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Effects of antidepressant drugs on synaptic protein levels and dendritic outgrowth in hippocampal neuronal cultures

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The alteration of hippocampal plasticity has been proposed to play a critical role in both the pathophysiology and treatment of depression. In this study, the ability of different classes of antidepressant drugs (escitalopram, fluoxetine, paroxetine, sertraline, imipramine, tranylcypromine, and tianeptine) to mediate the expression of synaptic proteins and dendritic outgrowth in rat hippocampal neurons was investigated under toxic conditions induced by B27 deprivation, which causes hippocampal cell death. Postsynaptic density protein-95 (PSD-95), brain-derived neurotrophic factor (BDNF), and synaptophysin (SYP) levels were evaluated using Western blot analyses. Additionally, dendritic outgrowth was examined to determine whether antidepressant drugs affect the dendritic morphology of hippocampal neurons in B27-deprived cultures. Escitalopram, fluoxetine, paroxetine, sertraline, imipramine, tranylcypromine, and tianeptine significantly prevented B27 deprivation-induced decreases in levels of PSD-95, BDNF, and SYP. Moreover, the independent application of fluoxetine, paroxetine, and sertraline significantly increased levels of BDNF under normal conditions. All antidepressant drugs significantly increased the total outgrowth of hippocampal dendrites under B27 deprivation. Specific inhibitors of calcium/calcmodulin kinase II (CaMKII), KN-93, protein kinase A (PKA), H-89, or phosphatidylinositol 3-kinase (PI3K), LY294002, significantly decreased the effects of antidepressant drugs on dendritic outgrowth, whereas this effect was observed only with tianeptine for the PI3K inhibitor. Taken together, these results suggest that certain antidepressant drugs can enhance synaptic protein levels and encourage dendritic outgrowth in hippocampal neurons. Furthermore, effects on dendritic outgrowth likely require CaMKII, PKA, or PI3K signaling pathways. The observed effects may be may be due to chronic treatment with antidepressant drugs.

1. Introduction

Recent studies have determined that depression may be related to impairments in structural and functional plasticity in several brain areas (Pittenger and Duman, 2008; Manji et al., 2001). More specifically, changes in hippocampal structure and function are evident in both depressed patients and animal models of depression (Campbell and Macqueen, 2004; Lucassen et al., 2001; Magariños et al., 1996; Watanabe et al., 1992). These results indicate that depression is associated with dendritic atrophy and the loss of synaptic connections in the hippocampus and that neuritic alteration might underpin hippocampus-related memory and cognitive deficits in depressed patients (Silva, 2003). Therefore, dysfunctional hippocampal plasticity may contribute to the pathophysiology of depression.

The mechanisms underlying the delayed therapeutic effect of antidepressant drugs are still unclear, but it has been suggested that these drugs induce changes involving neuronal plasticity and extensive network reorganization (Ampuero et al., 2010; Pittenger and Duman, 2008). Accumulating evidence indicates that dendritic spine formation and synaptogenesis are among these changes (Ampuero et al., 2010; Chen et al., 2008, 2010; Hajszan et al., 2005; Zheng et al., 2011). Fluoxetine increases the number of spine synapses in the CA1 and CA3 regions of the rat hippocampus (Hajszan...
et al., 2005; Zheng et al., 2011) as well as enhances the expression of synapse-associated proteins in the hippocampi of ovarioctomized female rats (O’Leary et al., 2009). However, imipramine does not have an effect on synaptic proteins in rats with learned helplessness, an animal model of depression (Iwata et al., 2006). Treatment with a high dose of desipramine decreases synaptic proteins levels in the rat hippocampus, whereas no change was observed following administration of paroxetine (Martínez-Turrillas et al., 2005). Thus, the effects of antidepressant drugs on synaptic plasticity remain controversial.

Postsynaptic density protein 95 (PSD-95) is preferentially located in dendritic spines and plays a critical role in the regulation of dendritic spine size and shape (Ehrlich et al., 2007; Han and Kim, 2008). Postsynaptic brain-derived neurotrophic factor (BDNF), the most abundant neurotrophin in the brain, contributes to axonal branching, dendritic differentiation, and connectivity among neurons (Ji et al., 2005; Lesmann et al., 2003; Poo, 2001). Synaptophysin (SYP), a major integral membrane protein of presynaptic vesicles, is required for vesicle formation and exocytosis (Valtorta et al., 2004). It is widely used as a marker for synapse activity.

Neurite outgrowth is regulated by several signaling molecules including calcium/calmodulin-dependent protein kinase II (CaMKII), cAMP-dependent kinase (PKA), phosphatidylinositol 3-kinase (PI3K)/Akt, and mitogen-activated protein kinase (MAPK)/extra-cellular signaling-regulated kinase (ERK) (Kobayashi et al., 1997; Sánchez et al., 2004; Vaillant et al., 2002). CaMKII is concentrated in the cytoplasm and dendrites and engages in extensive cross talk with SK2 (Sánchez et al., 2004; Vaillant et al., 2002). CaMKII is concentrated in the cytoplasm and dendrites and engages in extensive cross talk with CaMKII, PKA, and PI3K signaling increases the expression of cAMP responsive genes, including BDNF.

A central role of PI3K/Akt signaling in the determination of dendritic morphogenesis has been reported (Kumar et al., 2005). This suggests that this type of signaling is important for the action of antidepressant drugs on neuronal plasticity (Pittenger and Duman, 2008). Taken together, these findings suggest that the effects of antidepressant drugs on these signaling cascades induce morphological changes within hippocampal neurons. Thus, the identification of different classes of antidepressant drugs that affect hippocampal dendritic morphology via CaMKII, PKA, and PI3K is required.

It is proposed here that antidepressant drugs act as key regulators of synaptic proteins and neuronal plasticity. The present study included selective serotonin reuptake inhibitors (SSRIs), escitalopram, fluoxetine, paroxetine, sertraline, a selective serotonin reuptake enhancer (SSRE), tianeptine, the monoamine oxidase inhibitor (MAOI), tranylcypromine, and the tricyclic antidepressant (TCA), imipramine. Furthermore, the current study utilized a model of toxicity, namely the omission of B27 in the culture medium of primary hippocampal cells, which causes cell death (Bastianetto et al., 2006). Growth medium B27 facilitates the optimal growth and long-term survival of rat embryonic hippocampal neurons. It has been reported that a deprivation of growth medium N2, which consists of constituents similar to those in B27, induces hippocampal cell death by activating caspases 3, 8, and 9, which are enzymes that play a pivotal role in apoptosis-associated cell death (Bastianetto et al., 2006). Moreover, our laboratory has demonstrated a decrease in the expression of synaptic proteins following B27 deprivation in rat hippocampal cultures (Park et al., 2013). Within the toxic environment of B27 deprivation, this study assessed the expression of synaptic proteins in rat hippocampal neurons following the application of different classes of antidepressant drugs. Furthermore, the influence of these drugs on dendritic morphology and the involvement of various intracellular signaling pathways on hippocampal dendritic outgrowth were evaluated.

2. Material and methods

2.1. Drugs and reagents

This study utilized neurobasal medium, fetal bovine serum (FBS), horse serum (HS), B27 supplement, c-glutamine, penicillin-streptomycin, and trypsin (Intrazyme; Worthington, CA, USA). The antidepressant drugs used were fluoxetine, paroxetine, sertraline, tranylcypromine, tianeptine (Tocris Bioscience; Ellisville, MO, USA), escitalopram (Lundbeck; Copenhagen, DK), and imipramine (Sigma; St. Louis, MO, USA). Antibodies used for Western blotting included anti-synaptophysin (sc-7568), anti-BDNF (sc-546), anti-goat and anti-rabbit IgG-horseradish-peroxide conjugates (Santa Cruz Biotechnology; Santa Cruz, CA, USA), anti-α-tubulin and anti-mouse IgG, peroxidase conjugates (Sigma), and anti-PSD-95 (AB9634; Millipore; Temecula, CA, USA). Antibodies used for immunostaining included anti-MAP-2 (Millipore), Alexa Fluor 568 goat anti-mouse IgG, and Hoechst 33258 (Invitrogen). Specific kinase inhibitors included the PI3K inhibitor LY294002 (Cell Signaling Technology; Beverly, MA, USA), the PKA inhibitor H-89 (Calbiochem; San Diego, CA, USA), and the CaMKII inhibitor KN-93 (Sigma).

2.2. Primary hippocampal cell cultures

All animal manipulations were performed in accordance with the animal care guidelines of the US National Institutes of Health (NIH publication no. 23–85, revised 1996). This experiment was approved by the Committee for Animal Experimentation and the Institutional Animal Laboratory Review Board of Inje Medical (approval no. 2011-036). Primary cultures of hippocampal neurons were prepared from fetal brains (embryonic day 17; E17) obtained from Sprague–Dawley rats ( Orient Bio; Gyeonggi-Do, Korea) in a manner similar to that developed by Kaech and Banker (2006). Briefly, the brains were exposed, the hippocampi were carefully removed, and the same procedure was repeated in a neurobasal medium containing 0.03% trypsin for 20 min at 37 °C (5% CO2). Cells were suspended in the neurobasal medium supplemented with 1% FBS, 1% HS, 2% serum-free growth medium B27 (components: bovine, α-tocopherol acetate, α-tocopherol, vitamin A, bovine serum albumin, catalase, insulin, transferrin, superoxide dismutase, corticosterone, galactose, ethanolamine, glutathione, carnitine, linoleic acid, linolenic acid, progestrone, putrescine, selenium, and triodo-L-thyronine), 0.25% c-glutamine, and 50 U/ml penicillin-streptomycin; this was considered the control condition. In cultures using the neurobasal medium, glial cell growth was reduced to less than 0.5% to create a nearly pure neuronal population (Brewer et al., 1993). During the Western blotting procedure, neurons were plated in 6-well dishes coated with poly-L-lysine at a density of 2 × 10⁶ per well. For the neurite assay, neurons were plated in 12-well dishes at a density of 2 × 10⁶ per well. They were grown under control conditions for either 7 days (neurite assay) or 10 days (Western blotting). A preliminary experiment conducted by our laboratory found that hippocampal cells are reduced by approximately 32% under B27-deprived conditions (data not shown). Following incubation for either 7 or 10 days, the cells were treated with antidepressant drugs in the presence or absence of B27 for either 4 days (Western blotting) or 5 days (neurite assay) before being harvested for further analysis. The culture media and drugs were changed every 2 days.

2.3. Drug treatment

Antidepressant drugs (10 mM) were dissolved in dimethyl sulfoxide (DMSO) and diluted to various concentrations (final concentration of 1% DMSO) with neurobasal medium before use. For the purposes of Western blotting and the neurite assay, cells were cultured for 4 days and 5 days, respectively, with escitalopram (1, 10, and 50 μM), fluoxetine (0.1, 1, and 10 μM), paroxetine (0.1, 1, and 10 μM), sertraline (0.05, 0.1, and 1 μM), imipramine (0.1, 1, and 10 μM), tranylcypromine (1, 10, and 50 μM) or tianeptine (10, 50, and 100 μM) in the presence or absence of B27. Control cells were cultured without antidepressant drugs under control conditions. The concentrations of the drugs used in these experiments were based on observation that lower concentrations of drugs had no effect on the key proteins Akt or ERK responsible for the activation of signaling pathways that regulate neurite outgrowth and neuronal differentiation and the higher concentrations reduce the viability of hippocampal cells (Table 1).

2.4. Western blot analysis

The cells were washed twice with ice-cold phosphate-buffered saline (PBS), lysed buffer (20 mM Tris–HCl, 137 mM NaCl, 10% glycerol, 1% NonidetP-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 2 mM EDTA, and one tablet of complete protease inhibitor (Roche, Laval, Quebec, Canada) was then added, and the samples were centrifuged at 15,000 g and 4 °C after which the supernatants were boiled in lysis buffer. Equal amounts of protein (20 μg) derived from the cell extracts under each treatment condition were separated on SDS-polyacrylamide gels and transferred electrophoretically onto polyvinylidene fluoride membranes. The blots were blocked by incubation in 5% (w/v) non-fat milk in Tris-buffered saline (TBS) with 0.15% Tween 20 (TBS-T) for 1 h. After incubation with a primary antibody (anti-PSD-95, 1:1000; anti-BDNF, 1:1000; anti-synaptophysin, 1:1000; or anti-α-tubulin, 1:2000) in TBS-T at 4 °C overnight, the membranes were washed three times.
MTT assay was performed as described in detail elsewhere (Park et al., 2011a).

2.6. Statistical analysis

Western blotting, values from all samples on an individual blot were expressed as the percent change in the average of control cells. Two or three individual blots were performed for each specific protein. In the neurite assay, 300–400 cells obtained from five randomly picked areas were analyzed from each sample. Of all the cells in the 10 fields, 600–800 in total, were included.

Changes in protein expression were analyzed by a two-way analysis of variance (ANOVA) to determine the individual and interactive effects of drug treatment and the B27-deprived condition. Changes in dendritic outgrowth were analyzed with a one-way ANOVA. A Scheffe’s test was used for all post hoc comparisons, and P-values ≤0.05 were deemed to indicate statistical significance.

### 3. Results

3.1. Effects of antidepressant drugs on the expression of the postsynaptic proteins PSD-95 and BDNF and the presynaptic protein synaptophysin

Antidepressant drug-induced changes in the expression of the postsynaptic proteins PSD-95 and BDNF and the presynaptic protein SYP were measured in rat hippocampal neurons maintained separately in media with or without B27.

The results of the two-way ANOVA are summarized (Table 2). Significant individual effects of each drug were found on PSD-95, BDNF, and SYP levels (all p < 0.05). The individual effects of B27 deprivation were also significant (all p < 0.01). The analysis revealed a significant Drug × B27 deprivation interaction showing significant effects all antidepressant on PSD-95 and SYP levels (all p < 0.05) under the B27-deprivation condition; Escitalopram, imipramine, tranylcypromine, and tianeptine, but not fluoxetine, paroxetine, and sertraline, was found to significantly affect BDNF levels under the B27-deprivation condition (p < 0.05).

B27 deprivation significantly reduced the expression levels of PSD-95, BDNF, and SYP to approximately 37%, 38%, and 31% of control levels, respectively (all p < 0.01, Figs. 1–3).

All concentrations of escitalopram, paroxetine, sertraline, and tranylcypromine attenuated the B27 deprivation-induced decrease in PSD-95 (p < 0.05 or p < 0.01, Fig. 1A, C, D, and F) but not in control cultures. At concentrations up to 1 μM, fluoxetine and imipramine produced the same effect (p < 0.001, Fig. 1B and E), and tianeptine significantly increased PSD-95 levels in a concentration-dependent manner in B27-deprived cultures (p < 0.01, Fig. 1G). None of the antidepressant drugs affected PSD-95 levels under control conditions with B27.

Escitalopram, fluoxetine, paroxetine, and sertraline effectively prevented the B27 deprivation-induced decrease of BDNF levels (p < 0.001, Fig. 2A–D). Moreover, high concentrations of fluoxetine, paroxetine, and sertraline significantly increased protein levels of BDNF by 34%, 32%, and 28%, respectively, under control conditions (all p < 0.01). At concentration up to 1 μM, imipramine significantly increased BDNF levels under B27 deprivation (p < 0.01, Fig. 2E) but not under control conditions. Tranylcypromine and tianeptine significantly increased BDNF protein levels in a concentration-dependent manner in B27-deprived cultures (p < 0.05 or p < 0.01, Figs. 2F and G) but not in control cultures.

Escitalopram, fluoxetine, paroxetine, sertraline, tianeptine, and tranylcypromine significantly increased SYP protein levels in a concentration-dependent manner in B27-deprived cultures (p < 0.05 or p < 0.01, Fig. 3A–D, F and G) but not in control cultures. Concentrations of imipramine of 0.1 and 10 μM significantly increased SYP levels under B27-deprived (p < 0.01, Fig. 3E) but not control conditions. Optimum effects of imipramine were observed at a dose of 1 μM.

### 3.2. Effects of antidepressant drugs on dendritic outgrowth

A neurite outgrowth assay was performed to investigate whether antidepressant drugs regulate dendritic morphology in hippocampal neurons. Hippocampal cells were incubated for 5 days with different classes of antidepressant drugs in media with or without B27.

All antidepressant drugs in this experiment significantly increased dendritic outgrowth in hippocampal cells in a concentration-dependent manner under control conditions (F = 5.543, p < 0.001 for escitalopram; F = 9.328, p < 0.001 for fluoxetine; F = 11.553, p < 0.001 for paroxetine; F = 5.690, p = 0.006 for sertraline; F = 7.890, p < 0.001 for imipramine;...
Table 2

Summary of the two-way analysis of variance for the levels of PSD-95, BDNF, and synaptophysin.

<table>
<thead>
<tr>
<th>Synaptophysin</th>
<th>Drug</th>
<th>B27 deprivation</th>
<th>F</th>
<th>p-Value</th>
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<td>0.004</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>Sertraline</td>
<td>B27 deprivation</td>
<td>31.68</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Tranylcypromine</td>
<td>B27 deprivation</td>
<td>35.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
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<td>&lt;0.001</td>
</tr>
<tr>
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</tr>
<tr>
<td></td>
<td>Paroxetine</td>
<td>B27 deprivation</td>
<td>31.68</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Sertraline</td>
<td>B27 deprivation</td>
<td>31.68</td>
<td>&lt;0.001</td>
</tr>
<tr>
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<td>Tranylcypromine</td>
<td>B27 deprivation</td>
<td>35.2</td>
<td>&lt;0.001</td>
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<tr>
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<td>Tranylcypromine</td>
<td>B27 deprivation</td>
<td>13.20</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

F = 9.601, p < 0.001 for tranylcypromine; F = 13.532, p < 0.001 for tianeptine.

Hippocampal cells were photographed (Fig. 4A) and scored to quantify dendritic outgrowth induced by the application of different concentrations of antidepressant drugs under the B27-deprivation condition (Fig. 4B). Hippocampal cells treated with B27 exhibited modest dendritic differentiation and an average dendrite length of approximately 51 μm. B27-deprived cells exhibited decreased dendritic outgrowth compared with hippocampal cells treated with B27 (p < 0.05). Escitalopram, fluoxetine, paroxetine, sertraline, tranylcypromine, and tianeptine attenuated the B27 deprivation-induced decrease in dendritic outgrowth in a concentration-dependent manner (F = 20.755, p < 0.001 for escitalopram; F = 12.845, p < 0.05 for fluoxetine; F = 21.374, p < 0.01 for paroxetine; F = 10.136, p < 0.05 for sertraline; F = 17.516, p < 0.01 for tranylcypromine; F = 5.158, p < 0.05 for tianeptine).

Concentrations of imipramine of 0.1–10 μM increased dendritic outgrowth (F = 7.621, p < 0.05), but the maximum effect of imipramine was observed at 1 μM. This suggests that the optimum effect of imipramine on the upregulation of BDNF and SYP levels, as well as on dendritic outgrowth, is observed at 1 μM, not 10 μM, in B27-deprived cultures.

Furthermore, in the preliminary experiment, all antidepressant drugs significantly increased the dendritic outgrowth of hippocampal cells in a concentration-dependent manner in control cultures (data not shown).

To determine whether CaMKII, PKA, and PI3K/Akt signaling contribute to the enhancement of dendritic outgrowth induced by antidepressant drugs, the effects of the selective CaMKII inhibitor KN-93, the selective PKA inhibitor H-89, and the selective PI3K inhibitor LY294002 on hippocampal morphology were examined in hippocampal cells treated without B27. KN-93 and H-89 concentrations of 0.01–1 μM and LY294002 concentration of 0.1–10 μM had no significant effects on dendritic outgrowth and cell viability, but higher concentrations (>1 μM for KN-93 and H-89 and >10 μM for LY294002) reduced cell viability; KN-93 and H-89 were cytotoxic with 25% and 30%, respectively, inhibition of cell viability at 10 μM concentration; At 20 μM, LY209002 reduced cell viability to about 20%. These effects may due to cytotoxicity by overdose. Thus, the highest concentrations employed in this study (1 μM for KN-93 and H-89 and 10 μM for LY294002) did not affect dendritic outgrowth or cell viability (Fig. 5A–C). 50 μM for escitalopram, 10 μM for fluoxetine, 1 μM for paroxetine, 100 μM for imipramine, 50 μM for tranylcypromine, and 100 μM for tianeptine.

KN-93 and H-89 inhibited the enhancement of dendritic outgrowth induced by SSRIs, escitalopram, fluoxetine, paroxetine, sertraline, TCA, imipramine, and MAOI, tranylcypromine (KN-93: F = 34.017, p < 0.001 for escitalopram; F = 20.469, p < 0.001 for fluoxetine; F = 34.781, p < 0.001 for paroxetine; F = 15.022, p < 0.001 for sertraline; F = 12.517, p < 0.001 for imipramine; F = 32.087, p < 0.001 for tranylcypromine (Fig. 5A)); H-89: F = 28.064, p < 0.001 for escitalopram; F = 17.799, p < 0.001 for fluoxetine; F = 28.208, p < 0.001 for paroxetine; F = 13.399, p < 0.001 for sertraline; F = 9.693, p < 0.001 for imipramine; F = 32.087, p < 0.001 for tranylcypromine (Fig. 5B)). In contrast, these inhibitors did not alter the effects of tianeptine on dendritic outgrowth (KN-93: F = 6.527, p = 0.422 for tianeptine (Fig. 5A); H-89, F = 5.751, p = 0.094 for tianeptine (Fig. 5B)). These results suggest that CaMKII and PKA signaling are required for the enhancing effects of escitalopram, fluoxetine, paroxetine, sertraline, imipramine, and tranylcypromine, whereas the SSRE tianeptine does not influence this signaling. Furthermore, the potentiating effects of all antidepressant drugs were blocked by the PI3K inhibitor LY294002 (F = 18.188, p = 0.001 for
escitalopram; $F = 10.110$, $p = 0.016$ for fluoxetine; $F = 13.853$, $p = 0.036$ for paroxetine; $F = 7.556$, $p = 0.005$ for sertraline; $F = 6.566$, $p = 0.005$ for imipramine; $F = 15.137$, $p = 0.007$ for tranylcypromine; $F = 5.888$, $p = 0.009$ for tianeptine (Fig. 5C).

These data suggest that escitalopram, fluoxetine, paroxetine, sertraline, imipramine, tranylcypromine, and tianeptine could promote dendritic outgrowth in hippocampal neurons via PI3K signaling.

4. Discussion

In the present study, different classes of antidepressant drugs that differ in their primary mechanisms of action reversed a B27 deprivation-induced decrease in the expression of PSD-95, BDNF, and SYF and also exerted a dendritic outgrowth-promoting effect via CaMKII, PKA, or PI3K signaling in B27-deprived hippocampal cultures. In particular, the SSRE tianeptine resulted in an enhancement
of dendritic outgrowth via PI3K signaling. The effects of different classes of antidepressant drugs may elucidate the common pathways that contribute to enhanced synaptogenesis.

A primary finding of this study is that treatment with different classes of antidepressant drugs accelerates the recovery of B27 deprivation-induced decreases in hippocampal PSD-95 levels. Several studies have investigated the effects of fluoxetine on PSD-95. Chronic fluoxetine administration ameliorates the decrease in PSD-95 induced by exposure to learned helplessness, an animal model of depression, in the hippocampus (Reinés et al., 2008) and results in an increase of PSD-95 in the hippocampi of ovariectomized, but not sham, mice (O’Leary et al., 2009). This effect was blocked in transgenic TrkB.T1 mice overexpressing a truncated form of the TrkB receptor (O’Leary et al., 2009). This suggests that at least a portion of this effect requires TrkB signaling. In support of this, BDNF treatment in cultured neurons prompts the growth of spines via regulation of the formation of PSD-95-TrkB complexes (Yoshii and Constantine-Paton, 2010), and NMDA receptor-
dependent BDNF activation facilitates the rapid transport of PSD-95 to dendritic spines via PI3K signaling (Yoshii and Constantine-Paton, 2007). However, desipramine results in a decrease in PSD-95 levels in the rat hippocampus, and paroxetine has no effect (Martínez-Turrillas et al., 2005). This discrepancy might be attributable to differences in the experimental paradigms (i.e., dosage, duration of administration, stressful condition, and animals vs. cells) used in the present study compared with previous studies. An increase in PSD-95 protein levels may reflect an enhancement in the number and size of dendritic spines, which in turn results in an enhancement of the number of synapse. Thus, an increase in this protein at glutamate synapses may have a beneficial impact on information flow to other neurons. Given the role of PSD-95 in spine formation, the current results suggest that various antidepressant drugs may induce the promotion of new synapse formation in hippocampal neurons. However, further research examining

Fig. 3. Effects of antidepressant drugs on the expression of synaptophysin in hippocampal neurons. Cells were treated with different doses of antidepressant drugs for 4 days with (+B27) or without (-B27) B27. (A) escitalopram, (B) fluoxetine, (C) paroxetine, (D) sertraline, (E) imipramine, (F) tranylcypromine, and (G) tianeptine. For each condition, three independent experiments were performed. Cell lysates were analyzed by SDS-PAGE and Western blotting with anti-synaptophysin primary antibodies. Western blot analyses were repeated two or three times for each protein in three independent cultures. A representative image and quantitative analysis normalized to the α-tubulin band are shown. Values are means ± SEM expressed as percentage of values of the control cells (+B27, no drug treatment). *p < 0.05 vs. untreated control, **p < 0.01 vs. untreated control, #p < 0.05 vs. B27-deprived only, ##p < 0.01 vs. B27-deprived only.
the density and morphology of dendritic spine is required to confirm this hypothesis.

The importance of BDNF in neurotrophic hypotheses of depression and the action of antidepressant drugs is supported by many studies. First, the serum of depressed individuals contains decreased levels of BDNF, which can be reversed by antidepressant drugs, suggesting a positive correlation between BDNF levels and antidepressant action (Brunoni et al., 2008). Moreover, BDNF levels are upregulated by the chronic administration of different classes of antidepressant drugs including TCAs, MAOIs, and SSRIs (Pittenger and Duman, 2008). Second, BDNF mimics the action of antidepressant drugs. BDNF infusions produce an antidepressant-like effect in animal models of depression (Hoshaw et al., 2005), and reduced BDNF expression results in depressive-like behavior in animals (Monteggia et al., 2007). Third, BDNF modulates synaptic plasticity. The molecular mechanisms of synaptic plasticity and the molecular changes induced by antidepressant drugs overlap considerably. Major signaling pathways that converge on CREB, primarily PKA, PI3K, and CaMKII, are involved in synaptic plasticity and are influenced by antidepressant drugs (Pittenger and Duman, 2008). The transcription factor CREB induces the expression of BDNF via activation of the cAMP/PKA pathway, which contributes to the stabilization of synaptic plasticity. CREB activity is also elevated following treatment with different classes of antidepressant drugs (Duman and Monteggia, 2006).

Although BDNF is strongly associated with the effects of antidepressant drugs, BDNF in and of itself cannot fully explain the effects of antidepressant drugs. Studies employing CREB-deficient mouse models demonstrate that, although the behavioral and endocrinological effects of antidepressant drugs occur via CREB-independent mechanisms, CREB activation is upstream of BDNF in response to desipramine and fluoxetine (Conti et al., 2002). Additionally, desipramine prevents stress-induced behavioral changes, including ERK phosphorylation and BDNF mRNA reductions, in a restraint stress model. This suggests that the regulation of BDNF expression by stress and antidepressant treatments correlates with alterations in the MAPK/ERK signaling pathway involving activation of CREB and induction of BDNF (Bravo et al., 2009).

In the present study, all antidepressant drugs increased BDNF expression under the toxic conditions induced by B27 deprivation. Among SSRIs, fluoxetine, paroxetine, and sertraline increased BDNF levels under control conditions. This is consistent with previous studies that found that the expression of BDNF in the hippocampi of

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**Fig. 4. Effects of antidepressant drugs on dendritic outgrowth in hippocampal neurons.** Cells were incubated with different doses of antidepressant drugs for 5 days without (-B27) or with (+B27) B27 (control). For each condition, two independent experiments were performed. Cells were photographed (A) and scored (B) according to the methods described above. In total, 600–800 cells were analyzed from each sample. Data (mean ± SEM) are expressed as values of the control cells (+B27, no drug treatment). *p < 0.05  **p < 0.01 vs. control.

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normal rats and mice is increased following the chronic administration of these drugs (Alme et al., 2007; Martínez-Turrillas et al., 2005; Peng et al., 2008). In contrast, escitalopram, imipramine, tranylcypromine, and tianeptine may only regulate BDNF under stressful conditions. It has been reported that escitalopram, a SSRI with the highest selectivity for the serotonin transporter, increases BDNF levels in the hippocampi of ischemic animals (Alboni et al., 2010), whereas BDNF levels were unchanged in the hippocampi of normal rats (Jacobsen and Mørk, 2004). However, further research is required to confirm this finding. More recently, tianeptine, a SSRE, was shown to exert a strong influence on the expression of BDNF in the hippocampi of rats (Della et al., 2012). Unlike SSRIs, tianeptine stimulates the uptake of serotonin in the rat brain, and this indicates that the effects of different classes of...
antidepressant drugs on BDNF expression are independent from their alteration of serotonin levels. In spite of the fact that the primary action of most antidepressant drugs affects extracellular levels of monoamines, a major action of these drugs may be to influence synaptic plasticity via the modulation of BDNF expression.

The fusion of neurotransmitter-containing synaptic vesicles with the plasma membrane leads to the release of neurotransmitters into the synaptic cleft, and the maintenance of neurotransmission is dependent on the subsequent exocytosis and recycling of synaptic vesicles (Evans and Cousin, 2005). SYP, an integral Ca^{2+}-binding synaptic vesicle membrane protein, is required for vesicle fusion and neurotransmitter release (Valtorta et al., 2004). In the present study, all antidepressant drugs significantly increased SYP levels in B27-deprived hippocampal cells, suggesting that these drugs increase presynaptic activity. A reduced expression of SYP in the rat hippocampus in response to various stressors, including chronic unpredictable mild stress, learned helplessness, and restraint stress, has been reported (Li et al., 2012; Reines et al., 2008; Xu et al., 2004). This reduction following stressful conditions may represent the degree of impairment of hippocampal plasticity. In addition, loss of SYP in the hippocampus is correlated with the cognitive decline seen with Alzheimer’s disease, reflecting its role in cognitive functioning (Sze et al., 1997). Furthermore, fluoxetine and tranylcypromine lead to an increased expression of SYP in the rat hippocampus (Rapp et al., 2004). Taken together, the present findings suggest that the changes in synaptic plasticity induced by antidepressant drugs may be associated with levels of SYP expression in the hippocampus.

We found that the different classes of antidepressant drugs had no effect on PSD-95 and SYP levels under control conditions. However, these drugs reversed the B27 deprivation-induced decreases in proteins levels, suggesting that antidepressants may exert a regulatory effect only under toxic conditions. Under such conditions, fluoxetine and imipramine individually produced significant effects on PSD-95 and BDNF levels at doses from 0.1 to 1 µM but not at a dose of 10 µM; it appears that the 10 µM dose does not have an effect under either toxic or control conditions. Although the expressions of PSD-95 and BDNF were significantly decreased following the 10 µM dose without B27 compared with B27 (p < 0.01), lower doses of fluoxetine and imipramine did not result in a significant reduction in these proteins. It should be emphasized that fluoxetine or imipramine at doses up to 1 µM, but not 10 µM, might have optimal effects on PSD-95 or BDNF levels under conditions of B27 deprivation.

This study demonstrated that the application of different classes of antidepressant drugs enhances dendritic outgrowth in B27-deprived hippocampal cultures. The maximum effect of imipramine on dendritic outgrowth was observed at 1 µM, which is consistent with its action on BDNF and SYP levels.

We cannot draw conclusions about in vivo effects of antidepressant drugs from our in vitro data because it is unclear whether these drugs reached sufficient therapeutic concentrations to up-regulating synaptic proteins levels and dendritic outgrowth discussed herein. Although the doses of antidepressant drugs used were generally higher than those normally found in brain tissue, such high doses are routinely used in in vitro studies. The difference between complex brain structures and cell cultures may explain the need for higher drug concentrations in in vitro studies.

Dendrites are the primary site of synaptic connections (Dailey and Smith, 1996), and dendritic outgrowth and synapse formation are the two essential processes involved in synaptogenesis. In hippocampal neurons, dendritic branching is observed during periods of synaptogenesis (Ziv and Smith, 1996), and several studies have reported that imipramine or fluoxetine increases the number of hippocampal pyramidal synapses in rats (Chen et al., 2008, 2010; Hajszan et al., 2005). Thus, it is thus possible that antidepressant drugs promote dendritic outgrowth and thereby facilitate synapse formation. However, a detailed understanding of the molecular mechanisms underlying the effects of different classes of antidepressant drugs has yet to be achieved.

Neurite outgrowth is mediated by the activation of CaMKII, PKA, and PI3K signaling. Using specific inhibitors of signal transduction, the present study demonstrated that these signaling molecules partially participate in antidepressant drug-induced dendritic outgrowth in hippocampal neurons, whereas tianeptine-induced dendritic outgrowth involves only PI3K signaling.

Several studies have reported that tianeptine exerts a neurotrophic effect by recovering the dendritic atrophy of hippocampal neurons caused by stress or other damage (Czéh et al., 2001; McEwen et al., 1997; Uzbay, 2008). These studies are supported by the current in vitro data and indicate that tianeptine promotes dendritic outgrowth under toxic conditions. Some studies have reported that tianeptine can augment neurite outgrowth and promote synaptic contacts in cultured hippocampal neurons (Chu et al., 2010). The present study demonstrated that tianeptine upregulates syntenin-1, which is involved in dendritic outgrowth and may be involved in the tianeptine-induced increase in dendritic outgrowth. BDNF is believed to induce neuronal differentiation and synaptogenesis via binding to TrkB receptors and the activation of PI3K/Akt signaling (Ohira and Hayashi, 2009). Although various antidepressant drugs commonly increase the expression of BDNF, it appears that the major signaling pathway supporting tianeptine-induced dendritic outgrowth involves PI3K.

CaMKII has been extensively studied in relation to its role in dendritic development and function (Rezmond and Ghosh, 2005),
For example, CaMKIIa stabilizes dendritic growth, and CaMKIIß has a positive effect on the extension of dendritic filopodia, which are precursors of dendritic spines. It is known that motile dendritic filopodia are a largely active partner in synapse formation (Dailey and Smith, 1996) and that two different antidepressant drugs, desipramine and reboxetine, activate CaMKII in hippocampal cell bodies via increased phosphorylation of Thr286 (Tiraboschi et al., 2004). PKA is also upregulated by the chronic administration of different classes of antidepressant drugs including TCAs and MAOIs (Perez et al., 2000). An increase in the levels of CaMKII and PKA may involve the regulation of transcription factor CREB, a substrate of both CaMKII and PKA.

Taken together, our data suggest that these different classes of antidepressant drugs may play a role in elevating not only dendritic outgrowth but also synaptic protein expression via activation of these signaling cascades (Fig. 6).

5. Conclusions

The present study provides evidence that different classes of antidepressant drugs present synaptic plasticity via modulation of synaptic proteins levels and dendritic outgrowth in the hippocampus. These effects may represent the final common pathways for the intracellular actions triggered by antidepressant drugs. Furthermore, effects of the SSRE tianeptine are mediated only by PI3K signaling.

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