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Changes in 5-HT_{2A}-mediated behavior and 5-HT_{2A}- and 5-HT_{1A} receptor binding and expression in conditional BDNF knock-out mice

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Abstract

Changes in brain-derived neurotrophic factor (BDNF) expression have been implicated in the etiology of psychiatric disorders. To investigate pathological mechanisms elicited by perturbed BDNF signaling, we examined mutant mice with central depletion of BDNF (BDNF^{2L/2LCk-cre}). A severe impairment specific for the serotonin 2A receptor (5-HT_{2A}) in prefrontal cortex was described previously in these mice. This is of much interest, as 5-HT_{2A} receptors have been linked to neuropsychiatric disorders and in anxiety-related behavior. Here we further characterized the serotonin receptor alterations triggered by BDNF depletion. 5-HT_{2A} (³H-MDL100907) and 5-HT_{1A} (³H-WAY100635) receptor autoradiography revealed site-specific alterations in BDNF mutant mice. They exhibited lower 5-HT_{2A} receptor binding in frontal cortex but increased binding in hippocampus. Additionally, 5-HT_{1A} receptor binding was decreased in hippocampus of BDNF mutants, but unchanged in frontal cortex. Molecular analysis indicated corresponding changes in 5-HT_{2A} and _{1A} mRNA expression but normal 5-HT_{2C} content in these brain regions in BDNF^{2L/2LCk-cre} mice. We investigated whether the reduction in frontal 5-HT_{2A} receptor binding was reflected in reduced functional output in two 5-HT_{2A}-receptor mediated behavioral tests, the head-twitch response (HTR) and the ear-scratch response (ESR). BDNF^{2L/2LCk-cre} mutants treated with the 5-HT_{2A} receptor agonist DOI showed a clearly diminished ESR but no differences in HTR compared to wildtypes. These findings illustrate the context-dependent effects of deficient BDNF signaling on the 5-HT receptor system and 5-HT_{2A}-receptor functional output.

Keywords

BDNF; serotonin; 5-HT_{2A}; 5-HT_{1A}; 5-HT_{2C}; behavior

Brain-derived neurotrophic factor (BDNF) belongs to the family of neurotrophins and signals through the TrkB receptor. It is important for neuronal survival and differentiation during development and synaptic plasticity in adulthood. In clinical studies, reductions in BDNF levels have been linked to various psychiatric disorders including schizophrenia, bipolar disorder and major depression (Kozisek et al., 2008). Studies involving rodent

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models of BDNF deficiency also lend support to a chief role of BDNF in the control of affective behavior. For example, BDNF^{+/-} mice exhibited hyper aggressiveness, hyperphagia, excessive weight gain (Lyons et al., 1999; Kernie et al., 2000) and diminished responses to antidepressant treatment (Deltheil et al., 2008). In BDNF^{2L/2LCK-cre} mice, BDNF is depleted in all brain regions, except cerebellum. The depletion begins during the first post-natal week and is completed around P21, when BDNF mRNA levels in hippocampus, cortex and hypothalamus range from very low to non-detectable (Chan et al., 2006; Rios et al., 2001). The adult conditional BDNF mutants have similar behavioral alterations and show increases in depressive and anxiety-like behavior (Rios et al., 2001; Chan et al., 2006).

Alterations in the serotonin receptor system might underlie some of the behavioral changes observed in BDNF^{2L/2LCK-cre} mice. Deficient BDNF levels result in significant perturbations in serotonin receptor expression (Lyons et al., 1999) and in impairment of 5-HT_{2A}-mediated neurotransmission in the prefrontal cortex (Rios et al., 2006). 5-HT_{2A}R is a post-synaptic G-protein coupled receptor widely expressed in the CNS. It is located on glutamatergic and GABAergic neurons, and expressed at high densities in pyramidal neurons in frontal cortex, amygdala and hippocampus (for review, see Leysen, 2004). Importantly, changes in 5-HT_{2A}R binding have been associated with schizophrenia, Alzheimer's disease and depression (Naughton et al., 2000). Moreover, a recent study showed that cortical depletion of 5-HT_{2A}Rs in mice resulted in decreased anxiety-like behavior (Weisstaub et al., 2006). Together, the collective data support an important role of 5-HT_{2A}R in the regulation of affective behavior.

To further delve into the nature of the alterations in serotonin receptor system in the BDNF^{2L/2LCK-cre} mutant mice and the impact on receptor functionality, we conducted an analysis of various 5-HT receptors in the hippocampus and frontal cortex of these mice. Autoradiography and RT-qPCR were used to quantify differences in receptor binding and expression, respectively, in these brain regions. Besides 5-HT_{2A}Rs, we also examined 5-HT_{1A} and 5-HT_{2C} receptors. 5-HT_{1A}Rs have been implicated in depression and anxiety phenotypes (for review, see Savitz et al., 2009 and Akimova et al. 2009) and exert effects opposite to those of 5-HT_{2A}Rs (Yuen et al., 2008). 5-HT_{2C}Rs, for their part, resemble 5-HT_{2A} receptors in terms of functional activity and cellular distribution (Leysen et al., 2004). Levels of serotonin transporter were also measured in BDNF mutant mice to ascertain whether the alterations in the postsynaptic 5-HT receptors could be due to primary changes in serotonergic projection. Finally, we assessed 5-HT_{2A}-functional output *in vivo* by measuring DOI (DOI, ((±)-2,5-Dimethoxy-4-iodoamphetamine hydrochloride) induced head-twitch and ear-scratch responses in BDNF^{2L/2LCK-cre} mice. This was followed by an analysis of DOI-induced neuronal activation, measured by c-Fos protein induction, in frontal cortex and hippocampus. We found that central depletion of BDNF resulted in site-specific alterations in 5-HT receptor binding and expression. Furthermore, a deficit in 5-HT_{2A}-functional output was evident in BDNF mutants. The cumulative data indicate an important effect of BDNF depletion on serotonin receptor levels and 5-HT_{2A} functional output.

Experimental Procedures

The following procedures were in accordance with the guidelines from the Animal Care and Use Committee at Tufts University and with the NIH *Guide for the Care and Use of Laboratory Animals*. Every effort was made to minimize the number of animals.

Animals

BDNF^{2L/2LCK-Cre} mutants were generated as previously described (Rios et al., 2001). Briefly, mice containing floxed *Bdnf* alleles were crossed with transgenic mice in

which expression of cre recombinase was driven by the α -calcium/calmodulin protein kinase II (CamKII-cre93 mice). Animals used in the studies described here were in a mixed C57B16 and 129 background and wild type littermates used as controls to avoid problems interpreting the data due to differences in background. Animals were 10–14 weeks of age and the groups were age- and sex matched. All mice were individually housed in the Tufts University Behavioral Core Facility, habituated to the reverse 12-h light/dark cycle for a minimum of 1 week and had free access to water and standard chow. All of the procedures described here were approved by the Institutional Animal Care and Use Committee at Tufts University and were in compliance with the NIH guide for the care and use of laboratory animals.

Drugs and reagents

The 5-HT_{2A} agonist DOI ((±)-2,5-Dimethoxy-4-iodoamphetamine hydrochloride)(Sigma-Aldrich, St. Louis, MO, USA) was administered intraperitoneally in a volume of 10 μ L/g body weight. The dose used in the studies was 2.5 mg/kg. Saline was used as vehicle and injected in a corresponding volume in wild-types.

Western blotting

After cervical dislocation, the brain was quickly removed and hippocampus and frontal cortex dissected out (n=7–8 per group), homogenized and sonicated in ice-cold protein extraction buffer (Pierce, Rockford, IL) and protein inhibitor cocktail (Pierce). The protein concentration was measured with modified Lowry method (DC Protein Assay, BioRad Laboratories, Herlev, Denmark). Samples were protein-matched and dissolved in 2 \times SDS (sodium dodecyl sulphate) sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol), heated for 5 min, 100°C and run on 6–15 % SDS-polyacrylamide gel (60 minutes, 150 V, RT). The proteins were transferred onto a PVDF membrane (300 mA, 50 min, RT, semi-dry transfer, BioRad Laboratories, Herlev, Denmark) and the membrane was blocked for unspecific binding with 5% non-fat dry milk (1 h, RT) and then incubated overnight at 4°C with either primary antibody (β -actin, 1:10.000, Sigma; BDNF, 1:1000, sc-546, Santa Cruz, CA; 5-HT_{2C}, 1:1000, Abcam, MAP2, 1:2000, M4403, Sigma; synaptophysin, 1:5000, MAB368, Millipore, MA, PSD-95, 1:2000, Millipore; β -tubulin III, 1:10.000, Sigma, MO). Washed 3 \times 10 min in TBS, blocked with 5% BSA in TBS for 1 h at room temperature (RT), incubated with horseradish peroxidase-linked secondary antibody (G α M or G α R, DAKO Cytomation, Glostrup, Denmark) diluted at 1:5000 in blocking solution for 1 h at RT, and finally developed with ECL detection kit (Amersham Biosciences, RPN2106PL). Band intensity was quantified using the NIH Image J software. For each sample, the optical density of each band was normalized by dividing with the optical density of the corresponding band of β -tubulin III or β -actin.

Autoradiography

To determine 5-HT_{2A} receptor binding in conditional BDNF knock-out mice and wild-types, animals (n=4 per group) were decapitated and brains were quickly dissected out and stored at –80°C until sectioning. Brains were cut in 10 μ m sections and mounted on gelatine-coated slides and stored at –80°C until further processing. 5-HT_{2A} autoradiography was performed using ³H-MDL100907 [R(+)- α -(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)-ethyl]-4-piperidin-methanol] (specific activity; 64 Ci/mmol, a gift from C. Halldin, Karolinska Institute, Stockholm) and non-specific binding was determined using 10 μ M ketanserin tartrate ([3-[2-[4-(4-fluorobenzoyl)-1-piperidinyl]-ethyl]-2,4[1H, 3H]quinazolinedione tartrate)(Tocris Cookson Ltd, Bristol, UK). For 5-HT_{1A} autoradiography we used ³H-WAY100635 (GE Healthcare, UK) and measured non-specific binding with 10 μ M 5-HT (Sigma-Aldrich, Copenhagen, Denmark). Briefly, sections were allowed to thaw for 1 hr at room temperature (RT) and then pre-incubated at with 50 mM

Tris-HCl (Sigma), pH 7.4 containing 0.01% ascorbic acid (Sigma) and 10 μ M pargyline hydrochloride (N-methyl-N-2-propynylbenzylamine hydrochloride) (Research Biochemicals International, MA, USA) for 30 minutes at RT under constant gentle shaking. Sections were then incubated for 60 minutes at RT using the same buffer containing 0.4 nM of 3 H-MDL100907 (1.5 nM of 3 H-WAY100635 for 5-HT_{1A} binding). Following incubation, slides were washed 2 \times 20 sec in ice-cold 50 mM Tris-HCl, pH 7.4, and 1 \times 20 sec in ice-cold dH₂O, dried for 1 hr under a gentle stream of air.

The SERT autoradiography protocol was modified from Thomsen and Helboe (2003). Briefly, sections were pre-incubated for 30 minutes in 50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, pH 7.4 and subsequently incubated with 2.0 nM [3 H]-escitalopram (donated by H. Lundbeck A/S, Copenhagen, Denmark) for 60 minutes diluted in the same buffer. Nonspecific binding was determined in the presence of 10 μ M paroxetine. After incubation, sections were washed for 3 \times 2 minutes in 50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, pH 7.4 (4°C) and 1 \times 20 seconds in ice-cold dH₂O.

All sections were placed at 4°C overnight in a fixator containing paraformaldehyde vapour and then put in an excicator box for 3 hrs before slides were together with 3 H-microscales (GE Healthcare, UK) exposed to a BAS-TR2040 Imaging Plate (Science Imaging Scandinavia AB, Nacka, Sweden) for 14 days at 4°C. Finally, the imaging plate was scanned on a BAS-2500 scanner (Fujifilm Europe GmbH, Düsseldorf, Germany) and specific and non-specific binding was determined in frontal cortex and hippocampus using ImageJ (NIH) and expressed as fmol/mg tissue equivalents (TE).

RT-qPCR

For total RNA extraction the mice were sacrificed by cervical dislocation and the brain quickly removed and frontal cortex and hippocampus were dissected on ice (n=7). Samples were frozen at -80 °C before homogenization. Total RNA was isolated with TRIzol Reagent (Sigma) according to the manufacturer's directions. The RNA samples were dissolved in RNase-free water and quantified with UV-spectrophotometry at 260 nm.

Extracted RNA was reverse transcribed into cDNA using the procedure of the supplier (ImProm-IITM reverse transcription system, Promega, Madison, WI). The experimental RNA solution was combined with oligo(dT)₁₅ primers and heated at 70°C for 5 min. The reverse transcription reaction mixture contained of 20% ImProm-IITM 5x reaction buffer, 6 mM MgCl₂, 0.5 mM dNTP mix and 20 units RNase inhibitor. The reverse transcription reaction was performed at 42°C for 60 min, followed by heating at 70 °C for 15 min.

The real-time PCR reactions were performed by adding the sample cDNA to a reaction mixture consisting of 1x Brilliant II SYBR green mastermix (Stratagene, La Jolla, CA) and 15 pmol of each primer (DNA technology, Aarhus, Denmark) and adjusting the volume to 20 μ l with DNase free water (Invitrogen, Carlsbad, CA). PCR was performed with a 10 minutes preincubation at 94°C followed by 40 cycles of 30 seconds at 94°C, 45 seconds at 60°C and 90 seconds at 72 °C (Roche Light Cycler 480, Roche, Indianapolis, IN). The real-time PCR method was validated by using serially diluted cDNA to establish a standard curve. To quantify the gene expression profile of each sample, the efficiency of each standard curve was determined from its slope and comparative threshold according to the manufacturer's instructions. For each sample, the amount of targeted mRNA was normalized to that of the reference gene β -tubulin III. Primers for mouse 5-HT_{1A}, 5-HT_{2A} receptors and β -tubulin III were designed for real-time PCR. The sequence of the specific primers designed based on gene bank data were as follows: 5-HT_{2A}: F: 5-ccgctcaactccagaaccaaagc-3, R: 5-cttcgaatcatcctgtaccgaa-3; 5-HT_{2C}: F: 5-taatggtgaacctggcactgcgg-3, R: 5-taaaagtgtcagtactatagctgc, 5-HT_{1A}: F: 5-

ctgtttatgccctggatg-3, R: 5-atgagccaagtgcgagat-3; β -tubulin III, F: 5-atagggccaagtctgggagtc-3, R: 5-ctgggcacatactgtgagaggag-3

Behavioral studies

The head-twitch response (HTR) and ear-scratch response (ESR) scoring was performed as previously reported (Darmani 2001) with minor modifications. In brief, animals (n=7 per group) were kept in their standard home cage (15 × 24 cm), but enrichment material was removed during the test period. Animals were injected with either DOI (2.5 mg/kg, i.p.) or corresponding volume of saline 5 min prior to testing. The mice were video taped for 20 min and subsequently HTR and ESR were scored. One week later, the experiment was repeated so animals receiving DOI on the first test day, were given saline and vice versa.

Immunohistochemistry

Wildtype and cBDNF KO mice were injected either saline or DOI (2.5 mg/kg) and after 1 hr, transcardially perfused with 20 ml of cold saline followed by 50 ml of 4% paraformaldehyde (PFA). Brains were immediately removed, postfixed in 4% PFA overnight at 4°C, cryoprotected in a 30% sucrose solution, and frozen in mounting media (Tissue-Tek, Torrance, CA) until further use. 40 μ M coronal sections representing frontal cortex and hippocampus were obtained and c-fos immunohistochemistry was performed as previously described (Unger et al., 2007). In brief, endogenous peroxidase activity was quenched in free-floating sections with 0.5% H₂O₂ in PBS for 15 min, permeabilized with 0.5% Triton X-100 in PBS for 20 min, and blocked in 10% normal horse serum for 30 min at RT. Sections were then incubated for 48 h at 4°C with a rabbit primary antibody against c-fos (1:50,000; Ab5; Oncogene Science, Cambridge, MA), followed by washes and incubation with a biotinylated secondary antibody (1:200) and an avidin – biotin peroxidase complex as per the manufacturers instructions (ABC, Vector Elite Kit; Vector Laboratories, Burlingame, CA). Sections were then incubated with substrate solution (0.025% diaminobenzidine/0.15% nickel ammonium sulfate/0.0036% H₂O₂ in 0.05 M Tris buffer) for 5 min, mounted on slides and left to dry for 2 hrs before placing cover slips. c-fos positive neurons were quantified in frontal cortex and hippocampus using the computer assisted stereological toolbox software (CAST) module (Visiopharm, Denmark).

Statistical analysis

All analyses were performed using GraphPad 5. Data are presented as mean \pm SEM if not otherwise stated. The data were analyzed using Student's t-test if comparing the two groups, and two-way ANOVA with Bonferoni as post hoc test, when comparing the groups with more than one variable. The level of statistical significance was set to $P < 0.05$

Results

Total depletion of BDNF in frontal cortex and hippocampus of BDNF mutants

To confirm that adult BDNF^{2L/2Lck-cre} mutant mice were depleted of BDNF protein in our brain regions of interest, we measured levels of BDNF in the mutant frontal cortex and hippocampus. Whereas BDNF protein was clearly detectable by Western blotting in wildtype mice, BDNF levels in BDNF mutants were below detectable levels (Fig. 1A and B). These results demonstrate that BDNF^{2L/2Lck-cre} mice are depleted of BDNF protein in the frontal cortex and hippocampus.

5-HT_{2A} and 5-HT_{1A} receptor binding and expression is altered in BDNF mutant mice

The number of 5-HT_{2A} and 5-HT_{1A} receptors in frontal cortex and hippocampus was analyzed using autoradiography applying highly selective tritium-labeled ligands (³H-

MDL100907 and ^3H -WAY100635, respectively). 5-HT_{2A} binding in frontal cortex of BDNF^{2L/2LCK-cre} mice was lower when compared to wildtype littermates (45.6 ± 1.7 vs. 35.2 ± 1.6 fmol/mg/TE, respectively) ($P = 0.03$) (Fig. 2A). However, in hippocampus, 5-HT_{2A} receptor binding was increased by 31% in BDNF mutants (12.5 ± 0.7 vs. 16.4 ± 1.2 fmol/mg/TE, respectively) ($P = 0.02$) (Fig. 2B). In frontal cortex 5-HT_{1A} receptor binding was similar in the two groups (59.15 ± 2.9 (WT) vs 56.55 ± 3.2 (BDNF mutants) Fig. 3A), while in hippocampus there was a significant decrease in 5-HT_{1A} receptor binding in BDNF mutants compared to wildtypes (115.7 ± 2.5 vs. 100.2 ± 3.5 ; Fig 3B) ($P = 0.01$).

The effect of perturbed BDNF signaling on 5-HT_{1A} and 5-HT_{2A} receptors was further evaluated by gene expression studies. Using RT-qPCR, we observed a decrease in 5-HT_{2A} mRNA levels in the frontal cortex of BDNF^{2L/2LCK-cre} mutants that corresponded to the observed deficit in receptor binding (Fig. 4A). However, the increase in 5-HT_{2A} receptor binding in the BDNF mutant hippocampus was not associated with changes in 5-HT_{2A} mRNA levels (Fig 4D). Additionally, there was a trend towards a significant decrease in 5-HT_{1A} mRNA expression in the BDNF mutant hippocampus but it did not reach statistical significance ($P = 0.08$) (Fig 4E). No differences in 5-HT_{1A} mRNA levels were observed in the mutant frontal cortex (Fig. 4B). We also investigated whether transcript or protein levels of the 5-HT_{2C} receptor were altered in BDNF mutant animals. No between-group differences were observed in frontal cortex or hippocampus (Figs. 4C, 4F and 5). The cumulative data indicate that the changes in 5-HT_{2A} and 5-HT_{1A} receptor binding in BDNF mutants differ between brain regions and are apparently inversely regulated in hippocampus.

SERT binding is normal in BDNF^{2L/2LCK-cre} mutant mice

To determine whether the observed alterations in 5-HT receptor binding and expression were related to upstream changes in the serotonin transporter (SERT), we used ^3H -escitalopram autoradiography to quantify SERT binding sites. This analysis revealed that there were no differences in SERT binding between wildtypes and BDNF mutants either in frontal cortex (Fig. 6A) or hippocampus (Fig. 6B).

BDNF depletion leads to altered behavioral responses to 5-HT_{2A} receptor stimulation

Next, we tested the effect of the altered 5-HT_{2A} receptor expression in BDNF^{2L/2LCK-cre} mutant mice on 5-HT_{2A}-mediated behaviors. For this, we measured the ESR and HTR following saline or DOI (2.5mg/kg, i.p.) administration. There was a significant interaction of genotype and drug treatment in the ESR (F, 12.39 and $P = 0.002$, two-way ANOVA) (Fig. 7A). DOI induced a significant increase in ESR in wildtypes compared to their saline-treated counterparts (WT saline: 3 ± 1.5 ; WT DOI: 18 ± 5.5 head-twitches/20 min, Bonferroni post hoc test, $P < 0.001$). In contrast, DOI did not induce an increase in ESR in BDNF^{2L/2LCK-cre} mice compared to saline-treated mutants (Fig. 7A). However, DOI treatment induced similar levels of HTR in wild type and BDNF mutant mice (two-way ANOVA) (Fig. 7B). These results indicate that the 5-HT_{2A} mediated functional output measured by ESR is diminished in the BDNF mutant mice.

Neuronal activation following 5-HT_{2A} receptor stimulation in BDNF mutant mice

To further assess possible functional deficits in BDNF^{2L/2LCK-cre} mutants related to 5-HT_{2A} receptor alterations, we measured neuronal activation by c-fos immunoreactivity in frontal cortex and hippocampus 1 hr after a single DOI or saline injection. DOI elicited similar increases in number of c-Fos positive cells in frontal cortex in both wildtypes (saline: 1820; DOI: 4731 c-Fos+ cells; $P < 0.05$) and BDNF mutants (saline: 1198; DOI: 4783 cells; $P < 0.05$) (Fig. 8A) (two-way ANOVA, Bonferroni post hoc). In hippocampus, DOI did not induce a significant increase in c-Fos+ cells in either wild types (saline: 1930; DOI: 3257 cells), or BDNF mutants (saline: 4420; DOI: 4593 cells) (Fig. 8B). However, there was an

effect of genotype indicating that the cell activation response following 5-HT_{2A}R-mediated cell activation is higher in hippocampus of the BDNF mutants compared to wildtypes ($P = 0.04$). This difference between genotypes was mainly driven by high baseline activation in the BDNF mutants. Collectively, these data indicate that the 5-HT_{2A} receptor changes in frontal cortex are not reflected in 5-HT_{2A}R-mediated cell activation, while the hippocampal cell activation is higher for the BDNF mutants.

Expression of synaptic markers in hippocampus and frontal cortex of BDNF^{2L/2LCK-cre} mutant mice is normal

To examine the degree of synaptic integrity, we measured protein levels of synaptophysin (pre-synaptic), PSD-95 (post-synaptic) and MAP2 (dendritic) using western blotting. We found no change in protein levels between wildtypes and BDNF mutants neither in frontal cortex nor in hippocampus (Fig. 9)

Discussion

We hypothesized that alterations in the 5-HT system might underlie the behavioral alterations triggered by deficient BDNF signaling in the brain. One test of this hypothesis was to ascertain the viability of pivotal components of this neurotransmitter system in limbic regions of brain associated with anxiety and mood. We focused our investigations on 5-HT_{2A}, 5-HT_{2C} and 5-HT_{1A} receptors because human and animal model studies have linked them to behaviors known to be altered in BDNF mutant mice including anxiety-like behavior (Naughton et al., 2000; Heisler et al., 2007; Akimova et al., 2009). Our results confirm the previously described differences in cortical 5-HT_{2A} levels in BDNF mutant mice and reveal additional alterations in the 5-HT receptor system elicited by depleted BDNF stores. The decreased 5-HT_{2A} receptor content observed in frontal cortex of BDNF^{2L/2LCK-cre} mice using western blotting was also reflected in lower receptor binding in this area. Previously, we reported a 50–60% decrease in 5-HT_{2A} receptor protein levels in frontal cortex (Rios et al., 2006), and in the present paper we found a ~20% decrease in receptor binding in this region. Since the analyses were not conducted on the same tissue, the quantitative outcomes cannot be directly compared. Differences in the receptor detection methods utilized could, however, explain eventual discrepancies. Total protein measurements reflect the entire pool of 5-HT_{2A} receptors, while receptor autoradiography reflects the binding of the antagonist radioligand to the intact receptor binding pocket. A special characteristic of the 5-HT_{2A} receptor is its high turnover rate, the largest receptor fraction being located intracellularly (Cornea-Hebert et al., 1999; Gray & Roth, 2001). Our results suggest that in BDNF mutant animals, it is primarily the intracellular fraction of receptors not available for ligand binding that is diminished. This is supported by the mRNA quantification results showing that indeed the rate of receptor synthesis is decreased in BDNF mutant mice.

Contrary to our observations in frontal cortex, we found that there was an increase in 5-HT_{2A}R binding in the BDNF mutant hippocampus. This is in accordance with our *in vivo* findings from BDNF^{+/-} mice, where we also found increased hippocampal 5-HT_{2A}R protein levels (Trajkovska et al., 2009). The inverse observation, namely that exposure of BDNF to hippocampal cultures results in 5-HT_{2A}R downregulation is in also line with this (Trajkovska et al., 2009). The *in vivo* studies of BDNF^{2L/2LCK-cre} mice described here indicate that the effects of BDNF on 5-HT_{2A} receptor levels are context-dependent.

We extended our analysis to other 5-HT receptors to ascertain whether the effects of BDNF were restricted to the 5-HT_{2A} receptor. We found that 5-HT_{2C} receptor expression in frontal cortex and hippocampus of BDNF mutants was normal. We also measured expression and binding of the 5-HT_{1A} receptor in these brain regions. The 5-HT_{1A} receptor co-localizes

with the 5-HT_{2A} receptor in dendrites in prefrontal cortex (Feng et al., 2001). Central BDNF depletion did not impact 5-HT_{1A} receptor binding in frontal cortex, suggesting that the lower 5-HT_{2A} receptor binding was a result from structural reorganization or retraction of dendrites. In support of this assertion, we found that expression of synaptophysin, a synaptic marker and of MAP2, a dendritic marker, were normal in BDNF mutants. In contrast to our findings in frontal cortex, 5-HT_{1A} receptor binding was decreased in the BDNF mutant hippocampus. Furthermore, we showed previously that 5-HT_{1A} receptor protein levels were unchanged in dorsal raphe nucleus (DRN) in BDNF^{2L/2LCK-cre} mutants (Rios et al., 2006). The primary role of the 5-HT_{1A}Rs in DRN is as pre-synaptic autoreceptors, while in hippocampus they mainly exist as post-synaptic heteroreceptors (Riad et al., 2001). Therefore, the data shown here and previous findings provide additional examples of the context-dependent effects of BDNF. An intriguing study by Hensler et al. (2007) found no changes in hippocampal 5-HT_{1A} receptor binding using a tetracycline-inducible model of BDNF depletion (Hensler et al., 2007). It is important to note that whereas in those mutants BDNF was depleted in adulthood, in the mutant mice described here BDNF was gradually depleted during the first post-natal weeks (Rios et al., 2001). Therefore, the 5-HT_{1A} receptor alterations reported here might reflect the long-term effect of BDNF depletion, the effects of perturbing BDNF signaling during a critical developmental period or a compensatory response to the 5-HT_{2A} receptor changes also observed in these mice. A “functional antagonism” between these two receptors was proposed previously as a mechanism to circumvent challenges on the serotonin system and restore proper neurotransmission (Yuen et al., 2008). Nonetheless, this alteration in 5-HT_{1A} receptors might contribute to the complex behavioral phenotype of these mice.

By examining 5-HT_{2A}-mediated behavioral output, we showed that the decrease in 5-HT_{2A} receptor levels was accompanied by a diminished functional response to pharmacological stimulation of this receptor *in vivo*. DOI-induced ESR was significantly attenuated in BDNF mutants, indicating a reduction in the pool of functional 5-HT_{2A} receptors that mediate this response. Interestingly, the DOI-induced HTR was not affected in the mutants. This was surprising as it is well established that both HTR and ESR are mediated by the 5-HT_{2A} receptor (Gonzalez-Maeso et al., 2007; Willins & Meltzer, 1997). Accordingly, both HTR and ESR were completely abrogated in 5-HT_{2A} receptor knock-out mice (Gonzalez-Maeso et al., 2007, suppl.mat). We suggest that the ESR is more sensitive to small changes in 5-HT_{2A} receptor signaling, and that the 20% decrease in 5-HT_{2A} receptor binding in frontal cortex is too small to be reflected in the HTR. Indeed, the ESR was demonstrated to be 30 times more sensitive than HTR (Darmani, 2001). Alternatively, the HTR and ESR might be mediated by different brain circuits that are differentially affected by perturbed BDNF signaling. Unfortunately, little is known regarding the exact networks underlying the HTR and ESR. In a thorough study, Willins and Meltzer (1997) delivered DOI to different regions of the rat brain and established that HTR could only be induced when the medial prefrontal cortex was activated. The ESR was not examined as this response had not been described in rats. If other brain areas besides the medial prefrontal cortex such as the hippocampus are involved in the ESR, the observed increase in hippocampal 5-HT_{2A}R binding in the BDNF mutants might exacerbate the diminished 5-HT_{2A} frontal cortex output leading to impaired ESR. In support of this model, a previous study demonstrated a marked DOI-induced increase in motor-movements (head-bobs), which can be compared to HTR and ESR in rodents, in rabbits with increased 5-HT_{2A} receptor binding in the CA1 region of the hippocampus (Dave et al., 2004). Future studies with intracerebral site-directed injections of 5-HT_{2A} agonists in mice are needed to test this hypothesis.

Even though activation of the 5-HT_{2A} receptor signaling pathway is necessary for both ESR and HTR, it has been suggested that other receptor systems can modulate the response. For example, Darmani and colleagues showed that HTR and ESR induced by DOI can both be

inhibited by cannabinoids (e.g., CP55,940), but not to the same degree. This indicates that there are different thresholds for each response which can be modulated through other receptors. Other data suggest that 5-HT_{2A} mediated responses can be modulated by activation of the 5-HT_{1A} and 5-HT_{2C} receptors (Willins & Meltzer, 1997). Interestingly, we found that there was a significant decrease in hippocampal 5-HT_{1A} receptor binding in BDNF mutants. However, it remains to be elucidated whether this change is sufficient to impact the ESR and/or the HTR. Since BDNF depletion was not associated with changes in 5-HT_{2C} receptor expression, it is unlikely that deficits in this pathway contribute to the diminished ESR in BDNF mutants.

To further evaluate 5-HT_{2A} receptor functionality in the BDNF^{2L/2LCK-cre} mutant mice, we quantified DOI-induced c-Fos expression as a measure of 5-HT_{2A}-mediated neuronal activation. In spite of decreased frontal 5-HT_{2A}-receptor binding, BDNF mutants had the same DOI-induced c-Fos expression in frontal cortex, as the wildtype mice. However, in hippocampus, BDNF mutants had a different cell activation response. This was partly driven by high levels of c-fos+ cells in the hippocampus following the saline injection, suggesting that mutants might have elevated baseline levels of neuronal activity in this region at baseline. This may be mediated through the observed increase in 5-HT_{2A} receptors in hippocampus in these mice. Alternatively, BDNF mutants might have a higher basal level of arousal and are easily stressed when handled and accordingly, the saline injection *per se* may elicit a c-Fos response in this brain region.

In summary, we demonstrated that BDNF depletion results in context-dependent changes in 5-HT_{2A} and 5-HT_{1A} binding and expression. Furthermore, we showed that alterations in 5-HT_{2A} levels have functional consequences as illustrated by the diminished DOI-induced ESR in BDNF mutants. The diverse regulation of these receptors in BDNF^{2L/2LCK-cre} mice underlines the complexity of neurotrophin and serotonin signaling interaction.

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Abbreviations

BDNF	Brain-derived neurotrophic factor
CA1	cornu ammonis 1 of hippocampus
DOI	(±)-2,5-Dimethoxy-4-iodoamphetamine hydrochloride
DRN	dorsal raphe nucleus
ESR	ear-scratch response
5-HT	serotonin
5-HT _{1A} R	5-HT receptor 1A
5-HT _{2A} R	5-HT receptor 2A
5-HT _{2C} R	5-HT receptor 2C
HTR	head-twitch response
MAP2	microtubule associated protein 2

SERT	serotonin transporter
TrkB	tropomyosin-related kinase B

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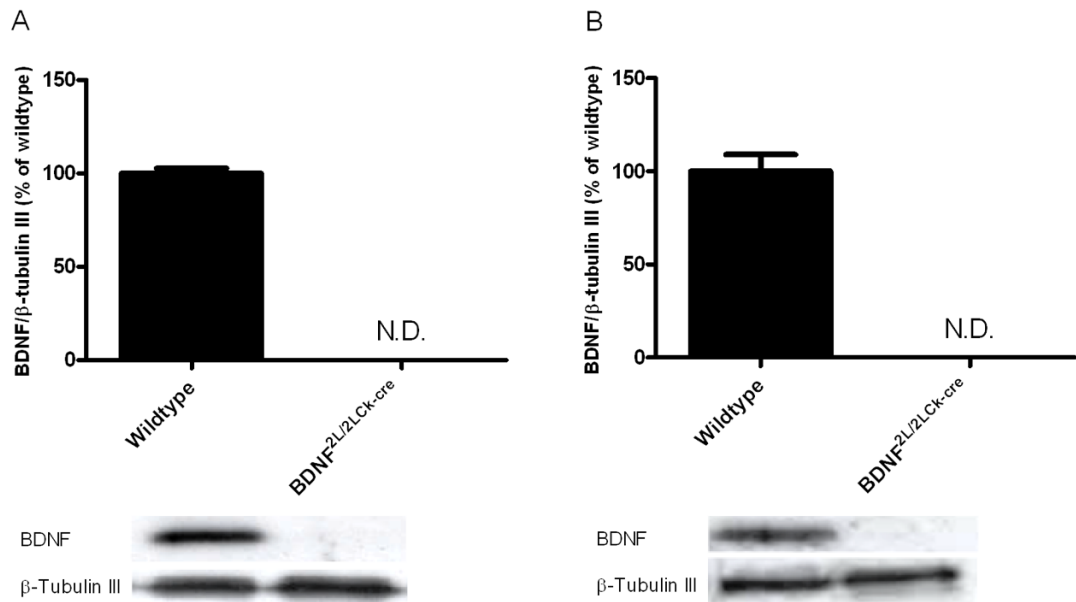


Figure 1.

BDNF protein levels in frontal cortex (A) and hippocampus (B) in wildtype and BDNF^{2L/2LCK-cre} mice. BDNF levels were not detectable in frontal cortex and hippocampus in the BDNF mutant mice using western blot, while wildtype levels corresponded to levels seen in other mice strains. BDNF levels were related to β-tubulin III levels (n=7). Representative bands for BDNF (14 kDa) and β-tubulin III (55 kDa) are shown below the corresponding column. N.D.: not detectable. Error bars indicate SEM.

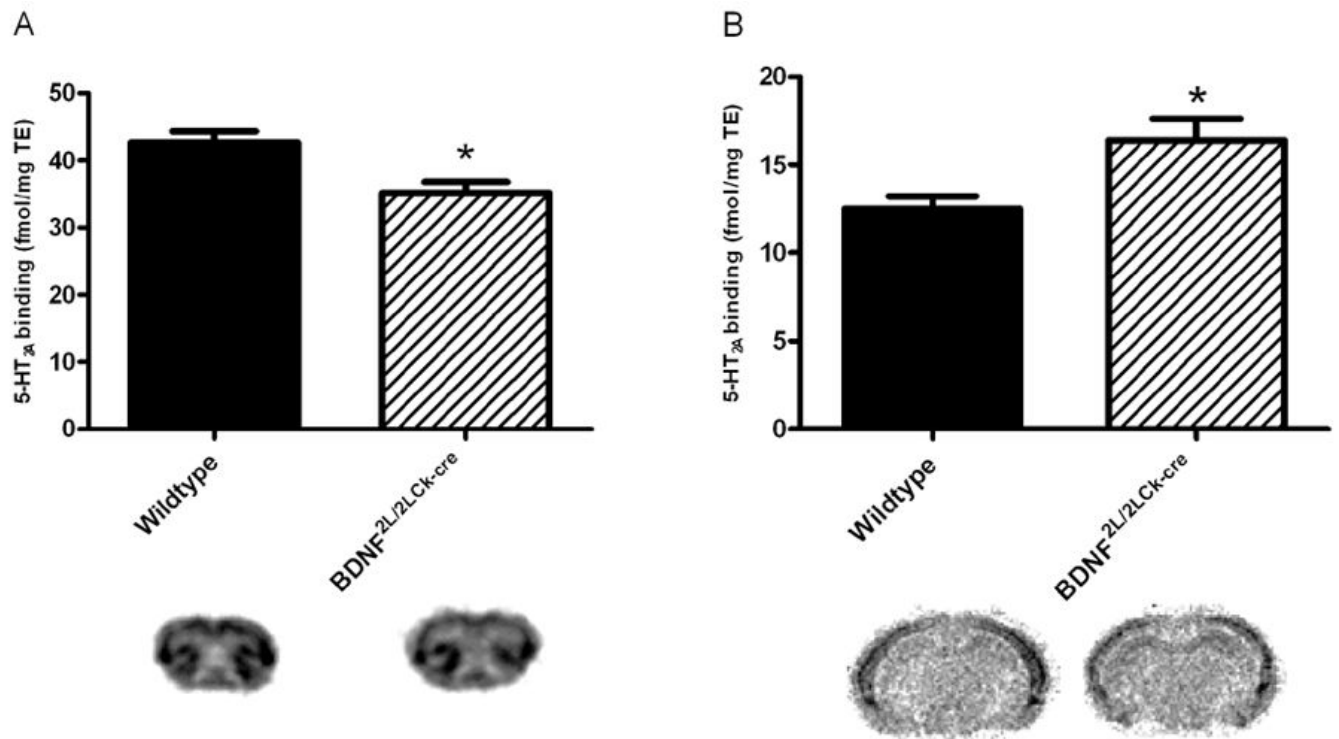


Figure 2. 5-HT_{2A} receptor binding (³H-MDL-100907) in BDNF^{2L/2LCK-cre} mice and wildtype littermates in frontal cortex (A) and dorsal hippocampus (B). There was a significant decrease in binding in frontal cortex in BDNF^{2L/2LCK-cre} mice vs wildtypes, while the opposite was observed for dorsal hippocampus. (n=4, Student's t-test, **P* < 0.05). Representative autoradiograms are shown below the corresponding column. Error bars indicate SEM

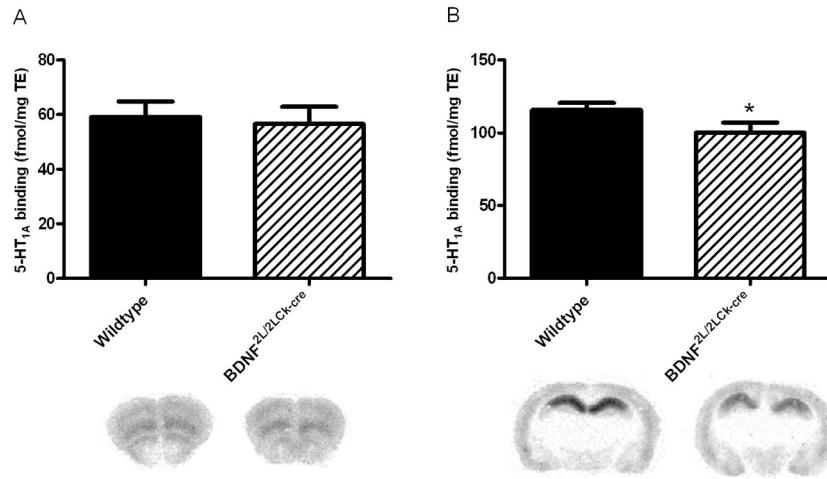


Figure 3. 5-HT_{1A} receptor binding (³H-WAY100635) in BDNF^{2L/2LCk-cre} mice and wildtype littermates in frontal cortex (A) and dorsal hippocampus (B). There was a significant decrease in binding in dorsal hippocampus in BDNF^{2L/2LCk-cre} mice vs wildtypes, while no change was observed in frontal cortex. (n=4, Student's t-test, **P* < 0.05). Representative autoradiograms are shown below the corresponding column. Error bars indicate SEM

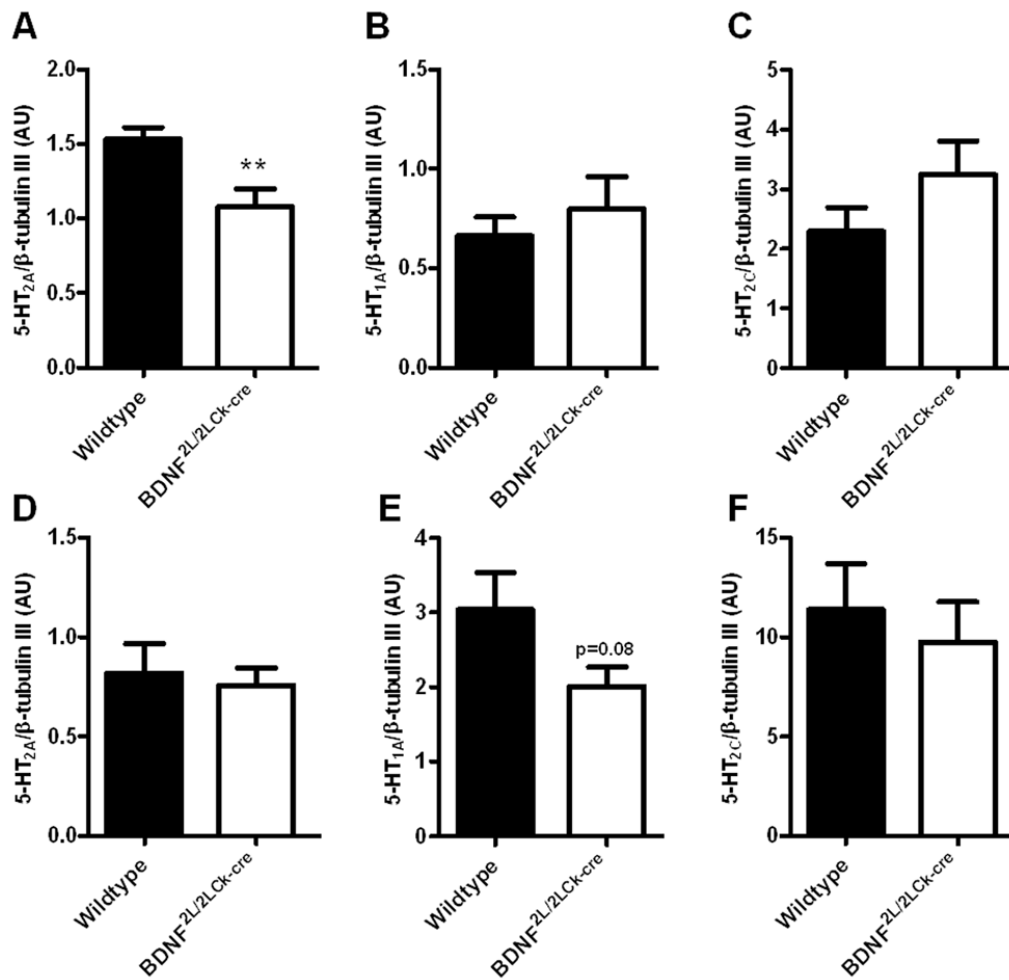


Figure 4.

RT-qPCR on 5-HT_{2A} (A, D), 5-HT_{1A} (B, E) and 5-HT_{2C} (C, F) mRNA in frontal cortex (upper panel) and hippocampus (lower panel) of wildtype and BDNF^{2L/2LCk-cre} mice. There was a significant lower 5-HT_{2A} mRNA expression in BDNF^{2L/2LCk-cre} mice in frontal cortex compared to wildtypes (A), while no differences could be observed in hippocampus (D) (n=7–8, Student's t-test, ** $P < 0.01$). For the 5-HT_{1A} receptor, there was a tendency towards a decrease in expression in BDNF^{2L/2LCk-cre} mice compared to wildtypes (E) ($P = 0.08$) for hippocampus, while no change was observed in frontal cortex (B). For the 5-HT_{2C} receptor we did not find any differences in expression of mRNA between BDNF^{2L/2LCk-cre} mice and wildtypes. (AU, arbitrary units, Error bars indicate SEM)

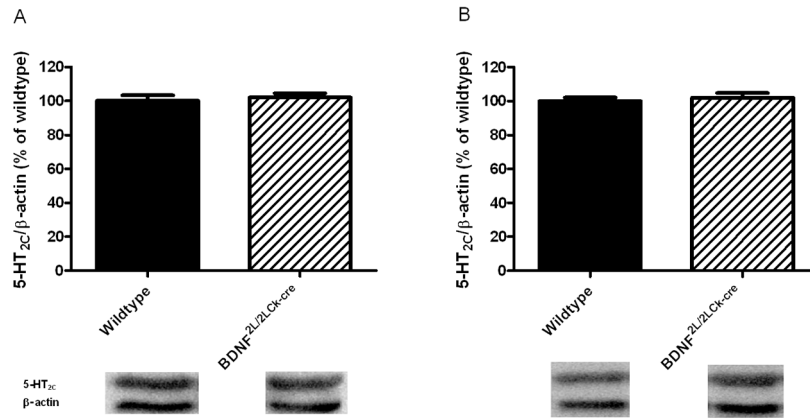


Figure 5. 5-HT_{2C} receptor protein levels in wildtype and BDNF^{2L/2LCk-cre} mice in frontal cortex (A) and hippocampus (B). No changes in protein levels of this serotonin receptor could be observed (n=7–8, Student's t-test). Representative bands for 5-HT_{2C} (55 kDa) and β-actin (40 kDa) are shown below the corresponding column. Error bars indicate SEM

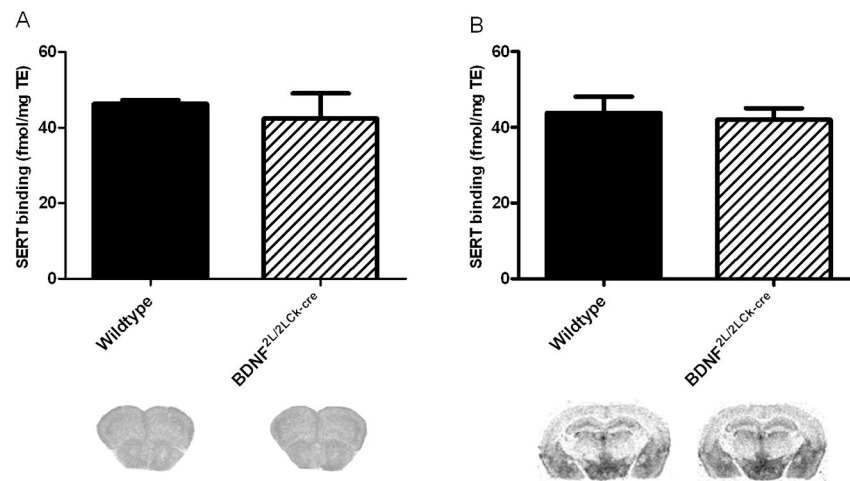


Figure 6. SERT binding performed with ^3H -escitalopram in frontal cortex (**A**) and hippocampus (**B**). We did not detect any differences in SERT binding between wildtypes and BDNF^{2L/2LCk-cre} mice. (n=4, Student's t-test). Representative autoradiograms are shown below the corresponding column. Error bars indicate SEM

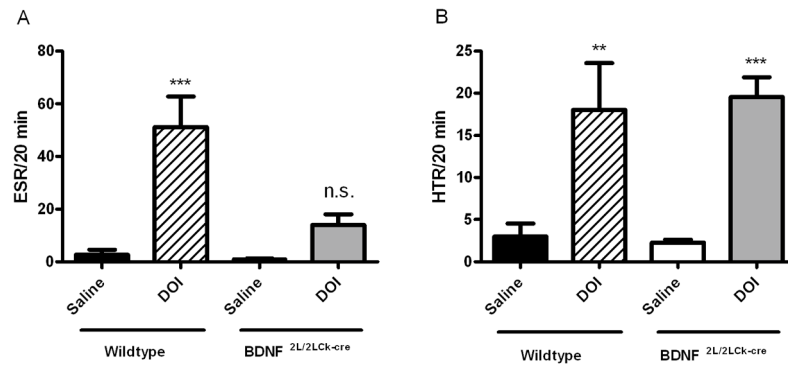


Figure 7. Ear-Scratch Reponse (ESR)(A) and Head-Twitch Reponse (HTR)(B) in BDNF mutant mice and wildtype littermates after administration of DOI (2.5 mg/kg, i.p.) or saline. The registration of ESR and HTR was started 5 min after administration and continued for 20 min. Bars: Black: wildtype, saline; Crossed: wildtype, DOI 2.5 mg/kg, i.p.; White: BDNF^{2L/2LCK-cre} mice, saline; Gray: BDNF^{2L/2LCK-cre} mice (n=7, two-way ANOVA, Bonferroni post test, n.s.=not significant, ** $P < 0.01$, *** $P < 0.001$). Error bars indicate SEM

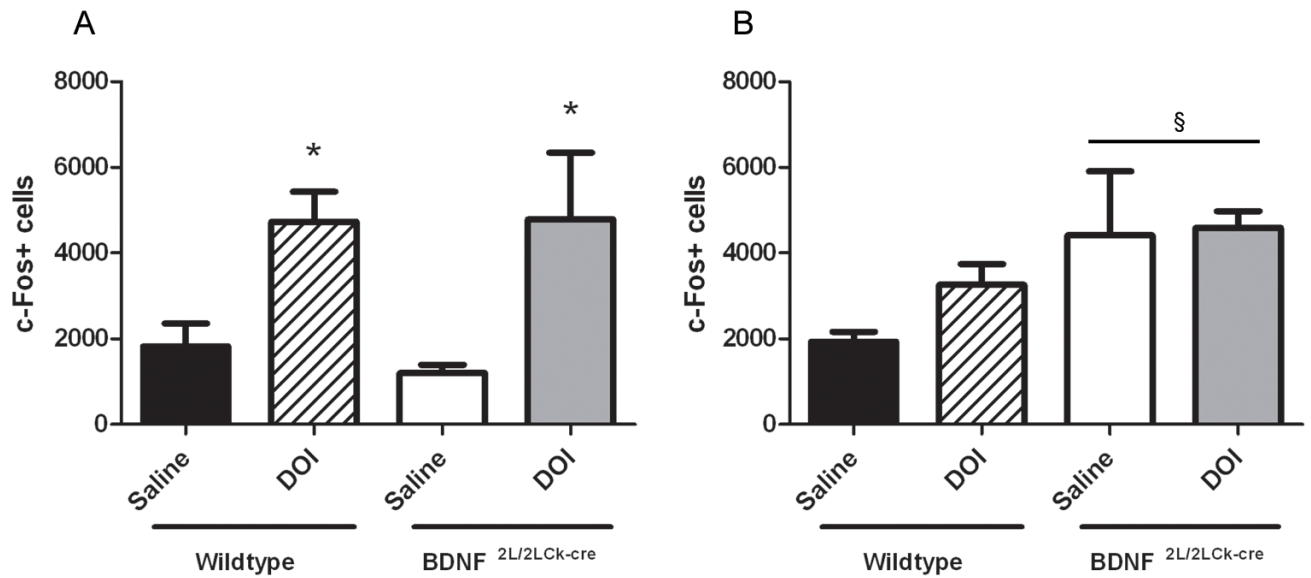


Figure 8. c-Fos immunopositive cells in frontal cortex (**A**) and hippocampus (**B**). A single DOI (2.5 mg/kg) injection significantly induced c-Fos immunoreactive cells compared to a single saline injection in frontal cortex in both wildtypes and BDNF^{2L/2LCK-cre} mice (**A**). There was a genotype effect in hippocampus (§) indicating higher cell activation response BDNF^{2L/2LCK-cre} mice compared to wildtypes (**B**) (n=4, two-way ANOVA, Bonferroni post hoc test, *P < 0.05. §P < 0.05. Error bars indicate SEM

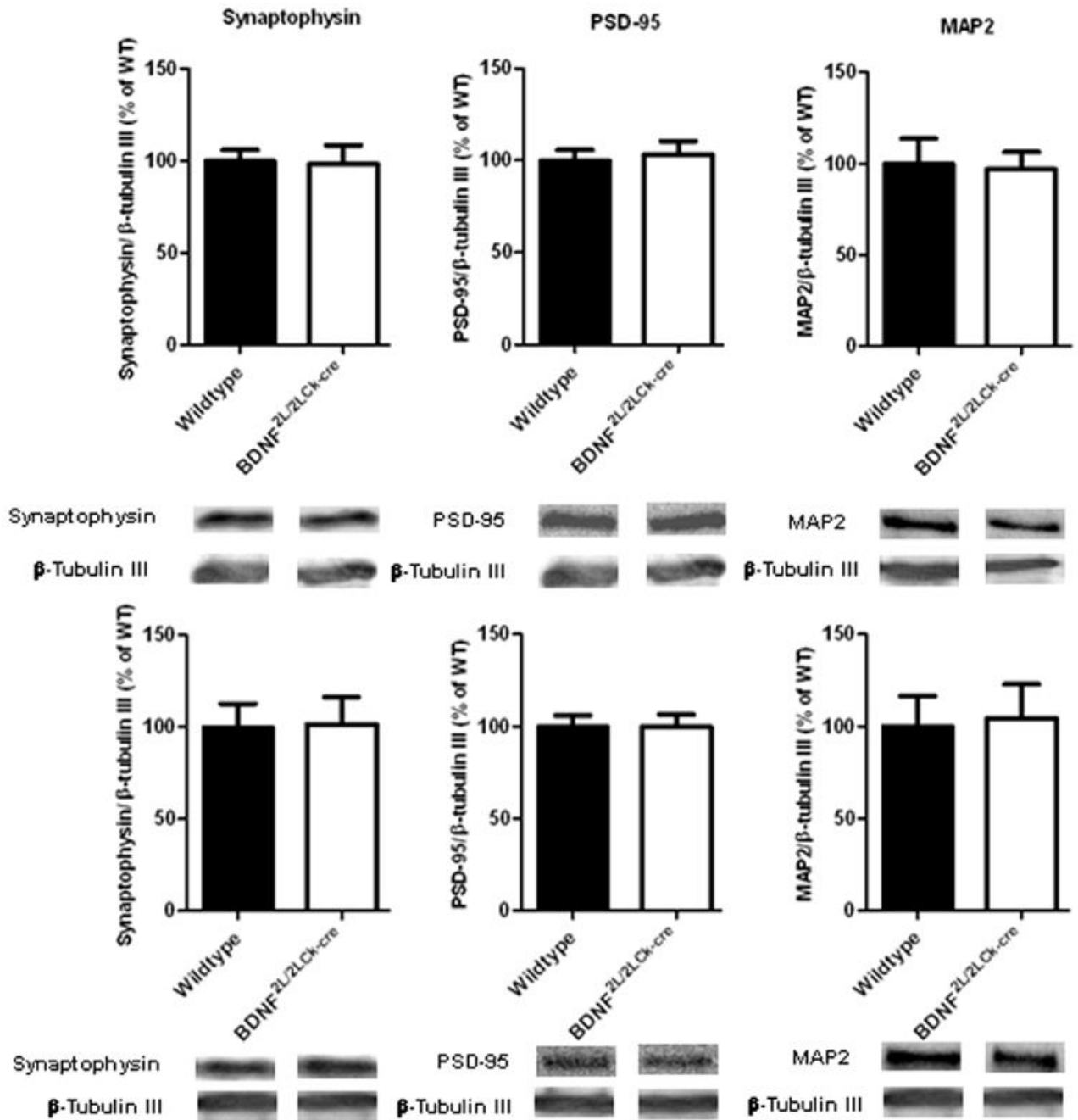


Figure 9.

Analysis of protein levels using western blotting for synaptophysin (38 kDa), PSD-95 (95 kDa) and MAP2 (250 kDa). Upper panel: frontal cortex, lower panel: hippocampus. BDNF mutants have normal contents of the synaptic markers synaptophysin and PSD-95 and the dendritic marker MAP2 when compared to wildtypes. β -tubulin III (55 kDa) was used as loading control. (n=7–8, Student's t-test). Error bars indicate SEM