Abstract: Background: Several lines of evidence implicate BDNF in the pathophysiology of psychiatric illness. BDNF polymorphisms have also been associated with the risk of schizophrenia and mood disorders. We investigated whether levels of (pro)BDNF and receptor proteins, TrkB and p75, are altered in hippocampus in schizophrenia and mood disorder and whether polymorphisms in each gene influenced protein expression. Methods: Formalin-fixed paraffin-embedded hippocampal sections from subjects with schizophrenia, major depressive disorder (MDD), bipolar disorder (BPD) and non-psychiatric controls were obtained from the Stanley Foundation Neuropathology Consortium. (pro)BDNF, TrkB(T1) and p75 protein densities were quantified by immunoaautoradiography and DNA extracted from each subject was used to determine the effect of genotype on protein expression. Results: In MDD, reductions in (pro)BDNF were seen in all layers of the right but not the left hippocampus with no changes in the dentate gyrus. The pattern was similar but less marked for BPD. In addition, BPD but not MDD patients, had bilateral reductions in p75 in hippocampal layers but not in dentate gyrus. No changes in TrkB(T1) density were seen in...
any diagnosis. Conclusions: These findings suggest MDD and BPD may share impairment in (pro)BDNF expression. However, BPD may involve impairments of both (pro)BDNF and p75 receptor, whereas MDD may involve impaired (pro)BDNF alone. Moreover, the lateralisation of changes may indicate a role of asymmetry in vulnerability to MDD. Hippocampal (pro)BDNF and receptor levels were also affected by genotype, suggesting that allelic variations are important in the hippocampal abnormalities seen in these psychiatric disorders.
Dear Dr Junkert

Enclosed is a revised copy of an original article entitled:

**Hippocampal Brain-Derived Neurotrophic Factor and its Receptors in Stanley Consortium Brains.**

by Jason S Dunham, JF William Deakin, Fabio Miyajima, Tony Payton and Carla T Toro.

I would like to thank you and the reviewers for your positive response to the changes we have made to the manuscript. I would also like to thank reviewer #2 for pointing us in the direction of the interesting and relevant article by Yang et al, 2009.

In response to the further comments made by Reviewer #2, we have made changes to the abstract and manuscript as summarised in the ‘Response to Reviewers’ file.

I hope that the much improved manuscript will be acceptable for publication and very much look forward to hearing whether you can accept this article.

Yours sincerely

Carla T Toro, PhD

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Response to Reviewers

In response to the further comments made by Reviewer #2, we have made the following changes to the abstract and manuscript:

- When referring to the protein measurements of (pro)BDNF we have ensured that (pro)BDNF (rather than BDNF) is used consistently
- We have made reference to the findings by Yang et al in the Discussion
- We have changed the first sentence of the Conclusions section to avoid confusing the reader
AUTHORS CONTRIBUTIONS

JS Dunham, CT Toro and JFW Deakin all contributed to the overall design of the study. CT Toro wrote the protocol for immunooautoradiography studies. F Miyajima and A Payton selected the SNPs and wrote the protocol for the genotyping. JS Dunham carried out the majority of the studies under the supervision of CT Toro. JS Dunham and JFW Deakin carried out all the statistical analysis. JS Dunham wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.
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CONFLICTS OF INTEREST

All authors declare that they have no conflicts of interest.
ROLE OF FUNDING SOURCE

Funding for this study was provided by an International Collaborating Centre grant from the Stanley Medical Research Institute and by NewMood EU Integrated Research Programme LSHM-CT-2004-503474. These funding bodies had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.
Expression of Hippocampal Brain-Derived Neurotrophic Factor and its Receptors in Stanley Consortium Brains

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Keywords: Neurotrophin, expression, polymorphism, postmortem, schizophrenia, mood disorders.

ABSTRACT

Several lines of evidence implicate BDNF in the pathophysiology of psychiatric illness. BDNF polymorphisms have also been associated with the risk of schizophrenia and mood disorders. We therefore investigated whether levels of (pro)BDNF and receptor proteins, TrkB and p75, are altered in hippocampus in schizophrenia and mood disorder and whether polymorphisms in each gene influenced protein expression. Formalin-fixed paraffin-embedded hippocampal sections from subjects with schizophrenia, major depressive disorder (MDD), bipolar disorder (BPD) and non-psychiatric controls were obtained from the Stanley Foundation Neuropathology Consortium. (pro)BDNF, TrkB(T1) and p75 protein densities
were quantified by immunoautoradiography and DNA extracted from each subject was used to determine the effect of genotype on protein expression. In MDD, reductions in (pro)BDNF were seen in all layers of the right but not the left hippocampus with no changes in the dentate gyrus. The pattern was similar but less marked for BPD. In addition, BPD but not MDD patients, had bilateral reductions in p75 in hippocampal layers but not in dentate gyrus. No changes in TrkB(T1) density were seen in any diagnosis. These findings suggest MDD and BPD may share impairment in (pro)BDNF expression. However, BPD may involve impairments of both (pro)BDNF and p75 receptor, whereas MDD may involve impaired (pro)BDNF alone. Moreover, the lateralisation of changes may indicate a role of asymmetry in vulnerability to MDD. Hippocampal (pro)BDNF and receptor levels were also affected by genotype, suggesting that allelic variations are important in the hippocampal abnormalities seen in these psychiatric disorders.

INTRODUCTION

Hippocampal volume reduction is one of the most consistent changes reported in structural MRI studies of schizophrenia (Nelson et al 1998; Wright et al 2000) and mood disorders (Videbech and Ravnkilde 2004). Postmortem studies suggest that hippocampal volume reduction in schizophrenia and possibly mood disorders may be a consequence of decreased neuropil and cell size rather than neuron loss (Harrison 2002; Harrison 2004; Heckers and Konradi 2002). As neurotrophins are important in the development and maintenance of neural connectivity in the hippocampus (Huang and Reichardt 2001; Lewin and Barde 1996), impaired neurotrophin function may be an important potential mechanism for loss of neuropil in psychosis and mood disorder (Hashimoto et al 2004; Thome et al 1998).

Brain-derived neurotrophic factor (BDNF) and its precursor, proBDNF, and receptors TrkB (coded by NTRK2) and p75 (coded by NGFR), are expressed in both the developing and adult hippocampus and also in other CNS regions implicated in schizophrenia and mood disorders (Allen et al 1994; Harrison 1999; Harrison 2002; Ozbas-Gerceker et al 2004;
Romanczyk et al. 2002; Webster et al. 2002, 2006; Yang et al. 2009). BDNF is also involved in the development and function of neurochemical systems that may be altered in these illnesses (Djalali et al. 2005; Guillin et al. 2004; Harrison 1999; Lessmann 1998). Impaired BDNF function could thus result in failure to develop and effectively maintain such neurotransmitter systems. Allelic variations may also affect BDNF and hippocampal function (Egan et al. 2003; Miyajima et al. 2007) and may be associated with schizophrenia and mood disorders (Levinson 2006; Neves-Pereira et al. 2005; Neves-Pereira et al. 2002; Sklar et al. 2002; Szekeres et al. 2003).

Decreased hippocampal BDNF mRNA has been reported in animal models of schizophrenia and mood disorders (Angelucci et al. 2005; Ashe et al. 2002; Lipska et al. 2001). However, postmortem studies of hippocampal BDNF mRNA and protein in subjects with schizophrenia have yielded inconsistent results (Brouha et al. 1996; Chen et al. 2001; Durany et al. 2001; Iritani et al. 2003; Knable et al. 2004; Takahashi et al. 2000; Webster et al. 2004, personal communication). Reductions in hippocampal TrkB protein and mRNA have been reported in single studies (Iritani et al. 2003 and Takahashi et al. 2000, respectively), particularly in the hilus (Webster et al. 2004, personal communication). Inconclusive results have also been reported in postmortem studies of BDNF and TrkB in MDD and BPD subjects (Chen et al. 2001; Knable et al. 2004; Webster et al. 2004, personal communication). There have been no expression studies of (pro)BDNF or the p75 receptor in schizophrenia or mood disorders.

Using hippocampal tissue from four groups (controls, schizophrenia, MDD and BPD), (pro)BDNF, TrkB and p75 protein density was quantified. In addition, DNA extracted from frozen cerebellum was used to determine whether polymorphisms within each gene influenced hippocampal protein expression.
METHODS AND MATERIALS

**Postmortem Brain Samples**

Formalin-fixed paraffin-embedded anterior hippocampal sections (10 µm) and frozen (-80º C) blocks of cerebellum were obtained from the Stanley Foundation Neuropathology Consortium which consists of subjects with schizophrenia, MDD, BPD and age and gender matched control subjects (n = 15 per group; however two cases were missing from the control group, resulting in n of 13). Frozen hippocampus from two cases were also obtained from the Stanley Consortium for characterising the specificity of antibodies using Western blotting. Diagnoses were retrospectively established by two senior psychiatrists using DSM-IV criteria. Detailed clinical information and diagnostic procedures are provided elsewhere (Dowlatshahi et al 1999).

**Western blots**

Frozen hippocampus from two cases obtained from the Stanley Consortium was homogenised in tris-HCl-sucrose buffer with a protease inhibitor cocktail (Sigma, UK) and centrifuged at 4ºC at 13000 rpm for 10 minutes. For (pro)BDNF (1:200; sc-546, Santa Cruz) and TrkB (1:500; MAB397, R&D Systems) antibodies, proteins were separated on 14% and 7% SDS gel respectively under reducing conditions, and transferred to a PVDF membrane which was incubated with the diluted primary antibody overnight at 4ºC, then with anti-rabbit (BDNF 1:4000) or anti-mouse (TrkB 1:2000) IgG peroxidase-labelled secondary antibody. For the p75 antibody (1:67; MAB367, R&D Systems), proteins were separated on
an 11% SDS gel under non-reducing conditions, transferred to a PVDF membrane which was incubated with the primary antibody overnight at 4°C, and then with anti-mouse IgG peroxidase-labelled secondary antibody (1:1000 dilution). Prior to incubations, all membranes were treated with TTBS (pH 7.4) blocking buffer (10mM Tris, 0.9% NaCl, 1% Tween-20 and 5% milk powder) for 1 hour at room temperature. Detection was achieved through the enhanced chemiluminescent (ECL) method (Amersham Biosciences, UK).

**Immunoautoradiography**

Two brain sections per case and per antibody were used for immunoautoradiography as described previously (Toro and Deakin, 2005). Briefly, sections were deparaffinised in xylene and rehydrated in an ethanol series. Sections were microwaved in citrate buffer (pH 6.0) for 20 minutes for antigen retrieval. Non-specific binding was blocked using 10% normal donkey/sheep serum (1.5 hours). This was followed by application of the antibody diluted in 0.1M phosphate-buffered saline (PBS) with 1% normal donkey/sheep serum and 0.3% Triton X-100. Primary antibody incubations at the following dilutions: BDNF (1:50), TrkB (1:60) and p75 (1:10), were carried out for 20 hours at 4°C. Sections were then washed in 0.1M PBS (3x10 minutes) and incubated at room temperature with 0.1µCi/µl Sulphur-35-labelled secondary antibody (1:800; Amersham, UK) for 1.5 hours. Finally, sections were washed in 0.1M PBS, air-dried and exposed to autoradiographic film (Kodak Biomax MR) for 6 days. Negative control sections were obtained by omitting the primary antibody.

**Genotyping**
All 60 subjects were genotyped for several SNPs located within the BDNF, NTRK2 and NGFR genes (table 6). SNP selection was based on a combination of haplotype-tagging strategy and SNPs reported in previous schizophrenia, mood disorder and behavioural association studies (Adams et al 2005; Egan et al 2003; Haga et al 2002; Kunugi et al 2004; Levinson 2006; Miyajima et al, 2007; Neves-Pereira et al 2005; Neves-Pereira et al 2002; Sklar et al 2002; Strauss et al 2004; Szekeres et al 2003), and criteria including validation by frequency and location within the gene.

Genomic DNA was extracted from frozen cerebellum using a modified version of a previously described method (Freeman et al 2003) that included 17 hour incubation in 0.5mg/ml proteinase K (Fisher Scientific, UK). Genomic DNA was resuspended in TE (10mM Tris-HCL, 1mM EDTA, pH 8), normalized to 5-10ng/µl and stored at -20°C. Genotyping was performed using iPLEX™ chemistry on a MALDI-TOF Mass Spectrometer (Sequenom Inc., San Diego, CA, USA). PCR was performed with 5-10ng of genomic DNA, 1.63mM MgCl₂, 0.1U Hot Start Taq polymerase, Hot Start Taq PCR buffer (1.25X), 100nM forward and reverse PCR primers, 500µM dNTPs and nuclease-free water in a total volume of 5µl. PCR thermal cycling was performed on a Peltier thermal cycler (PTC–225, MJ Research) at 95°C for 15 minutes, (95°C for 20 seconds, 56°C for 30 seconds, and 72°C for 60 seconds) for 45 cycles, and finally 72°C for 3 minutes. Dephosphorylation of unincorporated dNTPs was achieved by adding 2µl Shrimp Alkaline Phosphatase and buffer (0.3µl SAP enzyme, 0.17µl hME buffer and nuclease-free water) to PCR products, and incubating at 37°C for 20 minutes, followed by inactivation for 5 minutes at 85°C. The primer extension PCR reaction was performed in a final 9µl volume extension reaction containing 7µl of the cleaned product, 0.2µl of iPLEX termination mix, 0.2µl iPLEX buffer, 0.8µl primer plex, 0.04µl iPLEX enzyme and 0.76µl nuclease-free water. Samples were
denatured at 94°C, strands annealed at 52°C for 5 seconds and extended at 80°C for 5 seconds. This was repeated four more times and then looped back to a 94°C denaturing step for 5 seconds, before entering the five cycle annealing and extension loop again. The five annealing and extension steps with the single denaturing step were repeated an additional 39 times. A final extension was done at 72°C for 3 minutes. The iPLEX reaction products were desalted and dispensed onto a 384-well SpectroChip (Sequenom Inc.), processed and analysed in a mass spectrometer by MassARRAY Workstation (version 3.3) software (Sequenom Inc).

**Method of analysis**

*Immunoautoradiography*

Immunoautoradiographic images on all films were scanned at a resolution of 1000 pixels per inch using an A3 flatbed scanner (Microtek Scan Maker 9600xl). Using Bioquant Nova Prime analysis software and blind to diagnosis, optical density measurements were taken from the molecular layer (ML), granule cell layer (GCL) and hilus of the dentate gyrus and from stratum oriens (SO), pyramidal layer (PL) and stratum radiatum (SR) from CA1 to CA3. This was done for each antibody. However, some subregions could not be measured due to occasional artefacts on the autoradiographic films which affected a maximum of four subregional measures for p75, seven subregions for (pro)BDNF and sixteen subregions for TrkB-T1. The tables show data for all measures taken. The statistical analysis and the figures showing statistical effects include only cases with measures in all subfields. Absolute density was determined using a standard curve generated from optical density measurements of $^{14}$C
plastic standards. Sections were allocated to one of 6 anterior-posterior (AP) levels on the basis of the conformation of the GCL.

Statistical analysis

Immunoautoradiography

Data analyses were performed with SPSS v14.0 software (SPSS, Chicago, Illinois). All values are mean ± standard error (SEM) density. Effect of postmortem interval (PMI), brain pH, age and months in formalin were assessed by Spearman’s correlations in every subregion and every diagnostic group prior to the main analysis. Variables showing significant correlations were included as covariates in the main analysis.

Repeated measures analysis of variance (RM ANOVA) was used to identify overall effects of diagnosis (between subjects) and its interaction with subregion (within subjects) and side (left v right). Significant main effects of diagnosis ($p \leq 0.1$ confirmed by univariate Huyhn-Feldt corrected $p < 0.05$) were investigated by planned post-hoc contrasts. Significant interactions of diagnosis with subregion were investigated using multivariate ANOVA of subregion. This allowed the effects of diagnosis to be examined separately in each subregion, with planned comparisons within a single analysis.

To detect lateralised changes, side was included as a between subjects factor (only one hemisphere was fixed from each brain) and was included in the analysis if there was a significant effect or interaction with diagnosis. The possible influence of anterior-posterior
(AP) level (of the anterior hippocampus sections) on protein expression was investigated by including it as a covariate.

Fluphenazine equivalents were used to determine effects of antipsychotic treatment. Correlations of protein density with antipsychotics, age at onset and duration of illness were evaluated by Spearman’s rank order correlation within and across diagnostic groups, excluding controls. Effects of antidepressants, lithium, suicide, side and sex, were evaluated by re-running the repeated measures ANOVA with an additional 2-level drug, suicide, side or sex grouping factor. Controls were excluded from drug and suicide analysis since none were medicated and none committed suicide. To reproduce the analyses of antidepressant effects of Chen et al (2001) and Webster et al (2004) we additionally compared: i) all subjects on antidepressants with those off ignoring diagnosis although this risked the confound that all controls would be in the antidepressant group; and ii) controls vs patients on and off antidepressants (ie 3 groups).

Genotyping

Chi-squared tests were performed for each SNP assay to test for deviation from Hardy-Weinberg equilibrium and genotypic comparison was carried out using the dominant effect model. Potential stratification problems due to unequal representation of ethnic groups were not relevant to our sample, as all but one subject were white Caucasian. Subjects homozygous and heterozygous for the minor allele were grouped together and compared with subjects that were homozygous for the major allele. Mean (±SEM) protein density per subregion was compared between the two groups. Differences in mean protein density associated with genotype, regardless of diagnosis, were analyzed using RM ANOVA to
avoid artefacts of multiple comparisons (SPSS v14.0). The between-subjects factor was genotype (2 levels). The within-subjects factor was subregion (5/6 levels). Only main effects of genotype are considered significant since this indicates the effect of genotype is independent of subregion. Although not strictly necessary, separate t-tests were carried out to confirm effects of genotype in individual subregions. For all SNPs, mean differences between genotype groups, with regards to covariates (PMI, months in formalin and brain pH), were analysed with t-tests.

RESULTS

Western Blots

For the BDNF antibody, a 35kDa band corresponds to the precursor form of BDNF - (proBDNF), and a 42kDa band may correspond to a trimeric form of mature BDNF (Spires et al 2004). We have signified this co-reactivity as (pro)BDNF. For the TrkB antibody, a 95kDa band corresponds to the truncated isoform - TrkB T1, and a 116kDa band may correspond to a modification of either of the receptor isoforms (Connor et al 1996). We have signified this co-reactivity as TrkB (T1). For the p75 antibody, a single band was observed at 75kDa which corresponds to the p75 receptor. No bands were observed when each of the antibodies was omitted (figure 1).

(Figure 1 here)

(Pro)BDNF, TrkB (T1) and p75 Immunoautoradiography
Immunoautoradiographs (figure 2) show that the p75 density pattern appears partially complementary to (pro)BDNF density pattern. Within the CA axonal and dendritic layers (SO and SR), (pro)BDNF density is relatively high and p75 density is relatively low. In the GCL and PL, (pro)BDNF density is relatively low and p75 density relatively high. (pro)BDNF and p75 density is similar in the hilus and ML. TrkB (T1) density is generally lower than both (pro)BDNF and p75, in agreement with previous reports of low levels in adult human hippocampus (Webster et al 2006).

Figure 2 here.

Table 1 here

**Effect of demographic and perimortem variables on protein density**

In some subregions, greater months in formalin were significantly associated with decreased (pro)BDNF density but it was not a significant covariate in the overall analysis. Results without co varying for months in formalin are presented. However, all significant results were also significant with formalin as a covariate. There were no significant effects of potential confounds on TrkB (T1) and p75 density.

**Effect of diagnosis on hippocampal (pro)BDNF density**

Table 2 shows (pro)BDNF density (mean ± SEM) in individual hippocampal subregions of all diagnostic groups. A general decrease in (pro)BDNF density can be seen in the mood disorder groups. However, the main effect of diagnosis was not significant because the effect
of diagnosis depended on hippocampal subregion and side of the sample (diagnosis x subregion x side interaction, $p = 0.005$ and $p = 0.03$ after covarying for time in formalin). The multivariate analysis showed the interaction was due to effects of diagnosis in SO, PL and SR (figure 3) and the absence of effects in the dentate gyrus (ML, GCL/hilus; see table 2). Diagnosis x side interactions were significant in SO ($p = 0.002$) and SR ($p = 0.02$) and they remained of statistical interest after covarying for time in formalin ($p = 0.01$ and 0.06 respectively). In both layers, the interaction arose principally from reduced (pro)BDNF in right hippocampus from MDD subjects and to a lesser extent from the BPD group (figure 3) as revealed by separate multivariate ANOVAs for right and left sides. No differences from controls were seen in any subregion in the left hippocampus but highly significant reductions were seen in the right hippocampus in all three CA layers from MDD subjects (all $p < 0.01$) and in right SO and SR of BPD subjects (both $p < 0.03$). Furthermore, within the MDD group, (pro)BDNF densities in the right hippocampus were markedly reduced compared to the left in all three CA layers (SO and SR $p = 0.001$ and PL $p = 0.11$; 2-tailed t-tests). No left-right comparison in the other groups approached significance.

More anterior sections had greater (pro)BDNF density across all layers than posterior sections (see table 3). However, anterior and posterior sections were evenly distributed across groups and including it as covariate did not affect the statistical significance.

*Table 2 here*

*Table 3 here*

*Figure 3 here*

*Figure 4 here.*
**Effect of diagnosis on hippocampal TrkB (T1) and p75 density**

Table 4 shows TrkB (T1) density (mean ± SEM) in individual subregions of all diagnostic groups. There was no main effect of diagnosis or AP level and no interactions of diagnosis with side or layer.

Table 4 here

Table 5 shows p75 density (mean ±SEM) in individual subregions of all diagnostic groups. In every subregion, BPD subjects had lowest densities whereas MDD subjects had slightly greater densities, except in the granule cell layer. The overall effect of diagnosis was of borderline statistical significance (p=0.1) with or without formalin as a covariate. This was due to lower p75 values in BPD compared to MDD (p=0.02), schizophrenia (p=0.09) and control groups (p=0.18). No other group differences approached statistical significance. This pattern was seen to a significantly greater effect (diagnosis by subregion interaction p=0.02, RM ANOVA) in SO (p=0.02) and to some extent in PL and SR, than in the dentate gyrus layers (p>0.1). In SO, PL and SR, p75 reductions in BPD compared to the MDD group were all statistically significant (table 5). Reductions in BPD compared to the schizophrenia group were significant in two of the three layers and reductions compared to controls were trend significant (p=0.1), also in two layers.

Table 5 here.

Figure 5 here

**Effect of drug treatment and suicide on protein expression**
No effect of suicide, antipsychotic, antidepressant or lithium treatment was observed on (pro)BDNF, TrkB (T1) and p75 density in the main analyses. In the further analyses of antidepressant effects on (pro)BDNF, there were no trends to significance in those on (n=20) and off (n=31) antidepressants in any subfield or overall. Compared to controls, patients on (n=20) and off (n=21) antidepressants both tended have lower (pro)BDNF levels (p=.04 and p=.09 respectively) with no trends to significance between the patients on and off antidepressants. Within the MDD group there were no trends to increases in (pro)BDNF in the treated depressives (n=6 off vs 8 on) in any subfield or overall.

**Effect of genotype on protein expression**

Allele and genotype frequencies and chi-squared and p-values for nine, eight and fourteen SNPs for BDNF, NGFR and NTRK2 respectively were calculated. A summary of these are available by request from the authors. (pro)BDNF density was reduced in those subjects who carried the minor allele of SNPs rs12273363 and rs7127507 (main effect of genotype, p = 0.05; figure 5). TrkB (T1) density was decreased in subjects who carried the minor allele of NTRK2 SNPs rs1187323 and rs1187326 (main effect of genotype, p = 0.05; figure 6a). Reductions were seen in every subregion (figure 6c & 6d). p75 density was increased in subjects who carried the minor allele of rs11466117 (main effect of genotype, p = 0.03; Figure 6a). Increases were seen in every subregion (figure 7b).

*Figure 6 here*
DISCUSSION

Diagnosis-Specific Changes

In MDD, reductions in (pro)BDNF density were seen in the right hippocampal CA layers compared with right-sided controls and also when compared with left-sided MDD subjects. BPD subjects showed a similar but less marked pattern of right-sided changes. BPD subjects also showed reductions in p75 receptor density across all subregions compared to the other groups. The findings raise the possibility that BPD and MDD both involve lateralised impaired (pro)BDNF expression but that BPD additionally involves impaired p75 expression.

(Pro)BDNF

The interpretation of any effects of diagnosis must be tempered by the probable co-reactivity of the BDNF antibody. Future studies using antibodies that can differentiate between (pro)BDNF and mature BDNF are thus necessary. There was a reduction in (pro)BDNF density in right-sided hippocampal CA sublayers in MDD and to a lesser extent in BPD. Reductions in the ML and GCL/hilus were not statistically significant, in contrast to a study of the Stanley Consortium database, in which a significant reduction was observed in the hilus in BPD (Knable et al 2004). However, two other studies of consortium brains found no differences in MDD or BPD using a quantitative immunohistochemical method (Chen et al 2001) and in-situ hybridisation (Webster et al 2004, personal communication). However, in the latter study, subjects on antidepressant drugs at the time of death had similar levels of BDNF to unaffected controls, whereas those off antidepressant drugs had levels significantly
lower than controls. The present postmortem finding and others are compatible with the hypothesis from the animal literature that BDNF is involved in the pathophysiology and treatment of mood disorder.

In schizophrenia, although mean (pro)BDNF densities were lower than controls in most subregions, they did not reach significance. This is consistent with two previous studies (Chen et al 2001; Webster et al 2004, personal communication), although Webster measured mRNA, not protein, and in CA subfields rather than layers. Reductions in hilus have been reported in schizophrenia (Knable et al 2004) but this was not seen in the present study, most likely due to the fact the antibody used in the present study was specific to (pro)BDNF and quantitative immunoautoradiography rather than immunohistochemistry was used. Further studies are necessary to clarify this discrepancy. Our results also fail to corroborate increases in BDNF reported previously (Iritani et al 2003; Takahashi et al 2000) using non-quantitative immunohistochemistry and immunoassay, respectively. The latter finding however, was not replicated using similar methodology (Durany et al 2001). In summary it would appear from postmortem studies that major changes in hippocampal BDNF expression are not found in schizophrenia.

The TrkB Receptor

The co-reactivity of the TrkB antibody used in this study warrants attention, as different functions have been postulated for the different TrkB isoforms (Baxter et al 1997; Biffo et al 1995; Eide et al 1996). No significant differences versus controls or between diagnostic groups were seen for TrkB(T1). It is possible however that there might be group differences in TrkB isoforms that would not be revealed by this antibody. A role for both the full-length
and truncated receptor in depression and its treatment is implicated by findings from animal and postmortem studies (Nibuya et al 1995; Saarelainen et al 2003; Webster et al 2004, personal communication). Although Webster reported no change in MDD (and a reduction in hilus in BPD), they found that subjects on antidepressants at the time of death had similar levels of TrkB to controls, whereas those off antidepressants had levels significantly lower than controls.

In schizophrenia, one study reported a 44% reduction of TrkB in hippocampal extracts (Takahashi et al 2000). Others have reported a qualitative lack of TrkB-stained fibres (Iritani et al 2003) and a reduction in TrkB mRNA in the hilus (Webster et al 2004, personal communication). However, the present study casts doubt on the generality of a 44% reduction in schizophrenia.

The p75 Receptor

In contrast to the several TrkB studies, there have been no previous postmortem studies of the p75 receptor in schizophrenia, MDD or BPD. Like BDNF and TrkB, it is expressed during development and in the adult brain and has a role in cell death/survival, axon elongation and synaptic plasticity (Dechant and Barde 2002). In the present study, p75 was reduced in BPD compared to other groups in all hippocampal layers but not in dentate. However, this only achieved full statistical significance in comparison to the MDD group and to the schizophrenia group in two layers. Reductions compared to controls were not far short of significance. Overall the results suggest that while impaired BDNF expression is common to both BPD and MDD, there may be additional involvement of the p75 receptor in BPD which is clearly not shared by the less severe illness, MDD. Evidence for a role of
(pro)BDNF in neuronal signalling was extended recently by the finding that (pro)BDNF is released by neurons (Yang et al, 2009). This finding warrants further exploration of (pro)BDNF and p75 in BPD.

**Effect of Laterality**

Interestingly, a meta-analysis of imaging studies of hippocampal volume in unipolar depression showed reductions in both left and right hippocampus, though reductions were greater in the right. Furthermore, the total number of depressive episodes was significantly correlated with right, but not left hippocampal volume reduction (Videbech and Ravnkilde 2004). Two studies report that the antidepressant citalopram attenuates amygdalo-hippocampal responses to fearful faces on the right side (Del-Ben et al 2005, Norbury et al 2007). A comprehensive meta-analysis of amygdala activations in humans found evidence for a role of the right amygdala in rapid processing of visual emotional stimuli (Cosatfreda et al 2007). Therefore, right lateralised changes in BDNF signalling may determine vulnerability to mood disorder or could reflect the effect of stress. This may be right lateralised in humans because of hemispheric specialisation in emotion processing.

**Drug Treatment**

No effect of antipsychotic treatment on protein density was seen. However, data for antipsychotic treatment is expressed as fluphenazine equivalents. There were too few subjects to determine whether atypical antipsychotic drugs might differ from typical as suggested by animal studies (Bai et al 2003; Bai et al 2002; Chlan-Fourney et al 2002; Li et al 1999). BDNF may also be a mediator of both antidepressant and mood stabilizer action.
Despite the interesting findings from animal studies, there are few human studies of antidepressant effects on BDNF (Chen et al 2001; Webster et al 2004, personal communication). We were not able to confirm the antidepressant-associated increases in BDNF seen in two studies despite studying the same brains and using the same respective analyses. However, as (pro)BDNF was measured in the present study, this is likely to contribute to the discrepancy.

**Effect of Genotype**

Five of the 31 SNPs examined were associated with changes in protein density across all hippocampal subregions. These SNPs are located primarily in 5′ UTR and intronic regions and may affect protein levels by modulating gene expression, alternative splicing and protein translation mechanisms. These SNPs warrant further study in a larger cohort.

**Conclusion**

This postmortem study suggests that impaired (pro)BDNF expression, especially in the right side, may be common to both MDD and BPD. The present findings further suggest BPD may involve an additional impairment of (pro)BDNF signalling through the p75 receptor. In BPD, persistent depression is a far greater problem than the typically infrequent episodes of mania. It is tempting to speculate that a combined impairment of (pro)BDNF and p75 receptor function in BPD relates to the chronicity of depression rather than the occurrence of mania, however further larger-scale studies are necessary to test this theory. This study also provides evidence of five novel functional SNPs within the BDNF, TrkB and p75 genes, which affected levels of their respective protein. Each of these SNPs may play a role in the
development of hippocampal abnormalities and may thus increase the risk of developing schizophrenia or mood disorders. Again, larger-scale studies are necessary to clarify the effects of BDNF, TrkB and p75 gene SNPs on BDNF and (pro)BDNF signalling.

REFERENCES


Toro CT, Deakin JFW (2005): NMDA receptor subunit NR1 and postsynaptic protein PSD-95 in hippocampus and orbitofrontal cortex in schizophrenia and mood disorder. *Schizophr Res* 80: 323–330.


Webster MJ, Kleinman, JE., Weickert, CS. BDNF and TrkBTK+ mRNA Levels in the Prefrontal Cortex and Hippocampus of Individuals with Depression, Bipolar Disorder and Schizophrenia (personal communication).

TITLES AND LEGENDS TO FIGURES

Figure 1 Title. Antibody Specificity

Figure 1 Legend. Western blots showing the specificities of the neurotrophin/receptor antibodies. The blots were incubated with (A) anti-BDNF, (B) anti-trkB, and (C) anti-p75; Negative control blots (i.e. without the respective antibodies) showed no signal (not shown).

Figure 2 Title. Immunoautoradiographs

Figure 2 Legend. Representative immunoautoradiographic images of anterior hippocampal sections from all three studies showing density pattern of A. (pro)BDNF, B. p75 and C. TrkB (T1). Bottom row shows respective negative control sections (i.e. omission of primary antibody).

Figure 3 Title. Effects of diagnosis, subfield and side for (pro)BDNF

Figure 3 Legend. (pro)BDNF density (mean ± SEM) in individual hippocampal subregions, left and right sides in all diagnostic groups ** p<0.01, * p<0.05 compared to controls (from multivariate analysis of right side samples). + p<0.01 right v left side in major depressives group. C – Controls; S - Schizophrenia; D - Major Depressive Disorder; B - Bipolar Disorder.

Figure 4 Title. p75 density in Stratum Oriens showing bilateral change in bipolar disorder
**Figure 4 Legend.** * p < 0.01 B = Bipolars vs D = depressed group; p < 0.05 vs S = schizophrenia group; p = 0.07 vs C = controls, bilateral contrasts from multivariate analysis.

**Figure 5 Title.** Influence of Genotype on Hippocampal Brain Derived Neurotrophic Factor Density.

**Figure 5 Legend.** BDNF density (mean ± SEM) in all hippocampal subregions together of subjects that are homozygous for the major allele versus subjects that are homo/heterozygous for the minor allele of BDNF SNP rs12273363 and rs712507 [ * p < 0.05 (Repeated measures ANOVA)].

**Figure 6 Title.** Influence of NTRK2 and NGFR Genotype on hippocampal TrkB and p75 density.

**Figure 6 Legend.** (a) Receptor density (mean ± SEM) in all hippocampal regions of interest together of subjects that are homozygous for the major allele versus subjects that are homo/heterozygous for the minor allele of NGFR SNP rs11466117, and NTRK2 SNPs rs1187323 and rs1187326 [ * p < 0.05 (Repeated measures ANOVA)]. (b-d) Receptor density (mean ± SEM) in individual hippocampal regions of subjects homozygous for the major allele versus subjects homo/heterozygous for the minor allele of b) NGFR SNP rs11466117; c) NTRK2 SNP rs1187323; and d) NTRK2 SNP rs1187326. [** p < 0.01; * p < 0.05 (T-tests)]. ML - Molecular Layer; GCL - Granule Cell Layer; SO - Stratum Oriens; PL - Pyramidal Layer; SR - Stratum Radiatum.
Figure 1
Figure 2.

<table>
<thead>
<tr>
<th>a</th>
<th>b</th>
<th>c</th>
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</thead>
<tbody>
<tr>
<td>![Image A]</td>
<td>![Image B]</td>
<td>![Image C]</td>
</tr>
<tr>
<td>![Image D]</td>
<td>![Image E]</td>
<td>![Image F]</td>
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<td>![Image G]</td>
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<tr>
<td>![Image J]</td>
<td>![Image K]</td>
<td>![Image L]</td>
</tr>
</tbody>
</table>

*Note: Images A to L are not visible in the description.*
Figure 4

P75 density

nCi/g

C S D B C S D B
Left Right
S. Oriens
Figure 5

Rs12273363

Homozygous (Major allele)  
Homo/heterozygous (Minor allele)

Rs7127507

(pro)BDNF protein density (nCi/g)
Main effects of NTRK2 and NGFR genotypes

NGFR (rs11466117)

NTRK2 (rs1187323)

NTRK2 (rs1187326)
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<tr>
<th></th>
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<th>Bipolar Disorder</th>
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<td>N</td>
<td>13</td>
<td>15</td>
<td>15</td>
<td>15</td>
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<tr>
<td>Gender (F/M)</td>
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<td>6/9</td>
<td>5/10</td>
<td>6/9</td>
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<tr>
<td>Age</td>
<td>47.9 (±10.3)</td>
<td>44.5 (±13.1)</td>
<td>46.5 (± 9.3)</td>
<td>42.3 (±11.7)</td>
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<td>Age Onset</td>
<td>n/a</td>
<td>23.2 (± 8.0)</td>
<td>33.9 (±13.3)</td>
<td>21.5 (± 8.4)</td>
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<td>Duration of Illness (yrs)</td>
<td>n/a</td>
<td>21.3 (±11.4)</td>
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<td>20.1 (± 9.7)</td>
</tr>
<tr>
<td>PMI (hrs)</td>
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<td>33.7 (±14.6)</td>
<td>27.5 (±10.7)</td>
<td>32.5 (±16.1)</td>
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<td>6.2 (± 0.3)</td>
<td>6.2 (± 0.2)</td>
<td>6.2(± 0.2)</td>
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<tr>
<td>MthForm</td>
<td>4.2 (± 3.8)</td>
<td>11.2 (± 8.5) †</td>
<td>8.4 (± 6.6) †</td>
<td>9.7 (± 3.6) †</td>
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<tr>
<td>Side (L/R)</td>
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<td>9/6</td>
<td>9/6</td>
<td>7/8</td>
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<tr>
<td>Suicide</td>
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<td>4</td>
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<td>Antidepressants</td>
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<td>Antipsychotics</td>
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<td>Lithium</td>
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<td>2</td>
<td>2</td>
<td>4</td>
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</table>

† - different from controls, planned contrast p<0.05. PMI - Postmortem Interval; MthForm - Months in Formalin; F - Female; M - Male; L - Left; R - Right; Integers are numbers of cases.

Table 1. Clinical and demographical data of Stanley Foundation Neuropathology Consortium (mean ±SEM).
Table 2. Mean (± SEM) (pro)BDNF density (nCi/g/tissue) in individual hippocampal subregions of all 4 groups.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Control</th>
<th>N</th>
<th>SCZ</th>
<th>N</th>
<th>MDD</th>
<th>N</th>
<th>BPD</th>
<th>N</th>
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<td>234.0±29.3</td>
<td>10</td>
<td>210.3±26.7</td>
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<td>191.8±24.8</td>
<td>14</td>
<td>199.5±23.9</td>
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<tr>
<td>GCL-HIL</td>
<td>337.3±29.7</td>
<td>10</td>
<td>290.9±27.2</td>
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<td>305.8±25.1</td>
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<td>277.3±24.3</td>
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<tr>
<td>SO</td>
<td>627.6±31.0</td>
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<td>532.7±27.5</td>
<td>14</td>
<td>539.4±27.5</td>
<td>14</td>
<td>533.7±26.5</td>
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<tr>
<td>SP</td>
<td>296.2±30.6</td>
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<td>236.8±28.2</td>
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<td>183.9±27.1</td>
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<td>229.1±26.2</td>
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<tr>
<td>SR</td>
<td>461.6±32.4</td>
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<td>357.5±28.7</td>
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<td>405.4±28.7</td>
<td>14</td>
<td>369.1±27.7</td>
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<sup>a</sup><sup>b</sup><sup>c</sup> significance of main effect of diagnosis in each subregion and of planned contrasts vs control: <sup>a</sup> p ≤ 0.01, <sup>b</sup> p ≤ 0.05, <sup>c</sup> p ≤ 0.1 from multivariate analysis. SCZ - Schizophrenia; MDD - Major Depressive Disorder; BPD - Bipolar Disorder; N - Number; ML - Molecular Layer; GCL-HIL - Granule Cell Layer - Hilus; SO - Stratum Oriens; SP – Stratum Pyramidale; SR - Stratum Radiatum.
Table 3. Mean (±SEM) Protein Density in Anterior and Posterior Hippocampal Sections

<table>
<thead>
<tr>
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<th>Anterior</th>
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<th>Posterior</th>
<th>N</th>
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<tbody>
<tr>
<td>(pro)BDNF*</td>
<td>366.3±17.1</td>
<td>26</td>
<td>317.1±17.1</td>
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<tr>
<td>TrkB (T1)</td>
<td>73.2±5.5</td>
<td>22</td>
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<tr>
<td>P75</td>
<td>368.6±13.9</td>
<td>28</td>
<td>382.8±14.4</td>
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</table>

* Significant difference - p < 0.05 (Repeated Measures ANOVA). N - Number of Cases.
Table 4. Mean (± SEM) TrkB (T1) density (nCi/g/tissue) in individual hippocampal subregions of all 4 groups.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Control</th>
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<th>SCZ</th>
<th>N</th>
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<th>N</th>
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<tr>
<td>ML</td>
<td>51.1±8.8</td>
<td>9</td>
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<td>50.8±6.4</td>
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<td>GCL</td>
<td>67.1±10.4</td>
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<tr>
<td>HIL</td>
<td>54.2±10.5</td>
<td>10</td>
<td>66.3±6.0</td>
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<td>59.5±6.9</td>
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<td>71.8±8.2</td>
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<tr>
<td>SO</td>
<td>102.3±8.7</td>
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<td>110.7±11.7</td>
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<td>99.1±10.7</td>
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<td>109.9±10.9</td>
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<tr>
<td>SP</td>
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<td>53.2±7.7</td>
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<td>63.2±10.0</td>
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<tr>
<td>SR</td>
<td>67.2±7.6</td>
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<td>73.2±9.2</td>
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<td>62.9±7.5</td>
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<td>70.0±8.4</td>
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</table>

SCZ - Schizophrenia; MDD - Major Depressive Disorder; BPD - Bipolar Disorder; N - Number; ML - Molecular Layer; GCL - Granule Cell Layer; Hil - Hilus; SO - Stratum Oriens; SP – Stratum Pyramidale; SR - Stratum Radiatum.
Table 5. Mean (+ SEM) p75 density (nCi/g/tissue) in individual hippocampal subregions of all 4 groups.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Control</th>
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<th>N</th>
<th>MDD</th>
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<tbody>
<tr>
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<td>339.3±18.9</td>
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<td>360.4±18.2</td>
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<td>307.2±18.2</td>
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<tr>
<td>GCL</td>
<td>435.7±22.5</td>
<td>11</td>
<td>453.1±19.4</td>
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<tr>
<td>HIL</td>
<td>346.3±20.7</td>
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<td>356.3±18.3</td>
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<td>372.9±17.7</td>
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<tr>
<td>SO$^b$</td>
<td>426.5±28.4</td>
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<td>424.7±24.0</td>
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<td>467.6±23.2</td>
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<tr>
<td>SP$^c$</td>
<td>378.1±23.9</td>
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<td>403.0±19.5</td>
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<td>341.1±19.5$^{df}$</td>
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</tr>
<tr>
<td>SR$^c$</td>
<td>352.5±24.4</td>
<td>10</td>
<td>337.4±20.6</td>
<td>14</td>
<td>362.2±19.9</td>
<td>15</td>
<td>298.4±19.9$^{ce}$</td>
<td>15</td>
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</tbody>
</table>

$^b,c$ significance levels from multivariate analysis of effect of diagnosis in each layer and of planned contrasts within layers: versus controls- $^b$ p ≤ 0.05, $^c$ p ≤ 0.1; versus MDD- $^d$ p ≤ 0.01, $^e$ p ≤ 0.05; and versus schizophrenia- $^f$ p ≤ 0.05. SCZ - Schizophrenia; MDD - Major Depressive Disorder; BPD - Bipolar Disorder; N - Number; ML - Molecular Layer; GCL - Granule Cell Layer; HIL - Hilus; SO - Stratum Orien; SP - Stratum Pyramidale; SR - Stratum Radiatum.