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Hippocampal Brain-Derived Neurotrophic Factor Expression Following Treatment with Reboxetine, Citalopram, and Physical Exercise

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The antidepressants, reboxetine and citalopram, were used in conjunction with voluntary physical exercise (wheel running) in order to assess the contribution of noradrenergic and serotonergic activation to enhancements in hippocampal brain-derived neurotrophic factor (BDNF) expression resulting from antidepressant treatment and exercise. Reboxetine (40 mg/kg/day), citalopram (10 mg/kg/day), voluntary physical activity, and the combination of antidepressants with exercise were applied to rats for a range of treatment intervals (2 to 14 days). Hippocampal BDNF transcription levels (full-length BDNF, as well as exons I–IV) were then assessed via *in situ* hybridization.Reboxetine treatment led to a rapid (evident at 2 days) enhancement in BDNF transcription in several hippocampal regions. This increase was also observed when reboxetine treatment was combined with voluntary physical activity for 2 weeks. Treatment with citalopram led to an increase in BDNF mRNA in only one hippocampal region (CA2) after short-term (2 days) treatment, and when combined with exercise, increased BDNF mRNA in the CA4 and dentate gyrus after 2 weeks. As reported in previous studies, voluntary physical activity enhanced BDNF transcription in several hippocampal areas, both on its own and in combination with antidepressant treatments. Examination of the levels of individual BDNF transcript variants influenced by each of these antidepressants revealed distinct patterns of expression in response to the various treatments, and showed that exercise-plus-antidepressant produced significant changes where antidepressant alone failed. Overall, treatment with the norephinephrine-selective antidepressant, reboxetine, in combination with exercise, led to both rapid and sustained increases in hippocampal BDNF mRNA expression. The serotonergic agent, citalopram, appeared to require longer treatment intervals in order to influence BDNF expression positively.
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INTRODUCTION

Brain-derived neurotrophic factor (BDNF) is a molecule that influences not only neuronal survival, but also synaptic function and plasticity (Russo-Neustadt, 2003). Decreased BDNF expression is thought to play a role in the neurodegeneration and behavioral changes associated with chronic stress and depression, and restoring these levels may underlie the therapeutic responses to antidepressant medications (Duman *et al*, 1997). Much information has become available regarding the influence of physical exercise, antidepressant treatment, and other CNS-activating interventions on the expression of BDNF in the rat hippocampus. The combination of voluntary exercise and antidepressant treatment enhances the expression of hippocampal BDNF and that of its transcript variants in an additive manner (Russo-Neustadt *et al*, 1999), and evidence exists that monoaminergic activation is important for this effect (Garcia *et al*, 2003; Ivy *et al*, 2003). In the current study, we treated animals with the norepinephrine (NE)-selective antidepressant, reboxetine (Montgomery, 1999), and the highly serotonin (5-HT)-specific antidepressant, citalopram (Sanchez and Hyttel, 1999), in order to assess the relative contributions of NE and 5-HT activation to antidepressant and antidepressant/exercise-associated increases in hippocampal BDNF expression.

Much evidence exists that exercise activates monoaminergic transmission in multiple brain areas (Dey *et al*, 1992; Dunn *et al*, 1996; Dishman *et al*, 2000; Molteni *et al*, 2002). Recent studies from our laboratory have demonstrated that both an NE-specific lesion and temporary blockade of β-adrenergic receptors remove the BDNF mRNA-enhancing effects of voluntary exercise (Garcia *et al*, 2003; Ivy *et al*,

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2003). In addition, both NE- and 5-HT-selective receptor antagonists such as propranolol, ketanserin, and WAY-100635 reverse the BDNF-increasing effects of short-term treatment with the monoamine oxidase inhibitor, tranylcypromine (Ivy *et al*, 2003).

Acute forms of stress decrease the expression of hippocampal BDNF (Smith *et al*, 1995), and chronic, repeated stress leads to neurodegenerative changes and functional loss within the hippocampus (Kuroda and McEwen, 1998; Sapolsky, 2000). In one study from our laboratory, it was demonstrated that the combination of voluntary exercise with tranylcypromine treatment reversed the BDNF mRNA decline occurring with forced swimming, and also significantly increased total swimming time (Russo-Neustadt *et al*, 2001). In the Porsolt forced swim test, a variety of clinically efficacious antidepressants reverse the reduction in swimming time that occurs the day following the initial (15 min) exposure to this inescapable stress (Cryan *et al*, 2002). Reboxetine has been shown to increase the most active behaviors observed during the forced swim test, such as climbing the walls of the cylinder (Page *et al*, 2003).

In the current study, the effects of exercise, antidepressant medications, and the combination of exercise/antidepressants on hippocampal BDNF mRNA levels were examined via *in situ* hybridization after a range of treatment intervals (2–14 days). As transcription of the BDNF gene is directed by several distinct promoters (Timmusk *et al*, 1993), individual variants of BDNF mRNA (containing exons I–IV) were examined for distinct regulation by antidepressants and exercise. Exercise has been shown to lead to rapid increases in exon II, and more sustained increases in exon I in several hippocampal regions. Synergy was evident for the expression of one of these variants (exon I) when chronic exercise and tranylcypromine treatment were combined (Russo-Neustadt *et al*, 2000). In the current study, we determined the transcript expression patterns of each type of antidepressant and their combination with exercise.

MATERIALS AND METHODS

Subjects and Experimental Design

All animal use procedures described below were conducted in strict accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals (1996). All efforts were made to minimize the number of animals and any pain/distress they might incur. Male Sprague–Dawley rats ($n=115$, 350 g; Charles River) were housed singly in polyethylene cages ($48\times27\times20\,cm^3$) with food and water *ad libitum*, and a 12 : 12 h (06:00 to 18:00) light/dark cycle.

Voluntary physical activity entailed free access to running wheels throughout the experiment. After a week of initial acclimation to the vivarium, rats were placed in polyethylene cages equipped with running wheels (34.5 cm diameter; Nalgene, Oregon). Distance traveled on the running wheel per 24-h period was recorded by computer using Ratrun software (C. Hage Associates, CA).

Two-Day Study

Animals received injections of reboxetine (20 mg/kg, i.p., b.i.d. at 09:00 and 17:00), citalopram via osmotic minipump (10 mg/kg/day, see below), or comparable volume of saline by pump or injection, respectively (controls). As 06:00 has previously been determined to be the time of peak diurnal baseline expression of BDNF (Berchtold *et al*, 1997), rats were killed at this time of day following their last treatment day. Animals were decapitated, the brains quickly removed and quick-frozen in an isopentane/dry ice bath, and stored at $-80^{\circ}C$ until *in situ* hybridization experiments (see below). A total of six groups were used: saline, sedentary ($n=8$); saline, physical activity ($n=9$); reboxetine (20 mg/kg b.i.d., i.p.), sedentary ($n=5$); reboxetine, physical activity ($n=5$); citalopram (10 mg/kg/day, infusion), sedentary ($n=5$); and citalopram, physical activity ($n=4$).

Seven-Day Study

Conditions and parameters were identical to those in the 2-day study, except that an experimental duration of 7 days, instead of 2 days, was employed. Again, a total of six groups were used: saline, sedentary ($n=9$); saline, physical activity ($n=9$); reboxetine, sedentary ($n=5$); reboxetine, physical activity ($n=5$); citalopram, sedentary ($n=6$); and citalopram, physical activity ($n=7$).

Fourteen-Day Study

Adult male Sprague–Dawley rats were housed singly in cages as in the previous experiments, above. After 1 week of initial acclimation to the vivarium, rats were surgically implanted (09:00) in the mid-scapular region with osmotic minipumps (Alza, Palo Alto, CA), which continuously infused drug (saline, reboxetine, or citalopram) subcutaneously; citalopram (10 mg/kg/day) or reboxetine (40 mg/kg/day) was administered over 14 days. These rats were allowed free access to their running wheels for the duration of the experiment (14 days). A total of six groups were used: saline, sedentary ($n=5$); saline, physical activity ($n=7$); citalopram, sedentary ($n=6$); citalopram, physical activity ($n=7$); reboxetine, sedentary ($n=6$); and reboxetine, physical activity ($n=7$). Reboxetine was infused via osmotic minipump in this experiment because sores developed on injection sites when reboxetine was administered i.p. for more than 7 consecutive days (Pharmacia, personal communication).

cRNA probes, in situ *hybridization, and data analyses.* Construction of cRNA probes, *in situ* hybridization, and data analyses were performed as previously described (Russo-Neustadt *et al*, 2000). Statistical significance of results were determined using one-way ANOVA and subsequent Fisher's PLSD for multiple comparisons.

RESULTS

Two-Day Study

Within 2 days, reboxetine treatment led to a significant increase in total BDNF mRNA in all hippocampal regions

examined (Figure 1a): CA1 (125% of control, $F_{(3,24)} = 2.70$, $p = 0.04$); CA2 (165%, $F_{(3,24)} = 5.21$, $p = 0.003$); CA3 (156%, $F_{(3,24)} = 4.96$, $p = 0.007$); CA4 (168%, $F_{(3,24)} = 7.07$, $p = 0.004$); and dentate gyrus (DG) (156%, $F_{(3,24)} = 4.58$, $p = 0.003$). Reboxetine treatment also elevated exon II mRNA levels in the CA2 (Figure 1c: 300% of control, $F_{(3,18)} = 6.617$, $p < 0.001$).

Voluntary exercise for 2 days significantly increased full-length BDNF in the CA4 (139% of control, $F_{(3,24)} = 7.07$, $p = 0.041$) and DG (133%, $F_{(3,24)} = 4.58$, $p = 0.032$; Figure 1a). In all, 2 days of activity also elevated exon II levels in the CA4 (193%, $F_{(3,21)} = 3.09$, $p = 0.028$) and DG (165%, $F_{(3,21)} = 3.88$, $p = 0.026$; Figure 1c).

The combination of reboxetine and exercise increased total BDNF levels to a degree comparable to reboxetine alone in all hippocampal subregions examined after 2 days of treatment (Figure 1a). Exon I was elevated in the CA4 region with the combination of reboxetine and exercise, to a degree greater than either treatment alone (Figure 1b: 205% of control, $F_{(3,23)} = 6.517$, $p < 0.001$). Exon II showed significant increases in the CA1, CA2, CA4, and DG with reboxetine-plus-exercise: CA1 (265%, $F_{(3,18)} = 2.964$, $p = 0.017$); CA2 (261%, $F_{(3,18)} = 6.617$, $p = 0.006$); CA4 (226%, $F_{(3,21)} = 3.088$, $p = 0.016$); and DG (207%, $F_{(3,21)} = 3.88$, $p = 0.004$ (Figure 1c)). Exon III was increased in all hippocampal regions examined following 2 days of reboxetine-plus-exercise: CA1 (190%, $F_{(3,21)} = 2.728$, $p = 0.014$); CA2 (192%, $F_{(3,21)} = 2.738$, $p = 0.024$); CA3 (185%, $F_{(3,22)} = 6.471$, $p = 0.001$); CA4 (169%, $F_{(3,21)} = 1.957$, $p = 0.041$); and DG (204%, $F_{(3,22)} = 3.256$, $p = 0.006$ (Figure 1d)). The combination of reboxetine with exercise clearly led to a greater influence on the expression of BDNF transcript variants I–III than reboxetine alone at this time interval. On the other hand, the 2-day reboxetine treatments led to no significant changes in exon IV, with or without exercise (Figure 1e).

After 2 days of treatment, citalopram increased full-length BDNF mRNA levels only in the CA2 (208% of control, $F_{(3,19)} = 1.88$, $p = 0.037$; Figure 2a). Short-term citalopram treatment had a significant influence on the expression of the transcript variants, exons II and IV. Citalopram significantly decreased exon II levels in the CA1 (56% of control, $F_{(3,19)} = 9.40$, $p = 0.016$) and CA3 (62%, $F_{(3,19)} = 23.53$, $p = 0.007$; Figure 2c), and significantly elevated exon IV levels in the CA4 region (137%, $F_{(3,19)} = 5.71$, $p = 0.025$; Figure 2e).

In this group of animals, short-term exercise significantly elevated full-length BDNF levels in all hippocampal areas except the CA2 (Figure 2a): CA1 (252% of control, $F_{(3,19)} = 2.30$, $p = 0.019$); CA3 (178%, $F_{(3,19)} = 2.99$, $p = 0.022$); CA4 (246%, $F_{(3,19)} = 2.70$, $p = 0.014$); and DG (232%, $F_{(3,19)} = 3.09$, $p = 0.011$). There was also a striking elevation of exon I mRNA in the CA1 (456%, $F_{(3,19)} = 3.12$, $p = 0.027$), and significant increases in exon I in the CA4 (187%, $F_{(3,19)} = 4.54$, $p = 0.009$) and DG (288%, $F_{(3,19)} = 9.40$, $p < 0.001$; Figure 2b). In all, 2 days of exercise also elevated exon II in all hippocampal regions except for the CA2 in this group (Figure 2c): CA1 (139%, $F_{(3,19)} = 9.4$, $p = 0.020$); CA3 (148%, $F_{(3,19)} = 23.53$, $p < 0.001$); CA4 (172%, $F_{(3,19)} = 11.09$, $p < 0.001$) and DG (158%, $F_{(3,19)} = 9.42$, $p = 0.003$), and significantly increased exons III (198%, $F_{(3,19)} = 3.48$, $p = 0.016$) and IV (132%, $F_{(3,19)} = 3.56$, $p = 0.019$) in the DG (Figures 2d and e).

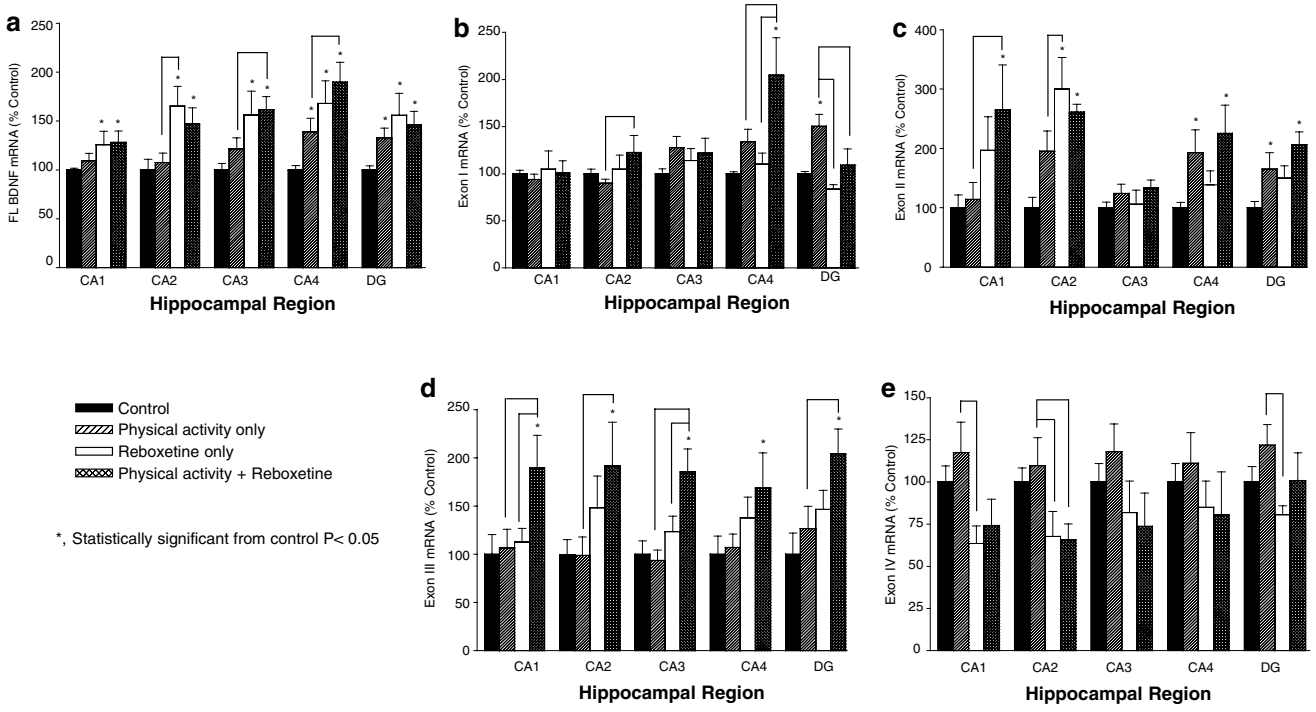

Figure 1 After 2 days, reboxetine and/or physical activity significantly elevated full-length BDNF (a), exon I (b), exon II (c), exon III (d), and exon IV (e) mRNA levels above those of controls in the indicated hippocampal regions (denoted by asterisks, which indicate statistically significant difference from controls at $p < 0.05$). Statistical significance was determined using one-way ANOVA and subsequent Fisher's PLSD for multiple comparisons. Brackets denote significant differences between the indicated groups ($p < 0.05$).

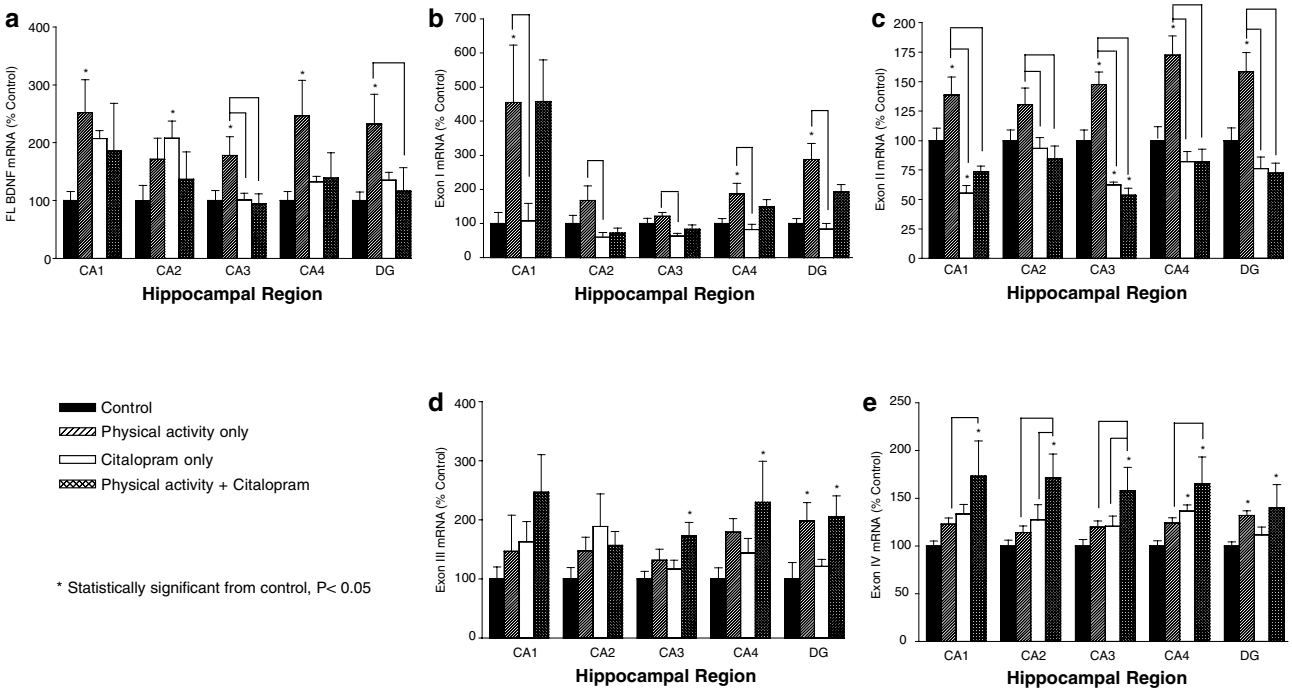

Figure 2 After 2 days, citalopram and/or physical activity significantly elevated full-length BDNF (a), exon I (b), exon II (c), exon III (d), and exon IV (e) mRNA levels above those of controls in the indicated hippocampal regions (denoted by asterisks, which indicate statistically significant difference from controls at $p<0.05$). Statistical significance was determined using one-way ANOVA and subsequent Fisher's PLSD for multiple comparisons. Brackets denote significant differences between the indicated groups ($p<0.05$).

The combination of citalopram-plus-exercise for 2 days had no significant influence on full-length BDNF mRNA levels (Figure 2a). On the other hand, citalopram-plus-exercise increased exon III levels in the CA3 (173% of control, $F_{(3,19)}=2.72$, $p=0.012$), CA4 (230%, $F_{(3,19)}=2.87$, $p=0.012$) and DG (205%, $F_{(3,19)}=3.48$, $p=0.025$; Figure 2d), and significantly elevated exon IV levels in all hippocampal regions examined (Figure 2e): CA1 (173%, $F_{(3,19)}=4.18$, $p=0.002$); CA2 (171%, $F_{(3,19)}=5.43$, $p<0.001$); CA3 (158%, $F_{(3,19)}=4.161$, $p=0.002$); CA4 (165%, $F_{(3,19)}=5.706$, $p<0.001$); and DG (140%, $F_{(3,19)}=3.56$, $p=0.013$).

In this 2-day experiment, all three active groups of rats ran comparably with an average running distance of (mean $\pm$ SEM) 1.08 ± 0.19 (saline, physical activity), 0.69 ± 0.19 (reboxetine, physical activity), and 0.65 ± 0.08 (citalopram, physical activity) kilometers per 24 h (Figure 7).

One-Week Study

After 1 week, the effects of reboxetine as a single treatment were not as widespread as those observed at 2 days. Reboxetine treatment upregulated full-length BDNF transcription only in the CA1 at this time point (130% of control, $F_{(3,24)}=6.03$, $p=0.016$; Figure 3a). Reboxetine led to an increase of exon III mRNA in the CA3 after 1 week (182%, $F_{(3,23)}=3.57$, $p=0.017$; Figure 3d), but significantly decreased exon I in the CA1 (58%, $F_{(3,24)}=5.35$, $p=0.006$; Figure 3b), and exon IV in all subregions examined (Figure 3e): CA1 (36%, $F_{(3,24)}=12.01$, $p<0.001$); CA2 (28%, $F_{(3,23)}=12.35$, $p<0.001$); CA3 (42%, $F_{(3,24)}=9.03$, $p<0.001$); CA4 (40%, $F_{(3,23)}=8.60$, $p<0.001$); and DG (40%, $F_{(3,24)}=9.75$, $p<0.001$).

The effects of voluntary exercise on full-length BDNF transcription were very evident after 1 week. Significant increases were observed in all hippocampal subregions except for the CA1 (Figure 3a): CA2 (144% of control, $F_{(3,24)}=4.34$, $p=0.009$); CA3 (149%, $F_{(3,24)}=4.87$, $p=0.005$); CA4 (148%, $F_{(3,24)}=3.32$, $p=0.016$); and DG (190%, $F_{(3,24)}=3.09$, $p=0.007$). Exercise also produced a significant increase in exon I mRNA in the DG (162%, $F_{(3,24)}=2.53$, $p=0.045$; Figure 3b).

As was observed after 2 days, full-length BDNF mRNA expression was enhanced in most regions examined following the reboxetine/exercise combination for one week (Figure 3a): CA1 (145% of control, $F_{(3,24)}=6.03$, $p=0.006$); CA2 (157%, $F_{(3,24)}=4.34$, $p=0.004$); CA3 (161%, $F_{(3,24)}=4.87$, $p=0.004$); and CA4 (159%, $F_{(3,24)}=3.32$, $p=0.012$). The transcript variant, exon I, was greatly increased by the physical activity/reboxetine combination at 1 week in the CA4 (283%, $F_{(3,24)}=4.09$, $p=0.004$; Figure 3b), and significantly higher levels were also evident in the DG (173%, $F_{(3,24)}=2.53$, $p=0.047$). The physical activity/reboxetine combination also enhanced the expression of exon II in the CA3 (158%, $F_{(3,24)}=2.06$, $p=0.034$); CA4 (185%, $F_{(3,21)}=3.94$, $p=0.005$); and DG (156%, $F_{(3,24)}=2.26$, $p=0.041$ (Figure 3c)). Exon III was also significantly elevated by reboxetine-plus-exercise in all hippocampal regions except for the CA2 (Figure 3d): CA1 (185% from control, $F_{(3,23)}=4.74$, $p=0.002$); CA3 (168%, $F_{(3,23)}=3.57$, $p=0.042$); CA4 (181%, $F_{(3,23)}=4.81$, $p=0.003$); and DG (183%, $F_{(3,23)}=4.31$, $p=0.003$). Once again, reboxetine-plus-exercise enhanced the expression of

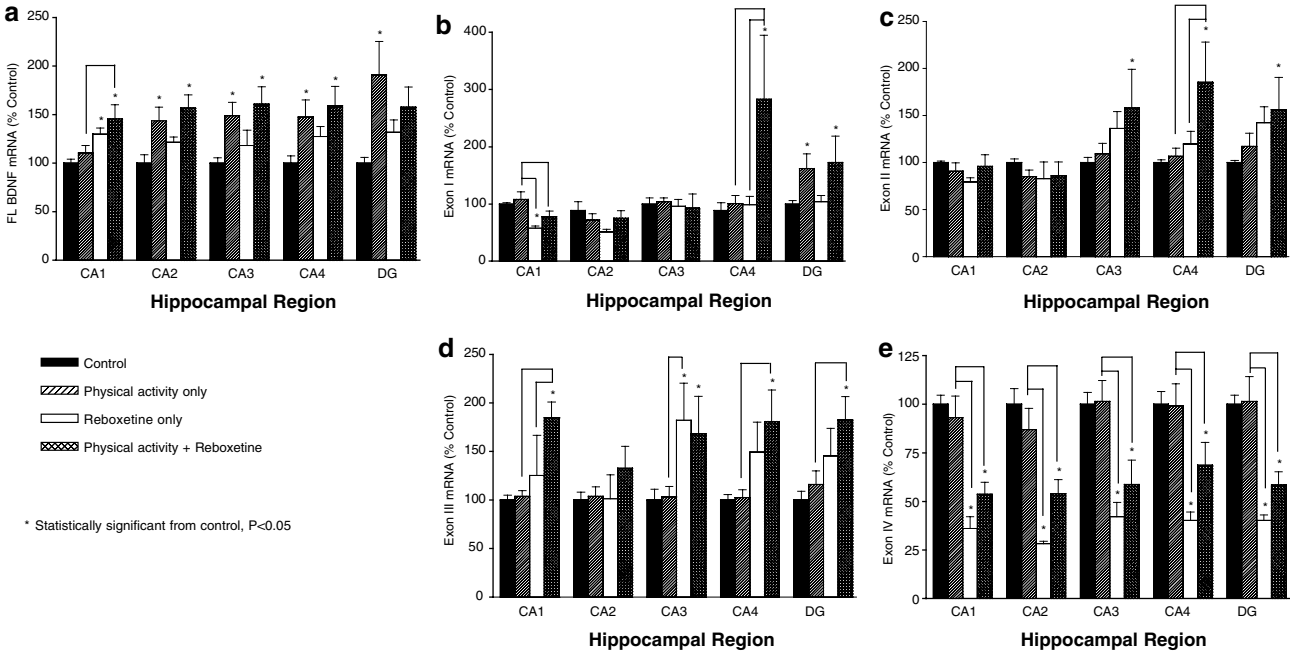

Figure 3 After 7 days, reboxetine and/or physical activity significantly elevated full-length BDNF (a), exon I (b), exon II (c), exon III (d), and exon IV (e) mRNA levels above those of controls in the indicated hippocampal regions (denoted by asterisks, which indicate statistically significant difference from controls at $p<0.05$). Statistical significance was determined using one-way ANOVA and subsequent Fisher's PLSD for multiple comparisons. Brackets denote significant differences between the indicated groups ($p<0.05$).

these transcript variants more than reboxetine alone after 1 week. In striking contrast to these results, exon IV levels were significantly decreased following reboxetine-plus-exercise treatments in all hippocampal regions examined (Figure 3e): CA1 (54% of control, $F_{(3,24)}=12.01$, $p=0.001$); CA2 (54%, $F_{(3,23)}=12.35$, $p=0.002$); CA3 (59%, $F_{(3,24)}=9.03$, $p=0.007$); CA4 (68%, $F_{(3,23)}=8.6$, $p=0.028$); and DG (58%, $F_{(3,24)}=9.75$, $p=0.006$).

In all, 1 week of treatment with citalopram had no effect on full-length BDNF mRNA levels, nor did it significantly affect the levels of any transcript variants (Figures 4a–e). Exercise alone enhanced full-length BDNF mRNA levels in the CA3 (152% of control, $F_{(3,23)}=9.79$, $p=0.002$) and CA4 (151%, $F_{(3,23)}=7.85$, $p=0.002$). Exercise also significantly increased exon I levels in the CA4 (142%, $F_{(3,23)}=8.35$, $p=0.024$) and DG (178%, $F_{(3,23)}=9.36$, $p=0.008$; Figure 4b), and exon II levels in the CA4 (127%, $F_{(3,23)}=4.57$, $p=0.034$; Figure 4c). Conversely, exon IV levels were significantly decreased in all hippocampal regions by one week of exercise in this group: CA1 (56% of control, $F_{(3,23)}=5.56$, $p=0.001$); CA2 (48%, $F_{(3,23)}=4.92$, $p=0.001$); CA3 (50%, $F_{(3,23)}=6.91$, $p<0.001$); CA4 (57%, $F_{(3,23)}=3.73$, $p=0.004$); and DG (51%, $F_{(3,23)}=4.98$, $p=0.001$; Figure 4e).

When citalopram was combined with exercise for 1 week, full-length BDNF levels were significantly increased in the CA2 (188% of control, $F_{(3,23)}=2.39$, $p=0.029$; Figure 4a), and exon I levels were elevated in the CA4 (182%, $F_{(3,23)}=8.35$, $p<0.001$) and DG (219%, $F_{(3,23)}=9.36$, $p<0.001$; Figure 4b). This combination treatment had no significant effect on exon II or III (Figures 4c and d). Exon IV levels were significantly decreased in the CA2 (67%, $F_{(3,23)}=4.92$, $p=0.024$), CA3 (70%, $F_{(3,23)}=6.91$, $p=0.015$), and DG (71%, $F_{(3,23)}=4.98$, $p=0.038$; Figure 4e).

There was no significant difference in average running distance between the three groups of animals: (mean $\pm$ SEM) 2.30 ± 0.31 (saline, physical activity), 1.35 ± 0.43 (reboxetine, physical activity), and 1.27 ± 0.211 (citalopram, physical activity) kilometers per 24 h (Figure 7).

Fourteen-Day Study

After 14 days of treatment, reboxetine no longer influenced full-length BDNF mRNA levels, but did increase exon IV levels in the CA2, CA3, and CA4 (Figure 5e): CA2 (138% of control, $F_{(3,23)}=2.32$,. $p=0.037$); CA3 (140%, $F_{(3,23)}=2.26$, $p=0.031$); and CA4 (141%, $F_{(3,23)}=2.55$, $p=0.049$). Physical exercise as a single treatment for 14 days increased only exon II in the CA4 (128%, $F_{(3,20)}=11.32$, $p=0.011$; Figure 5c). On the other hand, the combination of reboxetine-plus-exercise for this time interval led to significant increases in full-length BDNF mRNA in the CA3, CA4, and DG (Figure 5a): CA3 (149%, $F_{(3,21)}=2.76$, $p=0.040$); CA4 (230%, $F_{(3,21)}=5.05$, $p=0.003$); and DG (154%, $F_{(3.21)}=5.77$, $p=0.010$). Also, it should be noted that reboxetine-plus-exercise enhanced the levels of BDNF mRNA significantly over that of the exercise-only group in all regions except in the CA1. This combination treatment also led to striking increases in exon I in several hippocampal regions (Figure 5b): CA1 (269%, $F_{(3,21)}=1.88$, $p=0.038$); CA3 (212%, $F_{(3,21)}=5.84$, $p=0.003$); CA4 (155%, $F_{(3,21)}=3.31$, $p=0.048$); and DG (278%, $F_{(3,21)}=10.1$, $p<0.001$). Significant increases were also observed for exon II in the CA3 (125%, $F_{(3,20)}=5.02$,

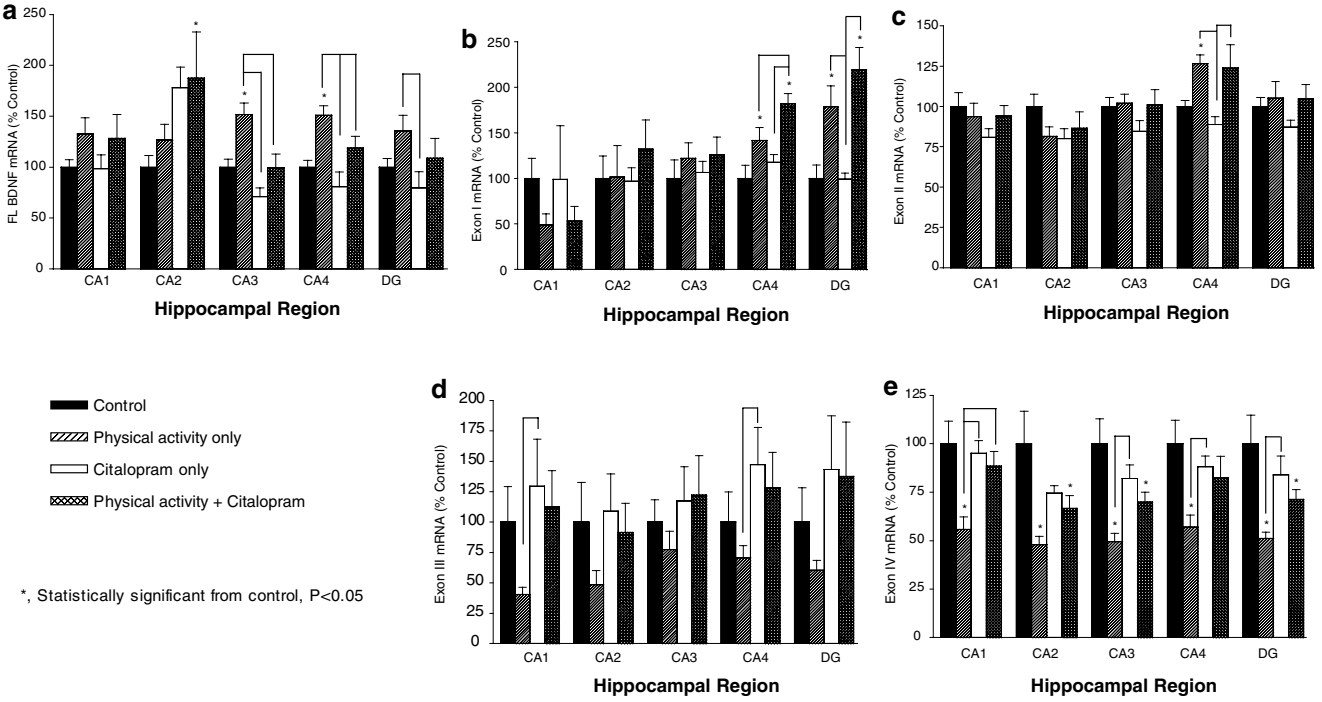

Figure 4 After 7 days, citalopram and/or physical activity significantly elevated full-length BDNF (a), exon I (b), exon II (c), exon III (d), and exon IV (e) mRNA levels above those of controls in the indicated hippocampal regions (denoted by asterisks, which indicate statistically significant difference from controls at $p<0.05$). Statistical significance was determined using one-way ANOVA and subsequent Fisher's PLSD for multiple comparisons. Brackets denote significant differences between the indicated groups ($p<0.05$).

$p=0.032$) and CA4 (147%, $F_{(3,20)}=11.32$, $p<0.001$; Figure 5c), and for exon IV in the CA4 (147%, $F_{(3,23)}=2.55$, $p=0.019$) and DG (138%, $F_{(3,23)}=2.36$, $p=0.021$; Figure 5e).

Citalopram alone did not increase full-length BDNF transcription relative to controls in any hippocampal region examined after 2 weeks of treatment. However, as was observed with reboxetine (above), the exon IV transcript variant was elevated in several hippocampal regions (Figure 6e), including CA1 (166% of control, $F_{(3,23)}=3.74$, $p=0.006$), CA3 (156%, $F_{(3,23)}=4.35$, $p=0.012$), CA4 (178%, $F_{(3,23)}=8.82$, $p<0.001$), and DG (154%, $F_{(3,23)}=7.84$, $p=0.001$). Similar to the results noted above, exercise alone for 2 weeks enhanced full-length BDNF (136% of control, $F_{(3,24)}=5.12$, $p=0.035$; Figure 6a) and exon II only in the CA4 (127%, $F_{(3,24)}=4.86$, $p=0.011$; Figure 6c). The combination of citalopram plus exercise, on the other hand, led to significant elevations in full-length BDNF mRNA in the CA4 and DG (Figure 6a): CA4 (158% of control, $F_{(3,21)}=5.12$, $p=0.002$); DG (134%, $F_{(3,21)}=8.41$, $p=0.008$), and increases in several transcript variants. Exon I was elevated by citalopram-plus-exercise in the CA1 and DG (Figure 6b): CA1 (280% of control, $F_{(3,21)}=3.81$, $p=0.005$); DG (225%, $F_{(3,21)}=8.51$, $p=0.002$). Exon II was increased in the CA4 (125%, $F_{(3,20)}=4.86$, $p=0.016$; Figure 6c), and exon IV was elevated in the CA3 (157%, $F_{(3,23)}=4.35$, $p=0.007$), CA4 (180%, $F_{(3,23)}=8.82$, $p<0.001$), and DG (160%, $F_{(3,23)}=7.84$, $p<0.001$; Figure 6e).

Rats allowed access to exercise during this 2-week study ran an average of (mean $\pm$ SEM) 0.853 ± 0.20 (saline, physical activity), 4.32 ± 0.16 (reboxetine, physical activity), and 1.99 ± 0.53 (citalopram, physical activity) kilometers per 24 h. There was a significant effect of treatment group on average running distance over the entire 14 days ($F_{(2,18)}=26.96$, $p<.0001$). In addition, two-way ANOVA revealed a significant effect for group ($F_{(2,241)}=97.08$, $p<0.0001$), time ($F_{(13,241)}=6.30$, $p<0.0001$), and the interaction between time and treatment group ($F_{(26,241)}=1.97$, $p=0.0045$). Note that rats treated with reboxetine ran, on average, more than double the citalopram group and 5 times more than animals given saline vehicle (Figure 7).

DISCUSSION

Evidence exists that NE stimulation is important for both exercise- and antidepressant-induced regulation of hippocampal BDNF expression (Garcia *et al*, 2003; Ivy *et al*, 2003). Therefore, it is possible that neuronal stimulation via NE may activate a common intracellular pathway for antidepressant medications and exercise, and participate in the additive effects of these two interventions (Russo-Neustadt *et al*, 2000). Furthermore, many antidepressants stimulate BDNF expression after chronic but not acute treatment (Nibuya *et al*, 1995; Nibuya *et al*, 1996), and the addition of exercise accelerates increases in BDNF expression with tranylcypromine treatment (Russo-Neustadt *et al*, 2000). Therefore, NE stimulation may exert a rapid influence on BDNF expression. Consistent with this idea is our current observation that reboxetine, a highly NE-selective antidepressant (Montgomery, 1999), led to rapid (within 2 days) increases in hippocampal BDNF mRNA. This effect was sustained over 2 weeks when reboxetine was combined with

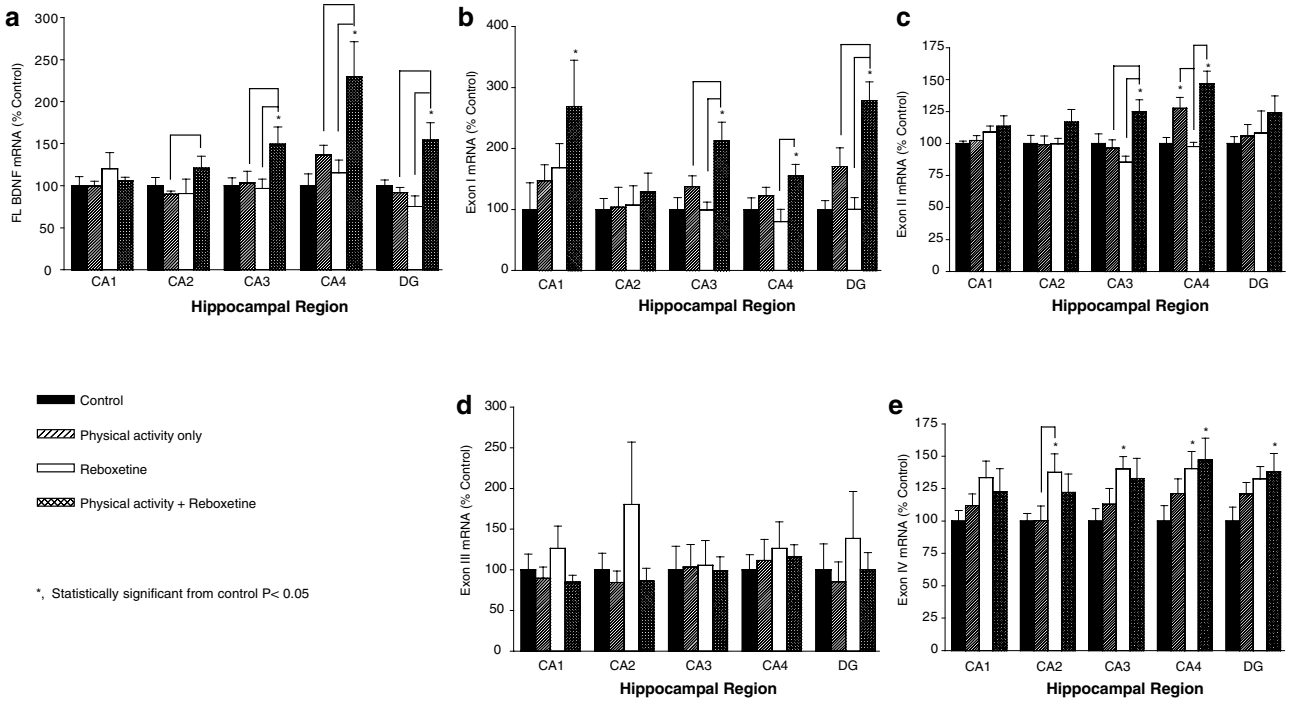

Figure 5 After 14 days, reboxetine and/or physical activity significantly elevated full-length BDNF (a), exon I (b), exon II (c), exon III (d), and exon IV (e) mRNA levels above those of controls in the indicated hippocampal regions (denoted by asterisks, which indicate statistically significant difference from controls at $p<0.05$). Statistical significance was determined using one-way ANOVA and subsequent Fisher's PLSD for multiple comparisons. Brackets denote significant differences between the indicated groups ($p<0.05$).

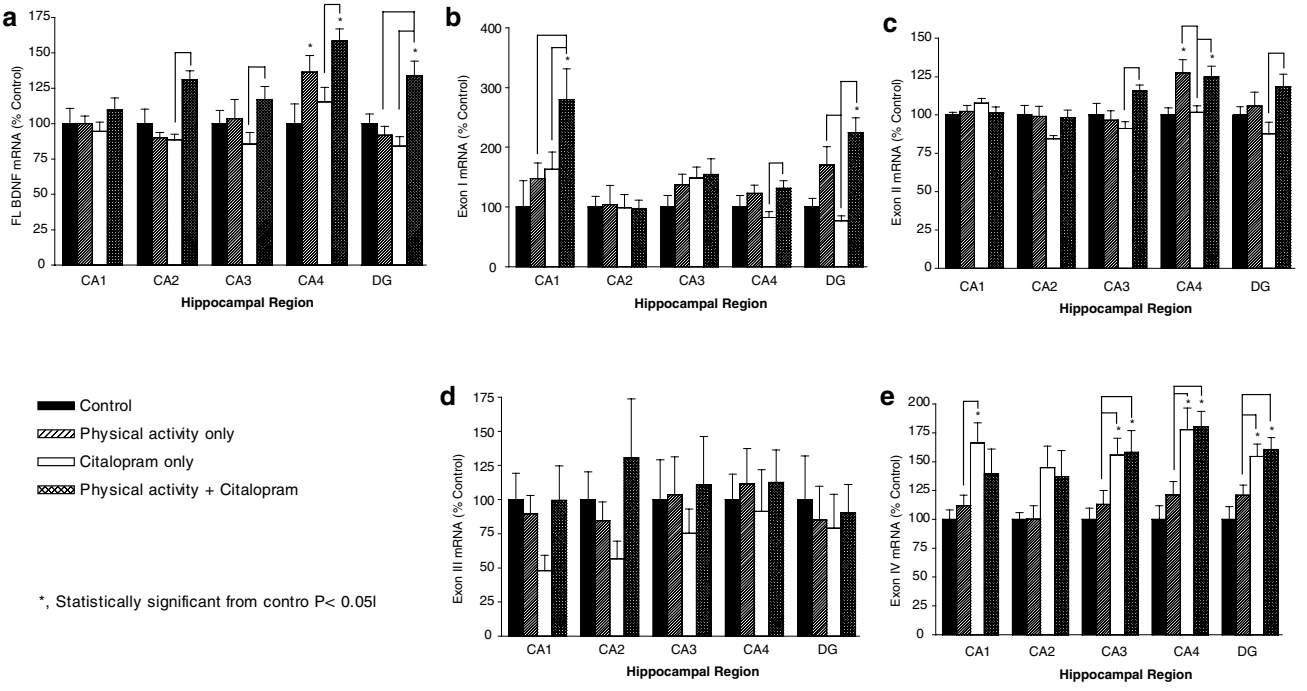

Figure 6 After 14 days, citalopram and/or physical activity significantly elevated full-length BDNF (a), exon I (b), exon II (c), exon III (d), and exon IV (e) mRNA levels above those of controls in the indicated hippocampal regions (denoted by asterisks, which indicate statistically significant difference from controls at $p<0.05$). Statistical significance was determined using one-way ANOVA and subsequent Fisher's PLSD for multiple comparisons. Brackets denote significant differences between the indicated groups ($p<0.05$).

exercise. It should be noted, also, that the combination of exercise with reboxetine increased the expression of several BDNF transcript variants that were not influenced by reboxetine alone. The addition of exercise therefore appeared to make the effect of reboxetine on BDNF expression more widespread and long-lasting. Importantly,

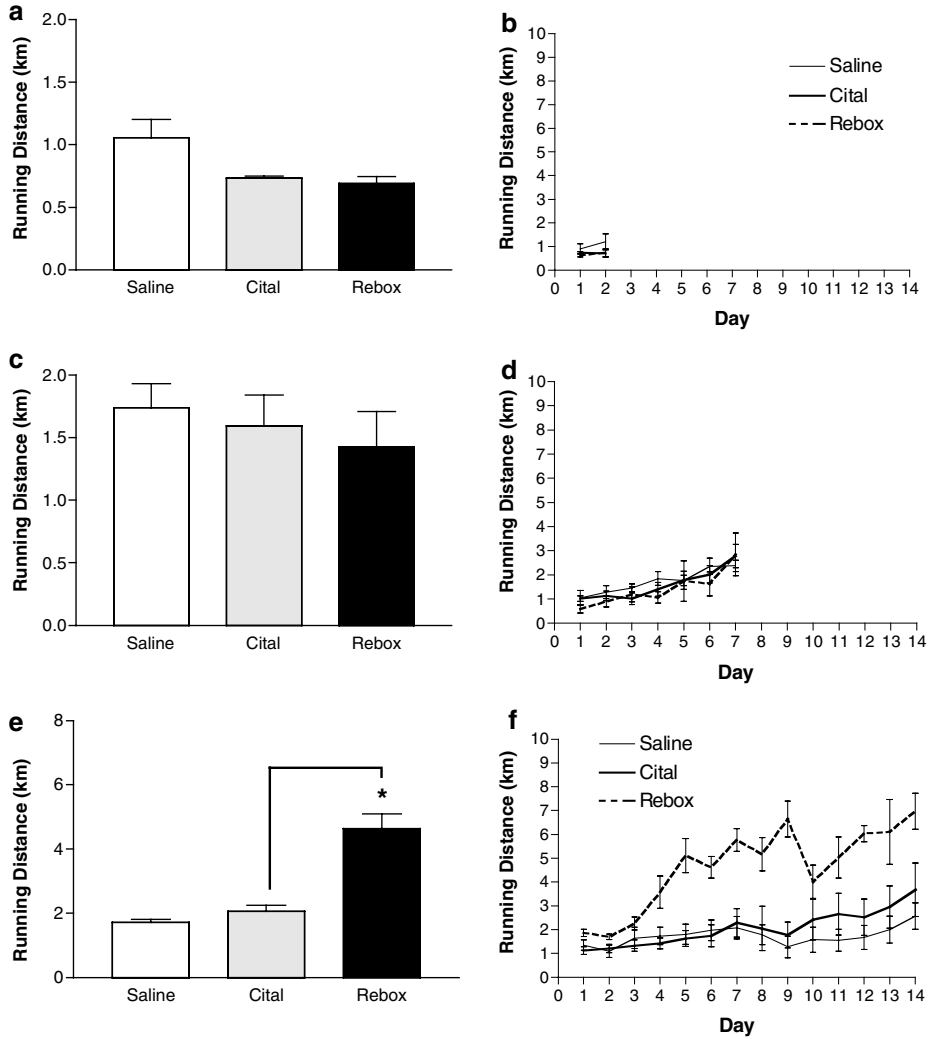

Figure 7 Longer-term treatment with reboxetine had a significant effect on wheel-running activity. Average running distance for each study is shown (a, c, e), and statistically significant differences from saline controls are denoted by asterisks ($p<0.05$). Statistical significance was determined using one-way ANOVA and subsequent Fisher's PLSD for multiple comparisons. Brackets denote significant differences between the indicated groups ($p<0.05$). The average running distance over time is also detailed for each study (b, d, f). A two-way ANOVA revealed a significant effect for group ($F_{(2,241)}=97.08$, $p<0.0001$), time ($F_{(13,241)}=6.30$, $p<0.0001$), and the interaction between time and treatment group ($F_{(26,241)}=1.97$, $p=0.0045$) for the 14-day study only (f).

however, long-term reboxetine treatment had an elevating effect on animal activity. Running distance was increased to a significant degree after 14 days of reboxetine treatment (Figure 7). Therefore, the robust increase in BDNF mRNA following the reboxetine/exercise combination at this time interval could be due to increased activity rather than a purely pharmacological effect.

Citalopram, a highly 5-HT-selective monoamine reuptake inhibitor (Sanchez and Hyttel, 1999), led to less consistent and widespread changes in hippocampal BDNF mRNA than reboxetine after short-term treatment. After 2 days, full-length BDNF mRNA levels were increased in only one hippocampal area, the CA2. Examination of individual transcript variants revealed that citalopram treatment led to a decrease in exon II and an increase in exon IV in several regions. These opposing effects of citalopram on individual subclasses of BDNF mRNA may explain the lack of a net effect on total BDNF mRNA expression in several hippocampal regions after short-term treatment. After long-term (14 days) treatment, on the other hand, citalopram enhanced total hippocampal BDNF transcription, as well as the expression of three transcript variants (exons I, II, and IV) when combined with physical activity. Citalpram treatment also independently enhanced the expression of exon IV with both short- (2 days) and long-term (14 days) treatment. Evidence suggests that 5-HT-selective antidepressant-induced changes in the expression of neurotrophins (Duman, 1998), as well as alterations in behavior (Detke *et al*, 1997; Lucki, 1998), require chronic treatment. It is possible, therefore, that treatment with citalopram may require longer intervals in order to influence the expression of most BDNF mRNA isoforms. Nevertheless, citalopram rapidly influenced the expression of the exon IV variant (this was especially widespread when combined with exericse), and this effect was even more evident with chronic (2 weeks) treatment. As was observed with

reboxetine, the addition of exercise to citalopram therapy led to more widespread and robust changes in the expression of several BDNF mRNA isoforms than treatment with citalopram (or exercise) alone. Examples include the effects of the citalopram/exercise combination on the expression of full-length BDNF, exon I and exon II after 2 weeks of treatment (Figure 5a–c).

It is important to note that, since different modes of administration were used for reboxetine (twice per day dosing i.p.) and citalopram (continuous infusion) during the 2- and 7-day treatment intervals, it is possible that this variation may have influenced the results. Several other investigators have revealed no significant differences in treatment responses to antidepressants and related compounds following these two modes of administration in rats. For example, no variation was evident in clovoxamine- or desipramine-induced downregulation of the 5-HT_2 receptor in the frontal cortex (Bradford *et al*, 1987). Additionally, neuronal responsiveness to amytriptyline (Gravel and de Montigny, 1987), antidepressant-induced upregulation of GABA-B binding (Lloyd *et al*, 1985), and 8-hydroxy-2-(di-*n*-propylamino)-tetralin-induced inhibition of 5-HT in the cortex and hippocampus (Bohmaker *et al*, 1993) did not vary with the two modes of administration. We investigated a possible variation in nondrug (exercise) responses with the two different control groups (saline administered via osmotic minipump *vs* i.p. injections). BDNF mRNA changes following exercise were compared using unpaired Student's *t*-tests. These statistical comparisons revealed significantly higher exercise-associated total BDNF levels in the hippocampal CA1 and DG with continuous infusion of saline compared to b.i.d. injection during the 2-day study. No differences were evident following the 7-day study. Similarly, exon I levels were significantly higher in the CA1, CA2, and DG following continuous infusion (2 days only). On the other hand, exon III levels were significantly higher in several regions (CA1, CA4, and DG) following i.p. injection as compared to continuous infusion (at 7 days only). Exon IV levels were also relatively elevated in all hippocampal regions following 7 days of i.p. injection. These results suggest that full-length BDNF and exon I responses might possibly be predisposed to a higher level with continuous infusion (citalopram administration) during the 2-day study, and that responses to exons III and IV may be greater with i.p. injection (reboxetine) after 7 days. In our experiments, full-length BDNF and exon I responses to 2 days of treatment were higher following i.p. reboxetine than continuously infused citalopram (Figures 1 and 2), and exon IV responses to i.p. reboxetine were lower than continuously infused citalopram after 1 week of treatment (Figures 3 and 4). Therefore, it could be kept in mind that some changes evident in our results may be slightly attenuated due to the different modes of administration for these two antidepressants.

Exercise leads to activation of several CNS neurotransmitter systems, including the NE, 5-HT, and glutamatergic systems (Dey *et al*, 1992; Dunn *et al*, 1996; Dishman *et al*, 2000; Molteni *et al*, 2002). Recent evidence has indicated that NE activation is essential for the BDNF expression-enhancing effects of exercise (Garcia *et al*, 2003), and that both NE and 5-HT stimulation are important for the elevation of BDNF mRNA occurring with tranylcypromine treatment (Ivy *et al*, 2003). Previous evidence exists that beneficial behavioral outcomes can result from exercise. In addition to enhancing hippocampal BDNF expression, long-term voluntary exercise has been shown to have anxiolytic effects in animals (Dishman, 1997), and antidepressant effects in humans (Morgan, 1985; Labbe *et al*, 1988; Hill *et al*, 1993). Chronic exercise has been shown to decrease depressive-like (learned helpless) behaviors in rat models, and alter the activity of serotonergic neurons in the dorsal raphe nucleus (Greenwood *et al*, 2003). Enhanced cognitive performance has been shown to result from exercise (Samorajski *et al*, 1985; Fordyce and Wehner, 1993). In addition, recent evidence indicates that physically active rodents demonstrate enhanced hippocampal neurogenesis (van Praag *et al*, 1999) and possess some resistance to the damaging effects of acute stressor exposure or brain injury. For example, exercise has been shown to enhance the expression of the heat shock protein HSP72 in the hippocampus and other brain areas (Campisi *et al*, 2003). In addition, laboratory rodents submitted to treadmill running near the time of experimental hippocampal lesions maintain intact spatial memory function (Carro *et al*, 2001).

The examination of individual transcript variants of BDNF revealed distinct patterns of expression following treatment with each of the different antidepressants. As might be expected, strong increases in the exon I variant were evident with short-term activity and activity/antidepressant treatments that were sustained with chronic combination therapy. Robust and long-lasting increases in this transcript have been reported following two other activating antidepressant treatments, tranylcypromine (Russo-Neustadt *et al*, 2000) and electroconvulsive stimulation (Dias *et al*, 2003). The exon II BDNF transcript variant, which has been shown to increase in the hippocampus following short-term exercise (Russo-Neustadt *et al*, 2000), was also increased following 2 days of reboxetine and reboxetine/activity, but was decreased with short-term citalopram. Significant increases in exon II expression were also evident following antidepressant/exercise combinations for 7 or 14 days. Consistent with an immediate early gene mode of expression (Lauterborn *et al*, 1996), hippocampal levels of the exon III variant were enhanced following short-term treatment only; no changes were evident following chronic treatment (14 days). It should be noted that short-term exercise/antidepressant combinations (using both reboxetine and citalopram) were particularly effective in elevating the expression of exon III (Figures 1d, 2d and 3d). The expression of exon IV appeared to be especially responsive to short-term citalopram treatment, which increased this variant in several hippocampal regions after 2 days. Surprisingly, subchronic (7 days) treatment with both antidepressants decreased expression of exon IV below baseline. After chronic (14 days) treatment, both antidepressants led to increases in exon IV in several hippocampal areas, with the most widespread effects due to citalopram. When taking a general overview of the results, it appears that subchronic (7 days) treatment produced less positive results than either short-term (2 days) or chronic (14 days) treatment. It is possible that the 7-day time interval may occur after the time of optimal NE activation and before the time of an optimal 5-HT-induced stimulus. On a similar vein, it was recently reported

that shorter-term treatment with some antidepressant agents led to decreases in some exon-specific BDNF transcripts (Dias *et al*, 2003). As noted earlier, all of the transcript variants of BDNF studied (containing exons I, II, III, or IV) are directed through distinct promoters. Therefore, distinct intracellular signaling pathways are likely activated in order to enhance the expression of each exon. Each mRNA variant is translated into the identical BDNF protein, as none of the exons are contained within the coding region for BDNF. The function of intervention-dependent, regionally and temporally distinct patterns of expression of these transcripts are therefore not clear. It is possible that they may reflect differential peri-transcriptional regulation, such as mRNA stability or localization, and therefore may lead to distinct effects upon neuronal growth and survival within the brain. Several investigators report region-specific and intervention-specific patterns of BDNF exon expression within the hippocampus, hypothalamus, and cerebral cortex. For example, immobilization stress rapidly increases hippocampal exon III expression, elevates exons I and II over a longer time course, and decreases exon IV (Marmigere *et al*, 2003). Osmotic stress elevates exon I in the hypothalamic supraoptic nucleus, and leads to a dramatic increase in exon II in the paraventricular nucleus (Aliaga *et al*, 2002).

It is important to note that hippocampal BDNF gene expression responses and subsequent protein transcription follow different time courses (Nawa *et al*, 1995). Therefore, it cannot be assumed that rapid changes in BDNF mRNA expression will correlate with parallel changes in hippocampal BDNF concentration. In our own laboratory, we have observed that BDNF protein increases at a later time than mRNA following exercise and exercise/antidepressant interventions. BDNF mRNA showed significant changes in as little as 2 days, but protein approached a significant increase after 7 days of an exercise/tranylcypromine combination, and was strikingly elevated after 14 days following exercise or exercise/tranylcypromine (unpublished data).

In conclusion, our results support a growing body of evidence that exercise augments antidepressant-associated increases in the expression of the hippocampal BDNF gene. Several transcript variants of BDNF that were not significantly influenced by a particular antidepressant were elevated when that antidepressant was combined with ongoing voluntary physical activity. In a very general sense, the impact of the NE-selective agent, reboxetine on hippocampal BDNF expression appeared to be rapid, but not long-lasting without concomitant exercise. The 5-HT-selective agent, citalopram, on the other hand, appeared to require more chronic treatment before significant effects on most BDNF transcripts became evident. It appears that exercise served to 'fill in the gap' for both of these; extending the time of treatment effectiveness and expanding the range of BDNF transcript variants elevated by either treatment. In several recent clinical trials, reboxetine was assessed to have equal total efficacy compared to 5-HT-selective antidepressants such as citalopram and fluoxetine (eg comparable scores on the Hamilton Depression Inventory; Andreoli *et al*, 1999), but was found to be superior for enhanced social functioning and activation (Massana, 1998; Montgomery and Schatzberg, 1998; Tse and Bond, 2002). This may be consistent with the enhanced activation observed following reboxetine treatment in the rat forced swim test (Page *et al*, 2003), and the effects we observed on wheel running activity following long-term reboxetine (Figure 7). To our knowledge, no formal studies have compared treatment onset for reboxetine and citalopram or other 5-HT-selective reuptake inhibitors.

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