Sensitivity of hippocampal 5-HT$_{1A}$ receptors to mild stress in BDNF-deficient mice

Teresa F. Burke$^1$, Tushar Advani$^1$, Megumi Adachi$^2$, Lisa M. Monteggia$^2$ and Julie G. Hensler$^1$

$^1$Department of Pharmacology, University of Texas Health Science Center, San Antonio, TX, USA
$^2$Department of Psychiatry, UT Southwestern Medical Center, Dallas, TX, USA

Abstract

Serotonin 1A (5-HT$_{1A}$) receptors in brain play an important role in cognitive and integrative functions, as well as emotional states. Decreased brain-derived neurotrophic factor (BDNF) expression and/or function, particularly in hippocampus, are implicated in the pathophysiology of stress-related disorders such as major depression. BDNF$^{+/+}$ mice are more vulnerable to stress than wild-type mice, exhibiting behavioural despair after mild handling stress. We examined the effect of mild handling stress on 5-HT$_{1A}$ receptor function, as measured by 8-OH-DPAT stimulated $[^{35}S]$GTP$^\gamma$S binding, in BDNF$^{+/+}$ mice and mice with a forebrain-specific reduction in BDNF (embryonic BDNF inducible knockout mice). Our data show a remarkable sensitivity of hippocampal 5-HT$_{1A}$ receptors to mild stress and a deficiency in BDNF. Other 5-HT$_{1A}$ receptor populations, specifically in frontal cortex and dorsal raphe, were resistant to the combined detrimental effects of mild stress and reductions in BDNF expression. Decreases in hippocampal 5-HT$_{1A}$ receptor function induced by mild stress in BDNF-deficient mice were prevented by administration of the selective serotonin reuptake inhibitor fluoxetine, which increased activation of TrkB, the high affinity receptor for BDNF, in wild-type and BDNF$^{+/+}$ mice. In hippocampal cultures, BDNF increased the capacity of 5-HT$_{1A}$ receptors to activate G proteins, an effect eliminated by the knockout of TrkB, confirming TrkB activation increases 5-HT$_{1A}$ receptor function. The mechanisms underlying the sensitivity of hippocampal 5-HT$_{1A}$ receptors to mild stress and decreased BDNF expression remain to be elucidated and may have important implications for the emotional and cognitive impairments associated with stress-related mental illness.

Key words: Antidepressant, frontal cortex, hippocampus, raphe, serotonin.

Introduction

The distribution of the serotonin 1A (5-HT$_{1A}$) receptor in brain is consistent with its role in cognitive or integrative functions, as well as in emotional states. 5-HT$_{1A}$ receptors are present in high density in cortical and limbic areas (Hensler et al. 1991; Kia et al. 1996; Verge et al. 1986). In these terminal field areas of serotonergic innervation, the 5-HT$_{1A}$ receptor is located post-synaptically to serotonergic neurons (Hensler et al. 1991; Riad et al. 2000; Verge et al. 1986). The 5-HT$_{1A}$ receptor is also present in high density in serotonergic cell body areas, particularly the dorsal and median raphe nuclei, where it is located on serotonergic cell bodies and dendrites (Riad et al. 2000; Sotelo et al. 1990) and functions as the somatodendritic autoreceptor (Aghajanian et al. 1990; de Montigny et al. 1984).

Central 5-HT$_{1A}$ receptors are important components of antidepressant drug action. 5-HT$_{1A}$ receptor antagonists block behavioural responses in animals produced by a variety of antidepressants (Berrocoso & Mico, 2009; Detke et al. 1995; Mayorga et al. 2001; Redrobe et al. 1996). Direct acting 5-HT$_{1A}$ receptor agonists have antidepressant-like effects in a variety of behavioural models used to assess antidepressant-like drug activity (e.g. de Vry, 1995; Koek et al. 1998; Lucki, 1991; Miyata et al. 2004). Increased sensitivity or stimulation of post-synaptic 5-HT$_{1A}$ receptor in the
hippocampus is thought to contribute antidepressant activity of a variety of effective therapies (see Savitz et al. 2009). In the hippocampus, 5-HTTA receptors appear to be particularly important for the maintenance of normal neuronal function and in mediating the effects of antidepressants on cell proliferation and survival (Fricker et al. 2005; Santarelli et al. 2003; Sarnyai et al. 2000).

Brain-derived neurotrophic factor (BDNF) promotes monoaminergic neurotransmission and has profound effects on neuronal structure and plasticity (Elmariah et al. 2005; Russo-Neustadt, 2003; Suciak et al. 1996, 1998). A decrease in BDNF expression and/or function, particularly in the hippocampus, can be induced by stress and has been implicated in the pathophysiology of major depression (Duman & Monteggia, 2006). Although BDNF+/− mice show unaltered emotional behaviour compared to wild-type mice in a variety of behavioural tests (Advani et al. 2009; Chourbaji et al. 2004; MacQueen et al. 2001), we have shown that BDNF+/− mice are more vulnerable to stress than wild-type mice and exhibit behavioural despair (i.e. increased immobility in the forced swim test) after mild stress (Advani et al. 2009).

Given the important role of 5-HTTA receptors in emotional states and the susceptibility of BDNF+/− mice to mild stress in behavioural studies (Advani et al. 2009), we examined the effect of mild handling stress on 5-HTTA receptor function in mice deficient in BDNF. To this end we utilized two model systems, i.e. heterozygous BDNF (BDNF+/-) mice and mice with a forebrain-specific reduction in BDNF (embryonic BDNF inducible knockout mice). 5-HTTA receptor function was measured at the level of receptor-G protein interaction using quantitative autoradiography of [35S]GTPγS binding stimulated by the 5-HTTA receptor agonist 8-OH-DPAT (Hensler, 2002; Hensler & Durgam, 2001). Our data show a remarkable sensitivity of hippocampal 5-HTTA receptors to mild stress and a concurrent deficiency in BDNF. Other 5-HTTA receptor populations, specifically in frontal cortex and serotonergic cell body areas, appear resistant to the combined detrimental effect of mild stress and reduced BDNF expression. Stress-induced reductions in hippocampal 5-HTTA receptor function in BDNF deficient mice were prevented by administration of the selective serotonin reuptake inhibitor (SSRI) fluoxetine, which increased activation of TrkB, the high affinity receptor for BDNF, in both wild-type and BDNF+/− mice. In vitro experiments in cultured primary hippocampal neurons confirmed that activation of TrkB increases 5-HTTA receptor function.

Method and materials

Materials

[35S]GTPγS (1250 Ci/mmol) was purchased from PerkinElmer (USA). [3H]8-OH-DPAT (210 Ci/mmol) was purchased from Amersham Biosciences (USA). Fluoxetine hydrochloride, 8-OH-DPAT hydrobromide and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) were purchased from Tocris (USA). R(+)-8-OH-DPAT, WAY 100635 maleate, guanosine 5′-diphosphate (GDP) disodium salt were purchased from Sigma/RBI (USA). GTPγS (tetralithium salt) was purchased from Roche/Boehringer-Manheim (USA).

Animals

BDNF heterozygous (BDNF+/-) and wild-type (BDNF+/-+) mice were bred at the University of Texas Health Science Center-San Antonio. Breeding pairs consisted of wild-type female (C57BL/6j) and heterozygote male (B6.129S4-Bdnftm1aj/J) mice (The Jackson Laboratory, USA). Mice were genotyped as recommended by The Jackson Laboratory. BDNF inducible knockout mice and littermate control mice were bred at the University of Texas Southwestern Medical Center and genotyped as previously described (Monteggia et al. 2004). BDNF inducible knockout mice were generated by crossing three lines of mice: the neuron-specific enolase–tetracycline transcriptional activator (NSE–tTA) line on a BL6/SJL × ICR background, TetOp-Cre line on an ICR background, and floxed BDNF mice on a BL6/sv129 background. NSE–tTA mice and TetOp-Cre mice were maintained as homozygotes then crossed to generate the bigenic mice. The bigenic NSE–tTA/TetOp-Cre mice were then crossed with the floxed BDNF mice to produce a neuron-targeted knockout of the bdnf gene in forebrain areas, specifically hippocampus, cortex, nucleus accumbens and basolateral amygdala (Hashimoto et al. 2005; Monteggia et al. 2004). For the present studies animals were bred in the absence of the tetracycline derivative doxycycline to produce an embryonic knockout.

The current study used male mice, 4–6 months old. Mice were group-housed and maintained on a 12-h day/night cycle (lights on 07:00 hours) with constant access to food and water. All procedures were approved by the University of Texas Health Science Center-San Antonio and the University of Texas Southwestern Medical Center, and carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health.
Mild handling stress

We used an i.p. injection of saline, administered using a volume of 10 ml/kg, as a mild stressor (Meijer et al. 2005; Ryabinin et al. 1999). We have previously shown that, although there is no difference between genotypes in plasma corticosterone levels at baseline, BDNF^+/− mice exhibit higher corticosterone levels than wild-type mice in response to a single injection of saline (Advani et al. 2009). Acute mild handling stress consisted of three saline injections over a 24 h period, administered 24, 4 and 1 h before animals were killed and brain tissue harvested for autoradiography. A separate cohort of animals received three injections of fluoxetine HCl (10 mg/kg) over 24 h, administered 24, 4 and 1 h before killing. Naive mice were un-handled except for routine cage changing.

Chronic mild handling stress consisted of a single daily injection of saline for 21 d. A separate cohort of animals received a single daily injection of fluoxetine HCl (10 mg/kg) for 21 d. In initial experiments, serum drug levels were determined after 21 d treatment from trunk blood of nine animals collected 24 h after the last injection. This dose of fluoxetine resulted in serum levels of fluoxetine (94 ± 36 ng/ml) and the active metabolite norfluoxetine (907 ± 145 ng/ml) that correspond to therapeutic drug level guidelines for patients (Clinical Psychopharmacology Laboratories, University of Texas Health Science Center, USA).

Tissue preparation for autoradiographic studies

Mice were killed by rapid decapitation 1 h after the last injection of saline vehicle or fluoxetine for acute experiments, or 24 h after the last injection for chronic experiments. Brains were rapidly removed, frozen on powdered dry ice and then stored at −80 °C. Coronal sections of 20 μm thickness were cut at −17 °C in a cryostat microtome at the level of the frontal cortex (plates 13 and 14), dorsal hippocampus (plates 46 and 47) or dorsal raphe nucleus (plates 68–70) according to the atlas of the mouse brain by Paxinos & Franklin (2001). Sections were thaw-mounted onto gelatin-coated glass slides, desiccated at 4 °C for 18 h under vacuum and then stored at −80 °C.

[^35S]GTPγS autoradiography

Autoradiography of 8-OH-DPAT-stimulated[^35S]-GTPγS binding was performed as previously described (Hensler, 2002; Hensler & Durgam, 2001; Rossi et al. 2006) with slight modifications. Briefly, slide-mounted sections were incubated in Hepes buffer containing GDP (2 mM), adenosine A1 receptor antagonist DPCPX (1 μM) and 40 pm[^35S]GTPγS, either in the absence or in the presence of 8-OH-DPAT (1 μM), for 60 min at 25 °C. This concentration of 8-OH-DPAT produces maximal stimulation of[^35S]GTPγS binding (Hensler & Durgam, 2001). 8-OH-DPAT, which has high affinity for 5-HT̂ receptors (K_i = 1 nM; e.g. Sprouse et al. 2004), also has moderate affinity for and agonist activity at 5-HT̂ receptors (K_i = 250 nM) (Duncan et al. 2004; Hagan et al. 2000; Hedlund et al. 2004; Sprouse et al. 2004). The stimulation of[^35S]GTPγS binding by (1 μM) 8-OH-DPAT is completely blocked by the 5-HT̂ receptor antagonist WAY 100635 (100 nM; Hensler & Durgam, 2001), but is not altered by the selective 5-HT̂ receptor antagonist SB 269970 (100 nM; Rossi et al. 2006). Basal[^35S]GTPγS binding was defined in the absence of 8-OH-DPAT.

Non-specific[^35S]GTPγS binding was defined in the absence of 8-OH-DPAT and in the presence of 10 μM GTPγS. The incubation was stopped by two washes for 5 min each in ice-cold 50 mM Hepes buffer (pH 7.4), followed by a brief immersion in ice-cold de-ionized water. Sections were dried on a slide warmer and exposed to Kodak Biomax MR film for 48 h.

[^3]H8-OH-DPAT autoradiography

The coupled, high-affinity agonist state of the 5-HT̂ receptors was measured by the binding of the agonist radioligand[^3]H8-OH-DPAT (Chamberlain, et al. 1993; Vergé et al. 1986). Autoradiography of the binding of[^3]H8-OH-DPAT to 5-HT̂ receptors in brain sections was performed as described with slight modification (Hensler et al. 1991; Vergé et al. 1986). Briefly, slide-mounted sections were thawed and desiccated at 4 °C for 1 h. Sections were pre-incubated for 30 min at 30 °C in assay buffer (170 mM Tris-HCl, pH 7.6) and then incubated in assay buffer containing 2 nM[^3]H8-OH-DPAT for 60 min at room temperature. Non-specific binding was defined by incubating adjacent sections in the presence of 10 μM WAY-100635. Incubation was terminated by two washes for 5 min each in ice-cold 170 mM Tris-HCl buffer (pH 7.6), followed by a dip in ice-cold de-ionized water. Sections were dried on a slide warmer and exposed to Kodak BioMax MR film for a period of 9 wk.

Image analysis

Analysis of the digitized autoradiograms was performed using the image analysis program ImageJ, version 1.42q (National Institutes of Health, USA). Tissue sections were stained with thionin and the brain areas identified using the atlas of the mouse brain by Paxinos & Franklin (2001). Autoradiograms
of [3H]8-OH-DPAT binding were quantified by the use of simultaneously exposed [3H] standards (ART-123; American Radioc, USA), which had been calibrated using brain-mash sections according to the method of Geary and colleagues (Geary et al. 1985; Geary & Wooten, 1983). The amount of ligand bound was determined by converting optical density measurements to femtomoles per mg protein. Specific binding was calculated by subtracting non-specific binding from total binding on adjacent sections. Autoradiograms of 8-OH-DPAT-stimulated [35S]GTPγS binding were quantified by the use of simultaneously exposed [35S] standards (ARC-146; American Radioc). Standard curves were fit to pixel data obtained from [35S] standards and tissue equivalent values (nCi/g) provided by American Radioc and were used to transform the actual regional densitometric values into relative radioactivity measures.

Primary hippocampal cultures

Primary hippocampal cultures from floxed TrkB mice in which both transcription initiation sites and the first coding exon of the TrkB gene are flanked with loxP sites (Luikart et al. 2005) were prepared as previously described (Kavalali et al. 1999; Mahgoub et al. 2006). Briefly, whole hippocampi were dissected from post-natal day 0–1 old pups and dissociated cells were plated on six-well plates. Hippocampal cultures at 8 d in vitro (DIV) were infected with either lentivirus expressing green fluorescent protein (LV-GFP) or Cre recombinase fused with GFP (LV-Cre.GFP) as previously described (Akhtar et al. 2009; Nelson et al. 2006). At DIV14, mouse hippocampal cultures were treated with BDNF (10 ng/ml) for 20 min and the cells harvested for Western blot analysis or [35S]GTPγS binding.

[35S]GTPγS binding

Cells were washed and harvested in ice-cold PBS containing phosphatase inhibitor cocktail (Sigma) and pelleted by centrifugation 14 000 rpm (15 996 g) in a table-top centrifuge at 4 °C. Cell pellets were then flash-frozen. The [35S]GTPγS binding assay was done as described previously with slight modification (Berg et al. 2007). Membranes were resuspended using a Tekmar Tissumizer (USA) in assay buffer [wash buffer plus GDP (100 μm) and dithireitol (DTT, 1 mm)] at a protein concentration of 60 μg/ml (mouse). Aliquots (100 μl) of the membrane suspension were pre-incubated in the absence (basal binding) or presence of R(+-8-OH-DPAT (10 μm) for 20 min, followed by the addition of [35S]GTPγS (0.3 nm, final) for 30 min at 37 °C in 96-well multiscreen filtration plates (Millipore, USA). Non-specific binding was defined in the absence of R(+-8-OH-DPAT and in the presence of 10 μM GTPγS. Binding was stopped by the addition of ice-cold wash buffer (20 mM Hapes, 3 mM MgCl2, 0.2 mM EGTA, and 100 mM NaCl, pH 7.4 at 23 °C) and rapid filtration, followed by several washes. Membrane protein content was measured by Bradford protein assay (Bio-Rad).

Western blot analysis

Wild-type and BDNF +/− mice were injected with saline or fluoxetine HCl (30 mg/kg i.p.; Rantamäki et al. 2007). Hippocampi were taken 30, 60 or 120 min post-injection and frozen on powdered dry ice. Thawed samples were sonicated in lysis buffer (50 mM Tris-HCl; 1 mM EDTA; 0.25% sodium deoxycholate; 150 mM NaCl; 1% Igepal; pH 7.4) supplemented with protease and phosphatase inhibitors (Sigma) and centrifuged at 4 °C for 30 min at 14 000 rpm (15 996 g). Twenty or 40 μg protein was resolved on a 10% SDS-PAGE gel and transferred to a PVDF membrane (Bio-Rad). Dilution of anti-pTrkB Y945/952 antibody (Cell Signaling Technology 4621) was 1:1000. Blots were reprobed using an antibody recognizing TrkB independent of phosphorylation state (total TrkB, TrkB_FL) (Neuromics GT15080, 1:2000). TrkB_FL was normalized to GAPDH (Calbiochem CB1001, 1:5000). The respective immunoreactive bands were detected by Amersham™ ECL Western Blotting Analysis System (GE Healthcare, UK) and visualized following exposure to Amersham™ Hyperfilm ECL (GE Healthcare). Mouse hippocampal cultures were harvested and proteins extracted in lysis buffer composed of 25 mM Hapes (pH 7.9), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 20 μM NaF, 1 mM EDTA, 0.1% NP40 and proteinase inhibitor cocktail (Sigma). Five μg protein were resolved on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Dilutions of primary antibodies were 1:3000 for TrkB antibody (Upstate/Millipore 07-225) and 1:10000 for actin (MP Biomedicals 08637931). Actin was used for normalization. Signals were detected by Amersham™ ECL Western Blotting Analysis System (GE Healthcare) and visualized following exposure to Amersham™ Hyperfilm ECL (GE Healthcare).

Data analysis

Statistical comparisons were made by two-way ANOVA with genotype and treatment as factors. F values reaching significance (p < 0.05) were evaluated further by post-hoc analysis using the Newman-Keuls
Acute mild handling stress resulted in a decrease in 5-HT$_{1A}$ receptor function, as measured by 8-OH-DPAT-stimulated $[^{35}S]$GTP$\gamma$S binding in hippocampus of BDNF$^{+/−}$ mice (Fig. 1a, c, e, see also Supplementary Fig. 1). In the CA1 region, two-factor analysis of variance (ANOVA) indicated no significant effect of genotype ($F_{1,32}=2.78$, $p=0.11$) or handling ($F_{1,32}=3.37$, $p=0.08$), but a significant interaction between factors ($F_{1,32}=6.05$, $p=0.02$). In the dentate gyrus there was no significant effect of genotype or handling and no significant interaction between factors ($F_{1,32}=2.16$, $p=0.15$). In the CA2/3 region we found no significant effect of genotype ($F_{1,32}=0.672$, $p=0.42$) or handling ($F_{1,32}=3.63$, $p=0.066$), but a significant interaction between factors ($F_{1,32}=9.17$, $p=0.005$). Post-hoc analyses indicated that there was a significant decrease in 8-OH-DPAT-stimulated $[^{35}S]$GTP$\gamma$S binding in CA1 and CA2/3 regions of hippocampus in BDNF$^{+/−}$ mice as a result of mild handling stress. These decreases in 5-HT$_{1A}$ receptor-stimulated $[^{35}S]$GTP$\gamma$S binding were not accompanied by changes in the number of 5-HT$_{1A}$ receptors in the high affinity agonist state, as measured by the binding of $[^{3}H]$8-OH-DPAT (CA1: $F_{1,32}=2.85$, $p=0.10$; Dentate gyrus: $F_{1,32}=0.26$, $p=0.61$; CA2/3: $F_{1,32}=1.29$, $p=0.26$) (Fig. 1b, d, f).

Other 5-HT$_{1A}$ receptor populations, specifically in frontal cortex and dorsal and median raphe, were...
resistant to the effects of mild stress and concurrent BDNF deficiency. In contrast to what was observed in hippocampus, acute mild handling stress did not alter 8-OH-DPAT-stimulated $[^{35}S]GTP\gamma S$ binding in frontal cortex of BDNF+/− mice (Supplementary Fig. 2A). There was also no effect of mild handling stress on 8-OH-DPAT-stimulated $[^{35}S]GTP\gamma S$ binding in the dorsal raphe or median raphe of wild-type or BDNF+/− mice (Supplementary Fig. 2C, E). Acute mild handling stress of either wild-type or BDNF+/− mice did not alter 5-HT$_{1A}$ receptors in these serotonergic cell body areas or in frontal cortex, as measured by the binding of $[^{3}H]$8-OH-DPAT (Supplementary Fig. 2B, D, F).

Administration of a variety of antidepressant drugs, including the SSRI fluoxetine, has been shown to increase activation of TrkB, the high affinity receptor for BDNF (Autry et al. 2011; Rantamäki et al. 2007; Saarelainen et al. 2003). We therefore asked whether the stress-induced impairment of hippocampal 5-HT$_{1A}$ receptor function in BDNF+/− mice could be prevented by administration of fluoxetine. To examine this question, mice were subjected to mild handling stress. A separate cohort received sub-chronic injections of fluoxetine (10 mg/kg, three times over a 24-h period) instead of receiving injections of saline. In BDNF+/− mice treated with fluoxetine, 8-OH-DPAT-stimulated $[^{35}S]GTP\gamma S$ binding in hippocampus was not significantly changed from wild-type control levels (Fig. 2). Thus, fluoxetine administration prevented the deficit in 5-HT$_{1A}$ receptor-stimulated $[^{35}S]GTP\gamma S$ binding observed in BDNF+/− mice subjected to mild stress. There was no effect of fluoxetine administration on 5-HT$_{1A}$ receptor-stimulated $[^{35}S]GTP\gamma S$ binding in wild-type mice, consistent with the literature indicating that 5-HT$_{1A}$ receptor function is not altered in wild-type animals by sub-chronic SSRI treatment (see Blier & de Montigny, 1994; Czachura & Rasmussen, 2000; Le Poul et al. 1995; Pilar-Cuéllar et al. 2012).

BDNF promotes activity-dependent synaptic plasticity and thus plays a role in the modulation of responses to repeated stress. BDNF+/− mice might not be expected to adapt or habituate to chronic mild stress. We therefore asked whether BDNF+/− mice exposed to chronic mild stress exhibit stress-induced impairment of hippocampal 5-HT$_{1A}$ receptor function. To address this question, mice were subjected to chronic mild handling stress (i.e. single daily injection of saline for 21 d). A separate cohort of mice received a once daily injection of the SSRI fluoxetine (10 mg/kg i.p.). Chronic mild handling stress resulted in a significant decrease in 5-HT$_{1A}$ receptor-stimulated $[^{35}S]GTP\gamma S$ binding in CA1 and CA2/3 regions of hippocampus of BDNF+/− mice, indicating a lack of adaptation or habituation to repeated stress. Moreover, the stress-induced decrease in 5-HT$_{1A}$ receptor function was prevented by chronic administration of fluoxetine (Fig. 3a, c, e). In CA1 region, two-factor ANOVA revealed a significant effect of genotype ($F_{1,38} = 19.5, p = 0.0001$) but not treatment ($F_{1,38} = 2.77, p = 0.104$) and a significant interaction between factors ($F_{1,38} = 10.16, p = 0.002$). In the dentate gyrus,
8-OH-DPAT-stimulated $[^{35}S]GTPgammaS$ binding was not significantly altered by chronic mild handling stress or by fluoxetine treatment ($F_{1,18} = 3.36, p > 0.07$). In the CA2/3 region, we found a significant effect of genotype ($F_{1,18} = 5.57, p = 0.023$) and treatment ($F_{1,18} = 6.73, p = 0.013$), but no significant interaction between factors ($F_{1,18} = 2.41, p = 0.13$). Post-hoc analyses indicated that there was a significant decrease in 8-OH-DPAT-stimulated $[^{35}S]GTPgammaS$ binding in CA1 and CA2/3 regions in BDNF$^{+/–}$ mice subjected to chronic handling stress, but not in those BDNF$^{+/–}$ mice treated with fluoxetine. Changes in 5-HT1A receptor-stimulated $[^{35}S]GTPgammaS$ binding in sub-regions of hippocampus were not accompanied by changes in the number of 5-HT1A receptors, as measured by the binding of $[^{3}H]8$-OH-DPAT (CA1: $F_{1,18} = 0.59, p > 0.45$; Dentate gyrus: $F_{1,18} = 0.65, p > 0.43$; CA2/3: $F_{1,18} = 1.05, p > 0.31$) (Fig. 3b, d, f).

To establish the generality of the interaction between mild stress and a deficiency in BDNF in the regulation of hippocampal 5-HT1A receptor function, we utilized a second model system, embryonic BDNF inducible knockout mice, in which 70–80% of BDNF mRNA is deleted in a forebrain-restricted manner (Hashimoto et al. 2005; Monteggia et al. 2004). In these inducible BDNF knockout mice, we found no effect of genotype on $[^{35}S]GTPgammaS$ binding stimulated by 8-OH-DPAT (1 $\mu$M) in CA1 region (controls: $110 \pm 6.4$% above basal; knockout: $112 \pm 6.9$% above basal), dentate gyrus (controls: $14.2 \pm 3.8$% above basal; knockout: $14.4 \pm 2.4$% above basal) or CA2/3 region (controls: $15.6 \pm 2.5$% above basal; knockout: $11.2 \pm 1.7$% above basal; $n = 9$ control mice; $n = 6$ knockout mice). As had been shown for BDNF$^{+/–}$ mice, chronic mild handling stress resulted in a significant decrease in 5-HT1A receptor-stimulated $[^{35}S]GTPgammaS$ binding in...
sub-regions of hippocampus, indicating a lack of adaptation or habituation to repeated stress. Moreover, the stress-induced decrease in 5-HT_{1A} receptor function was prevented by chronic administration of fluoxetine in CA2/3 region and dentate gyrus (Fig. 4a, c, e). In CA1 region, two-factor ANOVA revealed a significant effect of genotype (F_{1,32} = 5.252, p = 0.03), no effect of treatment (F_{1,32} = 1.44, p = 0.24) and no significant interaction between factors (F_{1,32} = 0.82, p = 0.37).

In dentate gyrus, we found a significant effect of genotype (F_{1,32} = 6.389, p = 0.02), no effect of treatment (F_{1,32} = 1.07, p = 0.31), but a significant interaction between factors (F_{1,32} = 5.25, p = 0.03). Similarly, in the CA2/3 region, we found a significant effect of genotype (F_{1,32} = 6.061, p = 0.02), no effect of fluoxetine treatment (F_{1,32} = 1.91, p = 0.18), but a significant interaction between the factors (F_{1,32} = 10.91, p = 0.002).

Post-hoc analyses indicated a significant decrease in 8-OH-DPAT-stimulated [35S]GTPγS binding in all sub-regions of hippocampus of mice subjected to chronic handling stress, which was prevented in the dentate gyrus and CA2/3 region by fluoxetine administration. Changes in 5-HT_{1A} receptor-stimulated [35S]GTPγS binding in sub-regions of hippocampus were not accompanied by changes in 5-HT_{1A} receptors binding (Fig. 4b, d, f).

In contrast to what was observed in hippocampus, cortical 5-HT_{1A} receptors appeared resistant to chronic mild stress in both control/wild-type and BDNF-deficient mice. 8-OH-DPAT-stimulated [35S]GTPγS binding in frontal cortex of BDNF^{+/−} mice and BDNF inducible knockout mice was not altered by chronic mild handling stress or by chronic fluoxetine administration (Supplementary Fig. 3A, C). There was no effect of chronic mild handling stress or fluoxetine treatment on 5-HT_{1A} receptors, as measured by...
the binding of \(^{3}H\)8-OH-DPAT (Supplementary Fig. 3B, D).

5-HT\(_{1A}\) receptors in the dorsal raphe nucleus also appeared resistant to chronic mild stress. Chronic mild handling stress did not alter 8-OH-DPAT-stimulated \(^{35}S\)GTP\(_{S}\) binding in the dorsal raphe of control/wild-type or BDNF-deficient mice (Supplementary Fig. 4A, C). However, chronic fluoxetine administration decreased 5-HT\(_{1A}\) receptor-stimulated \(^{35}S\)GTP\(_{S}\) binding in control/wild-type and BDNF-deficient mice (Supplementary Fig. 4A, C). In wild-type and BDNF \(^{+/–}\) mice, this decrease in 5-HT\(_{1A}\) receptor function was accompanied by a decrease in \(^{3}H\)8-OH-DPAT binding to 5-HT\(_{1A}\) receptors (Supplementary Fig. 4B). The attenuation of 5-HT\(_{1A}\) receptor function in the dorsal raphe by chronic fluoxetine treatment is consistent with the desensitization of somatodendritic autoreceptor function following chronic SSRI administration (Blier & de Montigny, 1994; Hensler, 2002, 2003; Rossi et al. 2008). The regulation of 5-HT\(_{1A}\) receptor function in the dorsal raphe nucleus by chronic fluoxetine administration was not altered by a reduction in BDNF expression.

Acute, as well as chronic antidepressant treatments have been shown to increase activation of TrkB, the high affinity receptor for BDNF (Autry et al. 2011; Rantamäki et al. 2007; Saarelainen et al. 2003). We asked whether acute fluoxetine administration increases the activation and therefore phosphorylation of the TrkB receptor in BDNF \(^{+/–}\) mice, as has been shown for wild-type mice (Autry et al. 2011; Rantamäki et al. 2007; Saarelainen et al. 2003). To this end, mice were injected with saline or fluoxetine and the phosphorylated state of TrkB measured by Western blot analysis (Fig. 5a). Fluoxetine increased the level of phospho-TrkB (pTrkB) protein, expressed as the ratio of pTrkB \(_{Y706}\) to total TrkB (TrkB\(_{FL}\)), to the same extent in wild-type and BDNF \(^{+/–}\) mice, although the time-course was somewhat different. Levels of total TrkB (TrkB\(_{FL}\)) were unchanged (Fig. 5b), indicating that the increase in pTrkB is due to increased phosphorylation of available protein and not due to an increase in total TrkB protein levels. Thus, fluoxetine administration results in activation of the TrkB receptor even in mice with reduced BDNF levels.

Our in vitro data suggested that the effect of mild handling stress on hippocampal 5-HT\(_{1A}\) receptor function could be prevented by antidepressant treatments shown to increase TrkB activation. Experiments in vitro were conducted to determine whether activation of TrkB can increase 5-HT\(_{1A}\) receptor function. To examine this potential mechanism further, cultured primary hippocampal neurons from floxed TrkB mice were infected with LV-GFP as a control or LV-Cre.GFP to induce the deletion of TrkB (Fig. 6a). Infection of hippocampal cultures with LV-Cre.GFP resulted in a 95% reduction of TrkB protein in comparison to those infected with LV-GFP, as determined by Western blot analysis (Fig. 6b). BDNF treatment of cultures infected with LV-GFP (i.e. with normal levels of TrkB) resulted in a significant increase in 5-HT\(_{1A}\) receptor-stimulated \(^{35}S\)GTP\(_{S}\) binding. In contrast, BDNF had no effect on 5-HT\(_{1A}\) receptor-stimulated \(^{35}S\)GTP\(_{S}\) binding in TrkB knockout cultures (floxed TrkB cultures infected with LV-Cre.GFP) (Fig. 6c). These data...
Discussion

Our data indicate that 5-HT1A receptors in hippocampus are remarkably sensitive to regulation by mild handling stress when combined with a deficiency in BDNF. Other 5-HT1A receptor populations, specifically in frontal cortex and in serotonergic cell body areas, appeared resistant to the combined detrimental effects of mild stress and a concurrent deficit in BDNF. Administration of the SSRI fluoxetine resulted in activation of TrkB in both wild-type and BDNF+/− mice and prevented the attenuation of hippocampal 5-HT1A receptor function induced by stress in BDNF-deficient mice. In hippocampal cultures, BDNF increased the capacity of 5-HT1A receptors to activate G proteins, an effect eliminated by the knockout of TrkB, confirming that TrkB activation increases 5-HT1A receptor function.

Our data in naive BDNF+/− mice indicate that the 50% reduction in BDNF (Kolbeck et al. 1999; Korte et al. 1995; Lyons et al. 1999) is not sufficient to reduce 5-HT1A receptor function in any of the brain regions examined. This in contrast to our previous report, in which we found a decrease in 5-HT1A receptor-stimulated [35S]GTPγS binding in hippocampus of BDNF+/− mice (Hensler et al. 2003). This apparent discrepancy may be related to differences in background strain, i.e. C57BL/6 in the present study, C57BL/6Cr in our previous study (Lino Tessarollo, personal communication). BDNF inducible knockout mice exhibit a neuron-specific 70–80% reduction in BDNF mRNA expression in hippocampus and frontal cortex (Hashimoto et al. 2005; Monteggia et al. 2004). The greater reduction in forebrain BDNF expression did not alter 5-HT1A receptor function in hippocampus. Thus, BDNF deficiency alone was not sufficient to alter 5-HT1A receptor function in hippocampus. This is in contrast to BDNF+/−/LCk−/− mice, in which BDNF is deleted in all brain regions except cerebellum; 5-HT1A receptor binding is decreased in hippocampus of these mice, but unchanged in frontal cortex (Klein et al. 2010).

Mild handling stress reduced 5-HT1A receptor function in hippocampus of BDNF deficient mice. This attenuation in 5-HT1A receptor-stimulated [35S]GTPγS binding as a consequence of mild stress was not accompanied by changes in 5-HT1A receptor binding, an indication that regulation occurred at the level of 5-HT1A receptor-G protein interaction, i.e. the capacity of the receptor to activate G proteins. Importantly, mild stress procedures had no effect on 5-HT1A receptor function or binding in hippocampus in wild-type or control mice. We have previously reported...
similar findings in embryonic BDNF inducible knockout mice subjected to the stress of subcutaneous sesame oil vehicle injection for 21 d; 5-HT$_1A$ receptors were not altered in control animals receiving the vehicle injection (Hensler et al. 2007). More severe stress paradigms, such as a colony model of chronic social stress (McKittrick et al. 1995), 14-d restraint stress (Watanabe et al. 1993), chronic unpredictable stress (Lopez et al. 1998) or repeated forced swim stress (Briones-Aranda et al. 2005), have been shown to down-regulate 5-HT$_1A$ receptor mRNA and binding sites throughout the dorsal hippocampus of wild-type animals.

Mild handling stress had no effect on 5-HT$_1A$ receptor function or binding in the frontal cortex or dorsal raphe of BDNF deficient mice (present study; Hensler et al. 2007). 5-HT$_1A$ receptor function or binding in these brain regions was also not altered by mild handling stress in wild-type or control mice. More severe stress paradigms, such as repeated exposure to novel or unpredictable environmental conditions, result in the desensitization of somatodendritic 5-HT$_1A$ autoreceptors in the dorsal raphe nucleus of wild-type animals (Froger et al. 2004; Laaris et al. 1999). 5-HT$_1A$ receptor function and expression in cortical areas of wild-type animals are reduced by corticosterone administration, a means to mimic excessive stress (Fernandes et al. 1997; Mendelson & McEwen, 1992).

The stress-induced decrease in hippocampal 5-HT$_1A$ receptor function in BDNF-deficient mice was prevented by fluoxetine administration. We and others have shown that chronic as well as acute administration of a variety of antidepressant drugs, including the SSRI fluoxetine, results in activation and auto-phosphorylation of TrkB, the high-affinity receptor for BDNF (Autry et al. 2011; Rantamäki et al. 2007, 2011; Saarelainen et al. 2003). In response to fluoxetine administration, the phosphorylated form of TrkB was increased in hippocampus of BDNF$^{+/−}$ mice to the same extent as in wild-type mice. Similarly, the tricyclic antidepressant imipramine has been reported to increase brain TrkB phosphorylation in BDNF$^{+/−}$ mice (Rantamäki et al. 2011). These observations suggest that increased TrkB activation may be one mechanism by which fluoxetine prevented the stress-induced reduction in hippocampal 5-HT$_1A$ receptor function in BDNF deficient mice. Although BDNF is the primary ligand for TrkB, recent evidence suggests that in vivo TrkB can be transactivated by antidepressants, independently of BDNF. Antidepressant-induced TrkB phosphorylation does not occur in cultured neurons or cells expressing TrkB, suggesting that intact tissue is necessary (Rantamäki et al. 2011).

Our in vitro data support a role for TrkB activation in enhancing hippocampal 5-HT$_1A$ receptor function. We found that BDNF treatment of hippocampal primary cultures increased 5-HT$_1A$ receptor-stimulated [$^{35}$S]GTP$γ$S binding, an effect eliminated by the knockdown of the TrkB receptor. The elucidation of the mechanism(s) underlying the regulation of 5-HT$_1A$ receptor function by TrkB signalling requires further investigation.

Although fluoxetine administration increased TrkB phosphorylation in hippocampus of both wild-type and BDNF$^{+/−}$ mice, we found no effect of fluoxetine administration on 5-HT$_1A$ receptor-stimulated [$^{35}$S]GTP$γ$S binding in wild-type mice. These observations are consistent with the literature, indicating that 5-HT$_1A$ receptor function is not altered in wild-type animals by sub-chronic SSRI treatment (see Blier & de Montigny, 1994; Czachura & Rasmussen, 2000; Le Poul et al. 1995; Pilar-Cueiller et al. 2012). Although some investigators have reported an increase in hippocampal 5HT$_1A$ receptor function with chronic administration of SSRIs to wild-type animals (Castro et al. 2003; Shen et al. 2002), this has not been a consistent observation (Blier & de Montigny, 1983; Hensler, 2002, 2003; Le Poul et al. 2000; Pejchal et al. 2002; Varrault et al. 1991). That fluoxetine administration prevented the stress-induced reduction in hippocampal 5-HT$_1A$ receptor function in BDNF deficient mice, but had no effect on this receptor population in wild-type mice, suggests that the developmental deficiency in BDNF alters how 5-HT$_1A$ receptors in hippocampus are regulated by antidepressant treatment.

In conclusion, our data show a remarkable sensitivity of hippocampal 5-HT$_1A$ receptors to mild stress and a concurrent deficiency in BDNF. These mild stress paradigms did not alter 5-HT$_1A$ receptor function in wild-type animals. In both of the mouse models used in the current study, BDNF expression was reduced throughout life. This developmental deficiency in BDNF not only altered how 5-HT$_1A$ receptors in hippocampus are regulated by antidepressant treatment, but also by neurogenesis and neuronal survival (Fricker et al. 2005; Gould, 1999; Santarelli et al. 2003), an increased understanding of how the sensitivity of this receptor population to mild...
stress is increased in BDNF-deficient mice may have important implications for emotional and cognitive impairments associated with stress-induced mental illness.

Supplementary material
For supplementary material accompanying this paper, visit http://dx.doi.org/10.1017/S1461145712000466

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Statement of Interest
None.

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