of T cells (Frick et al., 2008b). However, we observed differential events in these treatments. It seems that chronic stress impairs T helper immunity by reducing the total number of T $CD4^+$ cells. Instead, fluoxetine seems to alter T cell reactivity without any change in the total number of T lymphocytes.

The findings that fluoxetine alone does not affect the number of T $CD4^+$ cells, but stressed and fluoxetine-treated mice had no suppression of T $CD4^+$ cell number, as did the stressed alone mice, indicate that fluoxetine can act by compensatory and/or specific mechanisms modulating T helper immunity. The stress response involves important dysregulations of the neuroendocrine axis, including increased secretion of glucocorticoids and catecholamines (Calcagni and Elenkov, 2006). One possibility is that these stress hormones could be down-regulating the T helper cell component of immunity. However, fluoxetine, despite of its ability to restore hormonal levels under a stressful situation, is not able to modify them per se. The mechanisms underlying the effects of fluoxetine under normal and pathological situations remain still unknown and are now under study.

Another outstanding finding is the fact that fluoxetine per se plays an immunomodulatory role in normal animals. Others have shown that fluoxetine administration acts stimulating activity, cell cycle and proliferation in diverse tissues for example brain, muscle, bone and lymphoid cells among others (see for example Santarelli et al., 2003; Battaglino et al., 2007; Pacher et al., 2001). The fact that fluoxetine alone increases T cell proliferation as well as IL-2, IFN-γ and TNF-α expression above control levels may have important implications for the use of the this antidepressant drug in patients having pre-existing inflammatory conditions (Calcagni and

**Table 1**
Effects of stress and/or fluoxetine on cellular and humoral immunity

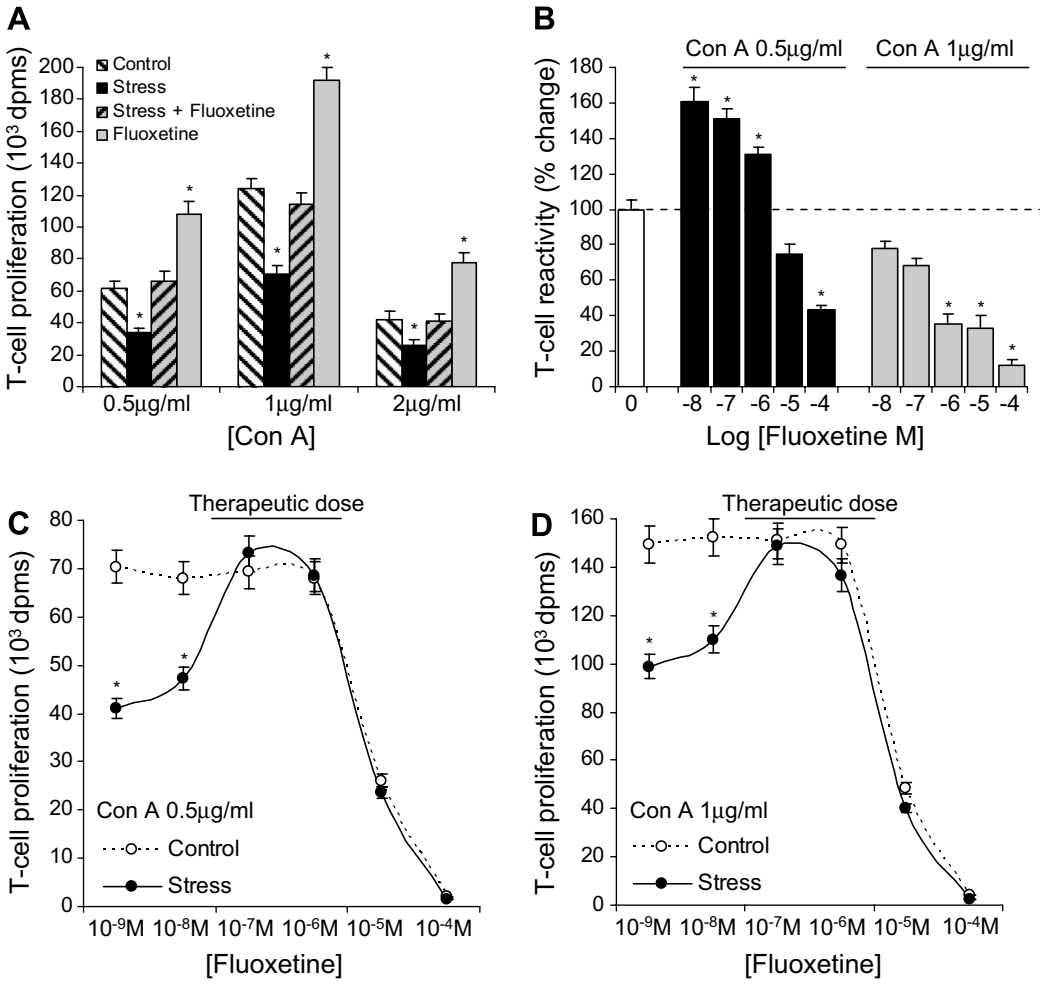
| | Control | Stress | Stress + fluoxetine | Fluoxetine |
|---|---|---|---|---|
| T cell proliferation to Con A | 73.8 ± 13.2 | 41.7 ± 8.3[a] | 67.9 ± 11.4[c] | 137.6 ± 16.7[a] |
| B cell proliferation to LPS | 7.39 ± 1.33 | 7.04 ± 1.62 | 7.38 ± 1.46 | 7.88 ± 2.15 |
| B cell proliferation to PWM | 8.59 ± 1.26 | 8.66 ± 1.65 | 7.72 ± 1.54 | 8.12 ± 1.56 |
| NK cell activity (lytic units) | 2.57 ± 0.51 | 2.70 ± 0.39 | 2.77 ± 0.54 | 2.62 ± 0.41 |
| IL-2 expression (mRNA quantity) | 3.46 ± 0.21 | 2.11 ± 0.19[a] | 3.79 ± 0.24[c] | 4.94 ± 0.29[a] |
| IFN-γ expression (mRNA quantity) | 1.27 ± 0.23 | 0.83 ± 0.18[a] | 1.19 ± 0.24[c] | 2.09 ± 0.49[a] |
| TNF-α expression (mRNA quantity) | 2.18 ± 0.55 | 1.19 ± 0.31[a] | 2.34 ± 0.53[c] | 4.27 ± 1.03[a] |
| $CD4^+$ T cells ($10^3$ events) | 9.02 ± 0.55 | 4.91 ± 0.23[b] | 8.44 ± 1.28[c] | 8.93 ± 1.18[c] |
| $CD8^+$ T cells ($10^3$ events) | 3.36 ± 0.66 | 3.28 ± 0.67 | 3.52 ± 0.73 | 3.57 ± 0.48 |

Mice were daily restrained for 3 weeks, treated daily with 15 mg/kg/day fluoxetine, or both. Proliferation indexes are shown for T and B cell proliferation to selective mitogens at optimal concentrations. NK activity is shown as lytic units/$10^6$ spleen cells. Gene expression is expressed as mRNA quantity of IL-2, TNF-α and IFN-γ normalized with β-actin. Total events of $CD4^+$ T helper and $CD8^+$ T-cytotoxic lymphocytes determined by flow cytometry are shown. Values are expressed as means ± standard deviation of three independent experiments ($n$ = 12–18 mice per group). Statistical significance was determined using one-way ANOVA followed by Dunnett's contrast for post hoc analysis.

[a] Differs from control group in $p < 0.001$.

[b] Differs from control group in $p < 0.01$.

[c] No significantly different from control group.




**E**

| [Fluoxetine] | Suboptimal Con A | |
|---|---|---|
| | Control | Stress |
| $10^{-9}$M | 70387 | 41089 |
| $10^{-8}$M | 108662 | 75521 |
| $10^{-7}$M | 104554 | 110395 |
| $10^{-6}$M | 89005 | 89873 |
| $10^{-5}$M | 19565 | 17679 |
| $10^{-4}$M | 3863 | 3867 |

**F**

| [Fluoxetine] | Optimal Con A | |
|---|---|---|
| | Control | Stress |
| $10^{-9}$M | 149405 | 98808 |
| $10^{-8}$M | 118764 | 85758 |
| $10^{-7}$M | 102642 | 101088 |
| $10^{-6}$M | 49230 | 45093 |
| $10^{-5}$M | 16898 | 14050 |
| $10^{-4}$M | 3467 | 3063 |

**Fig. 1.** Effects of fluoxetine on the proliferative response of T lymphocytes from normal and stressed mice. (A) T cell response of stressed mice, treated with 15 mg/kg/day fluoxetine, or both, to different doses of Con A was assayed. Total dpms are shown. (B) *In vitro* fluoxetine effect on normal T lymphocytes stimulated with suboptimal or optimal mitogen concentrations, expressed as percentage of change from control. Direct effects of fluoxetine on the stimulation of T cell proliferation induced by submitogenic (C) and mitogenic (D) Con A doses, obtained after subtracting the effects of fluoxetine alone (E and F). Values are expressed as means ± standard deviation of three independent experiments ($n = 12$ mice per group). Statistical significance was determined using one-way ANOVA followed by Dunnett's contrast for post hoc or unpaired *t*-test ($n = 12$ animals per group, $^{*}p < 0.01$).

Elenkov, 2006; Glaser and Kiecolt-Glaser, 2005). It is worth noting that some chronic inflammatory conditions such as multiple sclerosis, inflammatory bowel disease and psoriasis are also associated with stress and depression and the use of this drug may increase their inflammation.

A remarkable fact is that antidepressant route of administration conditions the effects further observed. Hence, it must be chosen carefully in order to avoid masking their effects due to an additional stressor that acts in the opposite direction. Nevertheless, it is worth noting that in either case, fluoxetine always improves the T cell mediated immunity, thus emphasizing the key importance of treating the negative symptoms of stress, and may help to elucidate the controversial findings of antidepressants in animal models of depression.

Fluoxetine can act at a central level (Pellegrino and Bayer, 1998, 2002), but it may also exert a direct regulation of peripheral cells (Frick et al., 2008b; Edgar et al., 1998, 1999). A direct effect of fluoxetine on normal and stressed T cells was demonstrated in this work. Although some components of this reversal might be indirect, we clearly demonstrated that fluoxetine directly modulates T

cell reactivity after *in vitro* exposure. It was previously shown that an oral dose between 10 and 25 mg/kg/day of fluoxetine administration in BALB/c mice results in plasma concentrations of about 170–1780 ng/ml, which correspond to $5 \times 10^{-7}$ M to $5 \times 10^{-6}$ M (Dulawa et al., 2004). Serum fluoxetine levels for the 10 mg/kg/day dose are toward the bottom of the range of plasma levels found in patients taking 20–80 mg/day Prozac (100–700 ng/ml) (Koran et al., 1996), whereas for 18 mg/kg/day dose (approximately 560 ng/ml) they are toward the high end of this range. The equivalent values in human serum are about to $2 \times 10^{-7}$–$3 \times 10^{-6}$ M. In these therapeutic doses we found that fluoxetine restores normal T cell proliferation in stressed mice both *in vivo* and *in vitro*. Our results also teach that antidepressant concentration is very important when assaying direct effects *in vitro*, given that cytotoxic/apoptotic phenomena can simply be due to high doses of these drugs, and these effects can not be paralleled to those occurring *in vivo*.

In a holistic conclusion, the present findings contribute to the growing evidences pointing to the relevance of pharmacological treatment of stress, and may help to open a new window to start exploring the mechanisms involved in direct and indirect effects of psychotropic drugs in non-neuronal cell types.

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