

# The BDNF val66met Polymorphism Affects Activity-Dependent Secretion of BDNF and Human Memory and Hippocampal Function

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## Summary

Brain-derived neurotrophic factor (BDNF) modulates hippocampal plasticity and hippocampal-dependent memory in cell models and in animals. We examined the effects of a valine (val) to methionine (met) substitution in the 5' pro-region of the human BDNF protein. In human subjects, the met allele was associated with poorer episodic memory, abnormal hippocampal activation assayed with fMRI, and lower hippocampal n-acetyl aspartate (NAA), assayed with MRI spectroscopy. Neurons transfected with met-BDNF-GFP showed lower depolarization-induced secretion, while constitutive secretion was unchanged. Furthermore, met-BDNF-GFP failed to localize to secretory granules or synapses. These results demonstrate a role for BDNF

and its val/met polymorphism in human memory and hippocampal function and suggest val/met exerts these effects by impacting intracellular trafficking and activity-dependent secretion of BDNF.

## Introduction

A remarkable feature of the human brain is its ability to store and recall a seemingly endless series of experiences many of which occur only once. Clinical studies suggest that this capacity, referred to as episodic memory, is critically dependent on the hippocampus and related mesial temporal lobe structures (Milner et al., 1998). Molecular studies of hippocampal synapses have revealed that memory-related modifications in synaptic transmission occur in stages. The first stage, known as early phase long-term potentiation (E-LTP), involves rapid increases of intracellular calcium concentrations and subsequent activation of protein kinases (Bliss and Collingridge, 1993). A second stage, referred to as late phase LTP (L-LTP), recruits the cAMP and CREB signaling pathway to direct protein synthesis-dependent changes in structure and function of hippocampal synapses (Kandel, 2001). One protein in particular, brain derived neurotrophic factor (BDNF), appears to play an important role in both (Lu and Gottschalk, 2000; Poo, 2001).

Evidence that BDNF is involved in hippocampal LTP, learning, and memory in nonhuman species is substantial (Lu and Gottschalk, 2000; Poo, 2001). BDNF gene expression is markedly enhanced by tetanic stimulation that induces LTP (Patterson et al., 1992) and during spatial memory tasks (Hall et al., 2000; Mizuno et al., 2000). BDNF application facilitates LTP while reduction of BDNF levels attenuates LTP (Figurov et al., 1996; Korte et al., 1995; Patterson et al., 1996). Studies using a variety of approaches demonstrate that BDNF enhances high frequency synaptic transmission by facilitating synaptic vesicle docking (Gottschalk et al., 1998, 1999; Jovanovic et al., 2000; Pozzo-Miller et al., 1999; Xu et al., 2000). BDNF also elicits rapid postsynaptic effects on ion channels and NMDA receptors (Levine et al., 1995). In addition to acute modulation of synaptic transmission and E-LTP, recent studies have demonstrated a role of BDNF in long-term changes in hippocampal synapses and L-LTP (Korte et al., 1998; Tartaglia et al., 2001). Inhibition of BDNF signaling in rodents by gene knockout or infusion of antisense BDNF impairs spatial learning and memory (Linnarsson et al., 1997; Minichiello et al., 1999; Mizuno et al., 2000). Despite substantial progress in animal studies, BDNF's relevance in human memory and hippocampal function has not been examined directly.

Unlike other growth factors, which are secreted primarily via a constitutive pathway, at least in hippocampal neurons, BDNF appears to be sorted into a regulated pathway that secretes BDNF in response to neuronal activity (Farhadi et al., 2000; Goodman et al., 1996; Mowla et al., 2001). Transfection experiments using BDNF-GFP (green fluorescence protein) fusion con-

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structs demonstrate that BDNF is packaged into secretory vesicles, which are transported to somatodendritic compartments (Kojima et al., 2001). Activity-dependent BDNF secretion from postsynaptic dendrites may serve as a retrograde or paracrine messenger that regulates hippocampal LTP (Poo, 2001). Indeed, electric stimulation of hippocampal neurons elicits BDNF-GFP secretion from dendrites (Hartmann et al., 2001). Taken together, these results strongly argue for a critical role of activity-dependent secretion of BDNF in hippocampus-based synaptic plasticity and learning and memory.

Based on these experimental data, one might predict that genetic interference of BDNF secretion would lead to deficits in hippocampal function, learning, and memory. The BDNF gene, like other peptide growth factors, encodes a precursor peptide (proBDNF), which is proteolytically cleaved to form the mature protein (Seidah et al., 1996). Only one frequent, nonconservative polymorphism in the human BDNF gene (dbSNP number rs6265) has been identified, a single nucleotide polymorphism (SNP) at nucleotide 196 (G/A) producing an amino acid substitution (valine to methionine) at codon 66 (val66met). We hypothesized that this SNP, though located in the 5' pro-BDNF sequence, and thus unlikely to alter the intrinsic biological activity of the mature protein, would affect intracellular processing and secretion of BDNF, leading to impairments in hippocampal function in humans. Because hippocampal dysfunction is associated with genetic risk for schizophrenia (Callicott et al., 1998; Egan et al., 2001a), we also hypothesized that BDNF genotype might be associated with genetic risk for schizophrenia. To test these hypotheses, we examined first the effects of the BDNF val66met substitution in a cohort of normal controls, patients with schizophrenia and their unaffected siblings, using three *in vivo* assays of human hippocampal function: (1) cognitive measures of episodic memory; (2) a neurophysiological measure of hippocampal activation during a memory task using fMRI; and (3) an intracellular neurochemical measure of hippocampal neuronal integrity and synaptic abundance assayed with proton magnetic resonance spectroscopic imaging (1H-MRSI). To investigate underlying molecular mechanisms, we transfected rodent hippocampal neurons with val- or met-BDNF fused with GFP and examined trafficking of val- and met-BDNF proteins under fluorescence microscopy. We also determined constitutive and regulated (i.e., depolarization-induced) BDNF secretion in these neuronal cultures. Our analyses revealed striking deficits in the cellular distribution and regulated secretion of BDNF caused by this SNP and corresponding alterations of human hippocampal function *in vivo*. While we found no relationship between BDNF genotype and schizophrenia, our results demonstrate that BDNF plays a role in hippocampal function and episodic memory in humans and provide insights into the mechanisms for intracellular trafficking and secretion of BDNF.

## Results

### Effect of BDNF Genotype on Episodic Memory

In the first experiment, we examined the effects of BDNF genotype on measures of episodic memory in a cohort

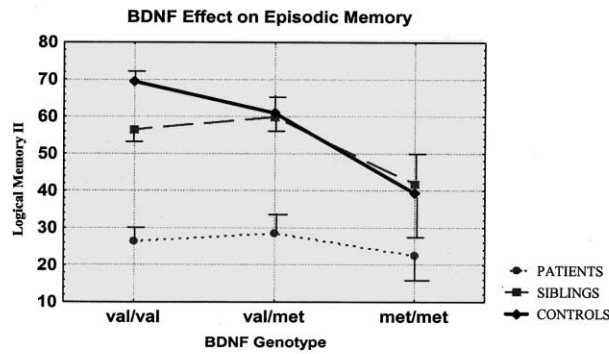
of 641 subjects, including normal controls, patients with schizophrenia, and their unaffected siblings, using measures from the Wechsler Memory Scale, revised version (WMS-R), a test of verbal episodic memory. Delayed recall scores from the WMS-R reflect the amount of information from two stories with 50 total elements that subjects are able to recall following a 0.5 hr delay. Patients with schizophrenia had substantially lower scores compared to controls, while siblings were intermediate between these groups, consistent with earlier reports (Egan et al., 2001b; Figure 1A). In the entire sample, BDNF genotype had a significant effect on these memory scores ( $F = 3.89$ ,  $df = 2$ ,  $591$ ,  $p = .02$ ). In the group of 133 controls alone, BDNF genotype also had a significant effect on memory scores ( $F = 5.04$ ,  $df = 2$ ,  $130$ ,  $p = .008$ ). While including patient and sibling groups did not substantially add to the results, both groups showed the same effect seen in controls with met/met subjects tending to score lower than other genotype groups (Figure 1A). Post hoc comparisons in the normal subjects alone showed that met/met homozygotes had lower scores compared to val/val ( $p < .005$ ) and val/met ( $p = .052$ ), while in the entire cohort, met/met homozygotes had lower scores compared to the other two genotype groups ( $p = .007$ ). Within each group (controls, siblings, patients), each genotype group was well matched on a variety of demographic parameters (see Figure 1B), suggesting they did not account for the effect of BDNF genotype. Similar effects of BDNF genotype were seen with immediate recall scores (of the stories described above) from the WMS-R (e.g., normal group,  $F = 3.65$ ,  $df = 2$ ,  $130$ ,  $p < .03$ ). Post hoc comparisons again showed that met/met homozygotes had lower scores compared to val/val ( $p < .01$ ) and val/met ( $p = .07$ ) groups.

BDNF genotype had no significant effect in either the normal subjects or the entire cohort on a second memory test which required recall of word lists, the California Verbal Learning Test (CVLT), immediately after hearing the list (1–5 summary scores,  $F = 0.79$ ,  $df = 2$ ,  $560$ ;  $p = .45$ ; post hoc comparisons  $p > .14$ ) and again 20 min later (long delay free recall scores,  $F = 0.23$ ,  $df = 2$ ,  $560$ ;  $p = .79$ ; post hoc comparisons  $p > .37$ ). The CVLT may have a larger “prefrontal component” (Kopelman et al., 1998) compared to the WMS-R, possibly reducing hippocampal and val66met related variance. No effect of BDNF genotype was seen on other types of memory, such as semantic memory ( $F = 0.02$ ,  $df = 2$ ,  $591$ ;  $p = .98$ ) or working memory/executive function (WCST,  $F = 0.33$ ,  $df = 2$ ,  $590$ ;  $p = .71$ ) (Figure 1B). These results suggest that the val66met polymorphism exerts its most robust effects on episodic memory.

### Effect of BDNF Genotype on Hippocampal Activation

To further characterize the effects of BDNF genotype, we performed an *in vivo* assay of hippocampal physiology using the blood oxygenation level dependent (BOLD) fMRI technique (Ogawa et al., 1992) in subjects performing the N-back working memory task. The brain network subserving the N-back task (which is not a test of episodic memory *per se*) is thought to involve primarily neocortical regions, particularly dorsolateral

A



B

Genotype Group (number of subjects)	Patients			Siblings			Controls		
	Val/val (138)	Val/met (56)	Met/met (9)	Val/val (206)	Val/met (85)	Met/met (14)	Val/val (91)	Val/met (36)	Met/met (6)
Age	36.8 (9.2)	35.7 (8.6)	32.5 (10.2)	37.2 (8.8)	35.8 (8.9)	34.7 (8.8)	35.0 (9.7)	34.1 (8.9)	39.3 (8.4)
Gender (M/F)	109/29	45/11	7/2	91/115	31/54	7/7	43/48 <sup>a</sup>	26/10 <sup>a</sup>	4/2
Education years	13.8 (2.2)	13.9 (2.6)	13.9 (2.8)	15.8 <sup>a</sup> (2.5)	15.1 <sup>a</sup> (2.4)	15.3 (2.6)	16.2 (2.4)	16.7 (3.2)	17.7 (4.3)
Reading Comprehension	101.3 (12.3)	100.9 (12.7)	105.1 (10.0)	106.5 (11.2)	104.7 (11.8)	104.5 (11.2)	106.3 (10.2)	106.2 (10.2)	104.7 (12.6)
IQ	92.9 (12.6)	91.1 (13.1)	92.3 (13.5)	106.2 (13.1)	106.1 (13.1)	104.7 (13.1)	107.6 (10.7)	109.1 (10.2)	105.7 (12.6)
Semantic Memory	33.5 (11.8)	33.9 (11.5)	33.2 (14.1)	42.1 (11.1)	39.2 (11.3)	41.7 (10.5)	44.11 (9.2)	48.0 (9.8)	44.3 (10.5)
Working Memory	37.0 (12.0)	37.4 (12.8)	42.0 (14.9)	44.0 (9.5)	44.7 (10.6)	43.4 (7.3)	47.3 (9.5)	46.1 (10.3)	40.1 (8.1)

Figure 1. Effect of BDNF val66met Genotype on Episodic Memory

(A) BDNF val66met genotype and episodic memory scores ( $\pm$  SE) from the WMS-R (delayed recall) in three samples studied. (B) Demographic and cognitive data by genotype. Means ( $\pm$  SD) are presented. Genotype groups are well matched except for <sup>a</sup> $p < .05$ , val/val versus val/met.

prefrontal cortex (Callicott et al., 1999). However, these tasks also produce a robust and reliable deactivation or disengagement of the hippocampus (Meyer-Lindenberg et al., 2001). Hippocampal deactivation has been reported to be disrupted in clinical pathological states (Callicott et al., 2000). We hypothesized, therefore, that the BDNF met allele would disrupt the normal hippocampal fMRI disengagement pattern during performance of the N-back working memory task.

We studied two independent cohorts of healthy subjects (patients were not included), each comprised of val/met and val/val genotype subgroups; no met/met subjects were included due to their low population frequency (< 5%). The members of the cohorts were selected so that N-back performance, age, and gender did not differ between the genotype subgroups. In the first cohort, val/met individuals had an abnormal pattern of increased activation of bilateral caudal hippocampus. In contrast, val/val subjects showed the characteristic hippocampal deactivation pattern. Directly comparing val/val and val/met subjects showed this difference to be statistically significant (see Figure 2). More specifically, restricting the analysis to the medial temporal lobes revealed two clusters of activation showing the inappro-

prate overactivation of val/met subjects in bilateral hippocampus that were significant at the set level ( $p < 0.05$ ,  $c = 2$ ) (Figure 2A). To replicate this unusual finding, we examined a second cohort of healthy subjects (patients not included). Val/met subjects again showed an abnormal pattern of increased bilateral hippocampal activation compared to baseline while val/val subjects again showed deactivation. Direct comparison of the two genotype groups again showed significant differences in hippocampal locales (Figure 2B).

Because these healthy subjects were matched prior to the experiment on task performance to remove this as a potential confounder, it cannot account for these findings (Figure 2C). Within each cohort, genotype groups were also matched on demographic variables, suggesting they did not account for the BDNF effects. One exception was a slight difference in full scale IQ and WMS-R scores in the second cohort (Figure 2C). However, adding IQ as a covariate again revealed significant bilateral clusters of differential caudal hippocampal activation. To ensure the differences between groups were not due to uneven sample sizes, all analyses were also performed using subsets with the same number of subjects in each genotype group. The results were

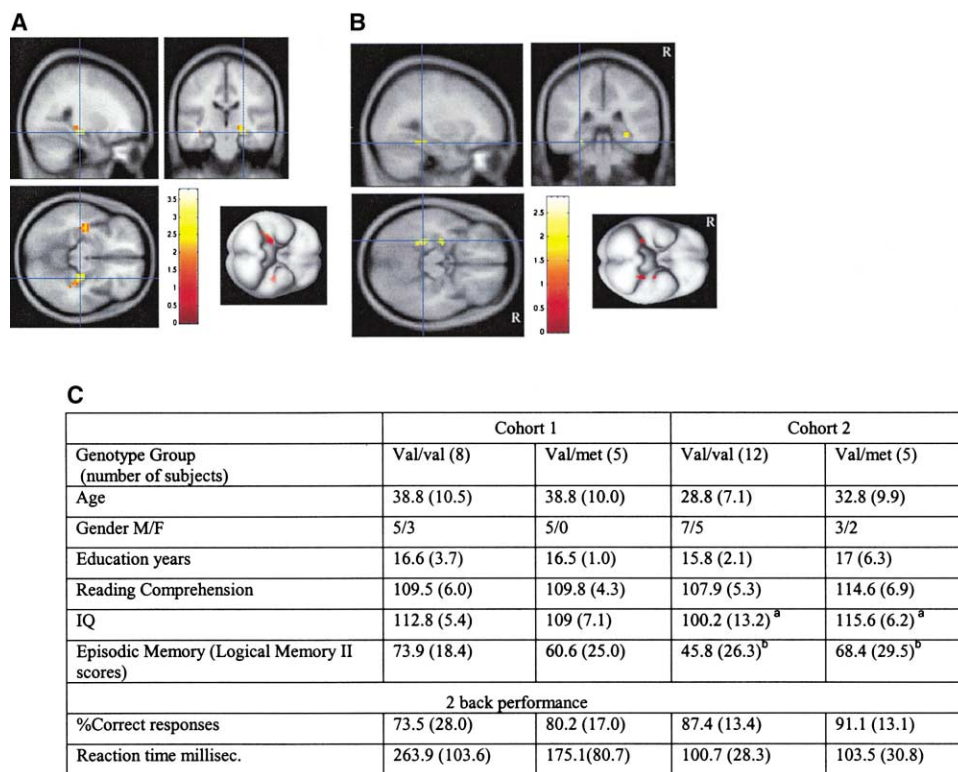


Figure 2. Effect of BDNF val66met Genotype on In Vivo Hippocampal fMRI Response

(A) Brain map showing locales where BDNF genotype groups differed in blood oxygenation, an indirect measure of neuronal activity, measured with fMRI during a working memory task. Regions marked in red are groups of voxels ("activation clusters") where subjects with the val/met genotype showed abnormal hippocampal activation and were significantly different when compared with val/val subjects (see Experimental Procedures for statistical model). The statistical results are rendered on a canonical averaged T1 brain image and localized according to the standard 3D stereotaxic space of Talairach and Tournoux. The maximally activated voxels are: right hippocampus ( $t = 3.77$ ,  $p < 0.01$ , cluster size ( $k$ ) = 25, 3D coordinates: 26 -22 -12); left hippocampus ( $t = 2.39$ ,  $p = 0.02$ ,  $k = 10$ , -38 -15 -12). Inset: these activation clusters are rendered on a canonical averaged smoothed 3D rendered brain. Color bar = T values. R = right hemisphere.

(B) BDNF genotype effect on hippocampal fMRI response in a second independent cohort. The activation clusters are those areas where BDNF val/met subjects again showed abnormal activation of bilateral hippocampi locales during the 2 back working memory task and were significantly different compared to val/val subjects. fMRI data rendered in the same manner as in (A) ( $p < 0.05$ , cluster size  $> 8$ ). Two clusters were identified in the left hippocampus (-30 -35 -16,  $t = 2.49$ ,  $p < 0.01$ ,  $k = 40$  and -30 -14 -13,  $t = 2.45$ ,  $p = 0.01$ ,  $k = 13$ ) and one in the right hippocampus (28 -31 -2,  $t = 2.55$ ,  $p = 0.01$ ,  $k = 37$ ).

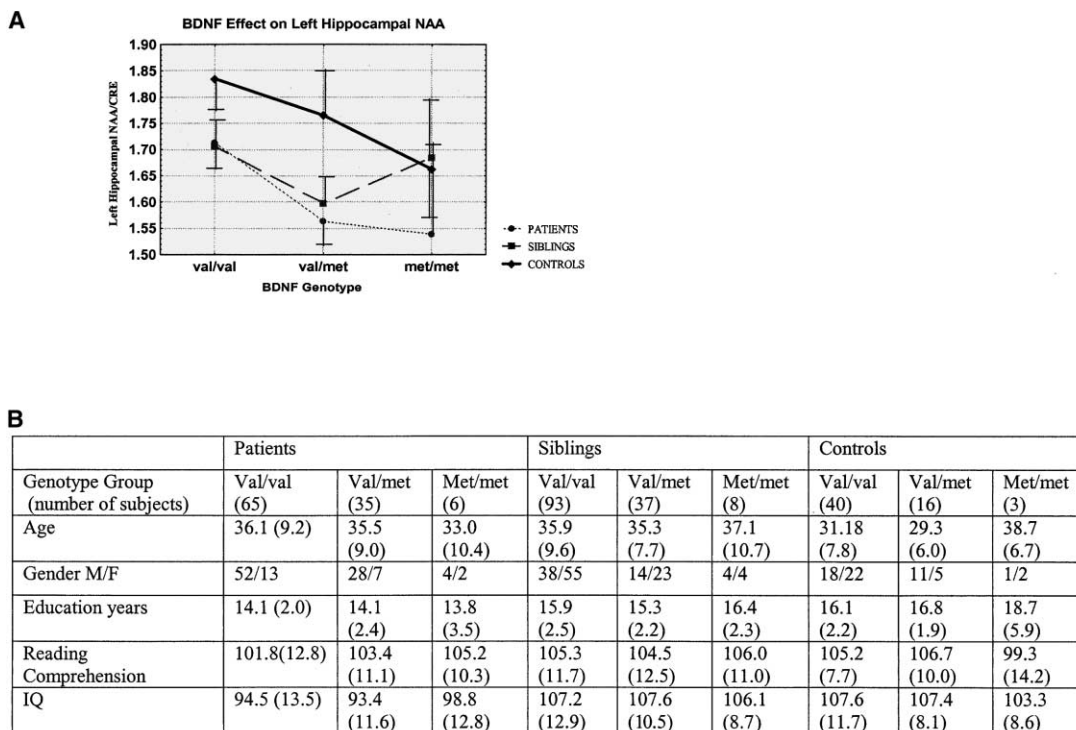
(C) Demographic and cognitive data for cohorts from (A) and (B) by genotype. Means ( $\pm$  SD) are presented. Genotype groups are well matched except for <sup>a</sup> $p < .05$  and <sup>b</sup> $p = .07$  (covariance analyses revealed no effects of these differences on hippocampal activation patterns).

essentially identical. Scatter plots did not reveal outliers. In summary, in two cohorts we found abnormal activation of hippocampus in val/met subjects compared to the normal pattern of deactivation in val/val subjects. Although this was seen in the context of a working memory task, which is typically more dependent on prefrontal function, and although the mechanism by which val66met might produce this effect is uncertain, the data indicate that the BDNF met allele relative to val is associated with a qualitatively altered hippocampal response.

#### Effects of BDNF Genotype on In Vivo n-acetyl-aspartate Measures

We next examined the effects of val66met on an in vivo measure of neuronal/synaptic activity in the human hippocampal formation. NAA is an intracellular marker of neuronal function, related to mitochondrial oxidative phosphorylation (Jenkins et al., 2000) and appears to be an indirect measure of neuronal integrity and synaptic

abundance (Maier et al., 1995). Patients and siblings both had lower NAA measures compared to controls, consistent with earlier studies (Callicott et al., 1998) (Figure 3A). A significant effect of BDNF val66met genotype was found in the entire cohort ( $F = 3.19$ ,  $df = 2$ , 280,  $p = .04$ ), accounting for 1.9% of the overall variance (Figure 3A). Post hoc comparisons indicated that heterozygotes (val/met) had lower NAA levels compared to val/val subjects ( $p < .02$ ). Using multiple regression analysis, a significant linear reduction in NAA levels with increasing number of met alleles was observed, indicating a possible allele dose effect ( $t(299) = -2.29$ ,  $p = .02$ ). BDNF genotype did not significantly affect right hippocampal NAA measures, although, again individuals with the met allele tended to have lower NAA signals (val/val versus val/met,  $t = 1.38$ ,  $p = .17$ ). ROI analyses of other brain regions (see Experimental Procedures) showed no effect of BDNF genotype on NAA measures (data not shown). Within each genotype group, subjects were well matched on demographic parameters (Figure



**Figure 3.** Effect of BDNF val66met Genotype on In Vivo Hippocampal NAA Measures  
**(A)** BDNF val66met genotype and NAA measures in the left hippocampal formation ( $\pm$  standard error, SE) in three samples studied.  
**(B)** Demographic data (means  $\pm$  SD) by genotype. Groups are well matched on all variables.

3B), suggesting they do not account for the effect of BDNF genotype. The results were similar in magnitude (i.e., effect size) but statistically slightly weaker ( $F = 2.35$ ,  $df = 2,254$ ,  $p = .097$ ; val/val versus val/met  $p = .04$ ) in more restricted analyses of one ethnic group alone (European Americans). Taken together, these results suggest that the val66met polymorphism elicits a specific reduction in hippocampal neuronal integrity/synaptic activity.

#### Val- and Met-BDNF Expression in Cultured Hippocampal Neurons

To understand how val66met elicits the in vivo phenotypes, we examined its impact on intracellular distribution, processing, and secretion of BDNF. We expressed constructs containing either vBDNF or mBDNF, followed by the green fluorescence protein (GFP), in cultured neurons using the sindbis virus expression system (Figure 4A). This approach allowed analysis of the expression, processing, biological activity, and release properties of the BDNF isoforms and to visualize their distribution in living neurons (Hartmann et al., 2001; Kojima et al., 2001; Mowla et al., 1999). The viral titer was controlled to 10% to avoid problems associated with overexpression of exogenous proteins (Figure 6A). Fluorescence microscopy revealed that vBDNF-GFP was localized to the cell body and distal processes (Figure 4B). Double staining using an antibody against the dendritic marker MAP2 showed that vBDNF-GFP fluorescence was mostly localized in dendrites (Figure 4B). In contrast, mBDNF-GFP was primarily localized in cell

bodies (Figure 4B). The green fluorescence extended, at best, to a few proximal dendrites. Quantitatively, the number of GFP-bearing varicosities on distal dendrites (within a 100  $\mu$ m diameter circle centered in the cell body) in mBDNF-expressing cells was dramatically reduced, compared to vBDNF-expressing cells (Figure 4C).

Confocal microscopy showed that vBDNF-GFP exhibited a striking punctate fluorescence, distributed throughout the cell body and extending to processes. mBDNF-GFP neurons, however, exhibited a diffuse fluorescence pattern mainly in cell bodies (Figure 5A). Green fluorescence appeared to accumulate as a large patch in the peri-nuclear region. Quantitative analysis showed a markedly reduced number of small fluorescence granules, suggesting an impairment in regulated secretion of BDNF protein in mBDNF cells.

#### Secretion of val BDNF and met BDNF

We used a modified two-site immunoenzyme assay to measure vBDNF-GFP or mBDNF-GFP secretion from virus-infected hippocampal neurons. To ensure that similar levels of vBDNF and mBDNF are expressed, we performed Northern blot using a GFP cDNA probe. No differences in levels of BDNF-GFP mRNAs were detected in hippocampal neurons infected with vBDNF-GFP and mBDNF-GFP (Figure 6D). Moreover, Western blot using an anti-GFP antibody indicated that levels of mBDNF proteins in mBDNF-GFP transfected cells (both the pro- and mature forms) were not different from those of vBDNF proteins in vBDNF-GFP transfected cells (Figure 6E).

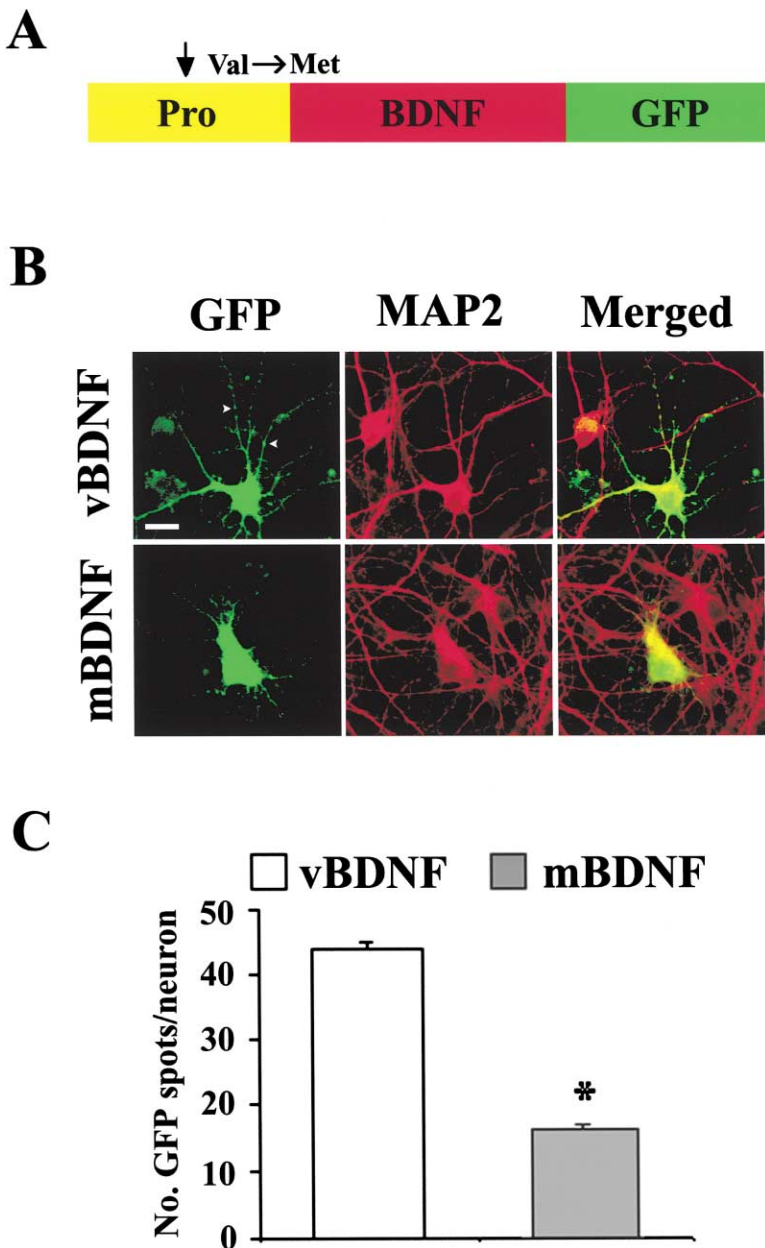


Figure 4. Abnormal Distribution of met BDNF in Dendrites of Hippocampal Neurons

(A) Diagram of BDNF constructs. A cDNA fragment for the entire coding sequence is fused with cDNA for GFP. Arrow = amino acid 66, which is val (vBDNF) or met (mBDNF).

(B) Representative low-power images of cultured hippocampal neurons expressing vBDNF-GFP or mBDNF-GFP. Twenty-four hrs after transfection, neurons were processed for MAP2 immunocytochemistry (red) and GFP imaging (green). GFP images were overexposed to improve visualization of GFP in dendrites. vBDNF-expressing neurons exhibit multiple labeled long processes while mBDNF-expressing cells have fewer and shorter labeled processes. Scale bar equals 10  $\mu$ m.

(C) Quantitation of BDNF dendritic expression. GFP-bearing varicosities (arrowheads in B) on dendrites. N = 50 neurons/genotype group. In this and all remaining figures, data are represented as means  $\pm$  SE \*:  $p < 0.001$ , student t test.

Conditioned media containing vBDNF-GFP or mBDNF-GFP were incubated in culture dishes precoated with anti-GFP monoclonal antibody. After extensive washes, levels of secreted BDNF in the media were determined by ELISA using an anti-BDNF antibody. vBDNF-GFP or mBDNF-GFP were virtually undetectable in a 10 min incubation period (Figure 6B). Neuronal depolarization by high concentration of KCl (55 mM) for 10 min induced a marked increase in vBDNF-GFP secretion (Figure 6B). The concentration of vBDNF-GFP reached approximately 30 ng/ml in the conditioned medium. In contrast, the activity-dependent release of mBDNF-GFP was severely reduced and sometimes not detectable. Of those experiments in which high K<sup>+</sup> did induce detectable release, the average concentration reached only 19 ng/ml. We next investigated constitutive secretion in neurons infected by vBDNF-GFP or mBDNF-GFP. Cultures

were washed, and the conditioned media were collected 2 hr later without high K<sup>+</sup>. As shown in Figure 6C, secretion of BDNF was similar in cells infected with either vBDNF-GFP or mBDNF-GFP. These data indicate that the val66met polymorphism in the pro-region significantly attenuated the activity-dependent form of BDNF secretion without affecting its constitutive secretion.

Finally, we examined whether the properties of secreted mBDNF had been changed due to some unforeseen consequence of the variation in the pro-region. COS cells were transfected with vBDNF-GFP or mBDNF-GFP. The sizes of the precursor as well as mature proteins did not differ, and the proBDNF-GFP/BDNF-GFP ratios were similar in the two groups (Figure 7A). Moreover, the magnitude of induced TrkB phosphorylation, as detected by Western blot using antibody against phosphorylated TrkB (pTrkB, Figure 7B), did not

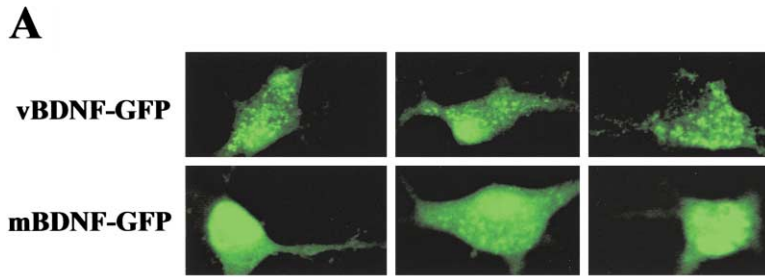
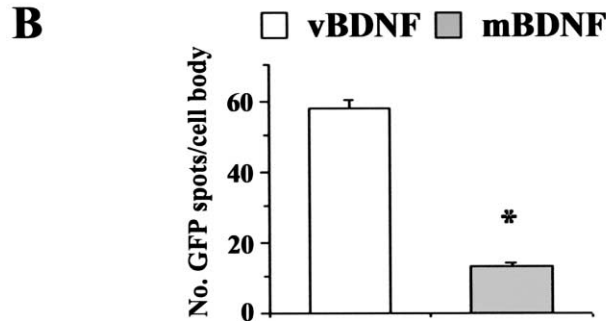


Figure 5. Abnormal Distribution of met BDNF in Cell Body of Hippocampal Neurons

(A) Higher power images (3 per construct) of cell body regions using confocal microscopy. Signals for vBDNF are punctate and extend into dendrites while those for mBDNF are diffuse and concentrated in the peri-nuclear region.

(B) Quantitation of fluorescent granules on the cell body of transfected neurons. N = 50 neurons in each genotype.



differ between vBDNF-GFP and mBDNF-GFP proteins. Further, the two proteins elicited comparable biological function, as measured by the neurite outgrowth assay using PC12 cells expressing TrkB receptors. Approximately 90% of the cells extended neurites longer than their cell bodies after treatment with BDNF or NGF, but not conditioned media from mocked transfected cells. In cultures treated with vBDNF-GFP or mBDNF-GFP proteins, similar percentage of the cells (90%) extended neurites (Figure 7C). Taken together, these results

strongly suggest that met substitution for val in the pro-region does not affect the structural and functional properties of the mature protein.

#### Subcellular Distribution of valBDNF-GFP and metBDNF-GFP

Differences in the distribution of vBDNF-GFP and mBDNF-GFP and in the depolarization-induced secretion of the proteins further suggest that this mutation may lead to errors in BDNF sorting into different secretory

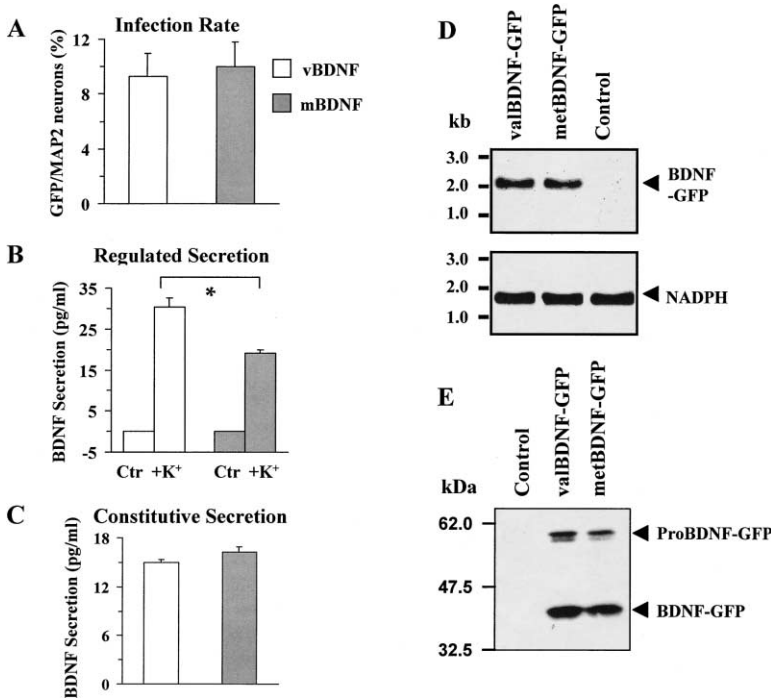


Figure 6. Activity-Dependent and Constitutive Secretion of vBDNF and mBDNF

(A) Infection rate, as the percentage of MAP2-positive neurons showing GFP fluorescence (approximately 10% in both).

(B) Activity-dependent secretion is markedly reduced in mBDNF-GFP neurons.

(C) Constitutive secretion. Amounts of vBDNF-GFP or mBDNF-GFP secreted into the media during 2 hr is similar. In (A-C), N = 4 independent experiments.

(D) Expression of vBDNF-GFP or mBDNF-GFP mRNAs. Northern blot was performed using total RNAs from neurons transfected with vBDNF-GFP or mBDNF-GFP. Control: RNA from untransfected cultures of the same density. Equal amounts of RNAs (10 µg) were loaded in each lane. A GFP cDNA probe was used to detect BDNF-GFP transcript (2 kb, upper image), and the expression of NADPH mRNA (1.7 kb, lower image) was used as the loading control.

(E) Expression of vBDNF-GFP or mBDNF-GFP proteins. Western blot probed with an anti-GFP antibody using proteins from transfected neurons. Equal amounts of proteins (10 µg) were loaded in each lane. Note that vBDNF and mBDNF proteins are expressed at the same level and processed in the same way.

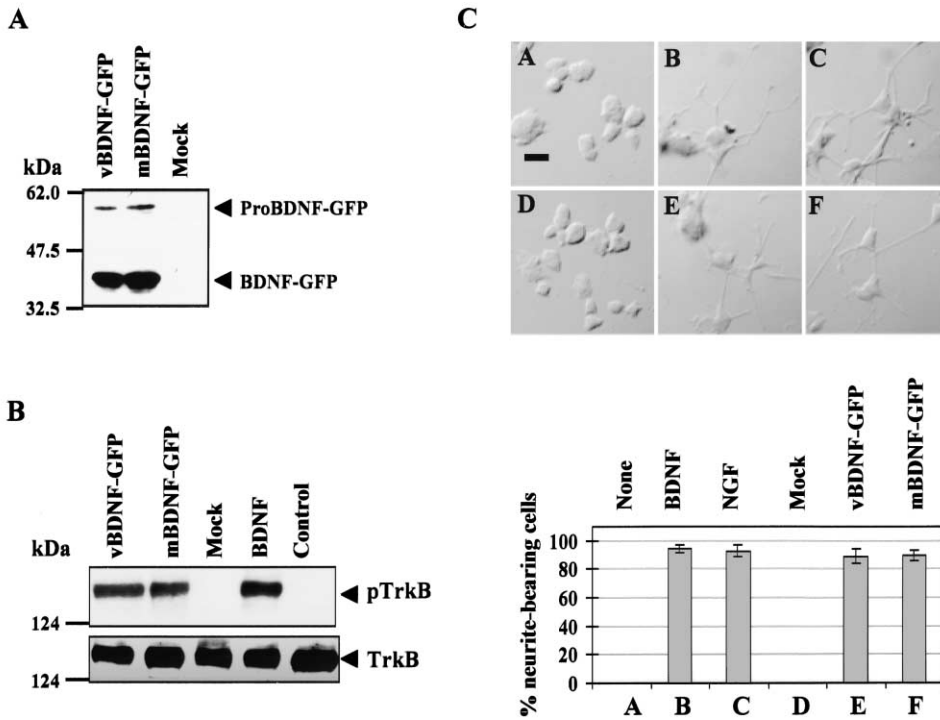


Figure 7. Characterization of Secreted vBDNF and mBDNF

(A) Secretion of BDNF in COS cells expressing BDNF-GFP isoforms. Conditioned media from COS-7 cells transfected with vBDNF-GFP or mBDNF-GFP genes were collected and concentrated. Note that the molecular weights of both pro- and mature proteins are similar in the two groups. Moreover, the ProBDNF-GFP/BDNF-GFP ratios are similar in both groups, suggesting that mBDNF-GFP is successfully processed and secreted.

(B) Activation of TrkB receptor by vBDNF-GFP and mBDNF-GFP. Cultured hippocampal neurons were treated for 5 min with the conditioned media (100 ng/ml) derived from vBDNF-GFP, mBDNF-GFP, or mock-transfected COS-7 cells. Recombinant BDNF and BSA (50 ng/ml) were used as positive and negative controls, respectively. Western blots were performed using an antibody against phospho-TrkB (pTrkB; upper image) or TrkB (lower image).

(C) Effects of vBDNF-GFP and mBDNF-GFP on neurite outgrowth. PC12 cells expressing TrkB (PC12-pAB1) were cultured for three days in the absence or presence of various factors. Upper image: examples of treated PC12-pAB1 cells. Lower image: quantitation of percentage of neurite-bearing cells. The cells were treated with nothing (A), recombinant BDNF (B, 50 ng/ml), recombinant NGF (C, 50 ng/ml). Conditioned media from mock transfected cells (D), or cells transfected with either vBDNF-GFP (E, 100 ng/ml) or mBDNF-GFP (F, 100 ng/ml) were also used. Note that mBDNF-GFP exhibits the same biological activity as vBDNF-GFP.

pathways. To test this, we attempted to determine the subcellular localization of vBDNF-GFP and mBDNF-GFP by double-staining techniques. Hippocampal neurons expressing vBDNF-GFP or mBDNF-GFP were stained using antibodies for markers of intracellular organelles. Some vBDNF-GFP and mBDNF-GFP signals colocalized well with the Golgi marker TGN38, suggesting that both are successfully targeted into Golgi (Figure 8A). Many fluorescent vBDNF-GFP particles, but not mBDNF-GFP particles, were associated with the secretory vesicle marker secretogranin II (SecII) (Figure 8B). This strongly implies that met BDNF is not sorted from Golgi to secretory granules. Both val BDNF and met BDNF failed to colocalize with the large dense core marker chromogranin A (CGA) (Figure 8C), indicating that BDNF-containing particles are not large dense core vesicles. In almost all cases, mBDNF-GFP formed large clusters in the peri-nuclear region (Figures 8A–8C). While it is unclear to what subcellular constituents these clusters belong, failures in sorting met BDNF into the appropriate secretory granules may explain the deficits in activity-dependent secretion of met BDNF. Finally, it appeared

that vBDNF-containing particles often superimposed with punctate staining for the synapse marker synaptophysin (Figure 8D). Although we cannot resolve whether vBDNF is located pre- or postsynaptically at the light microscopic levels, the synaptic localization of vBDNF obviously would help its secretion locally at synapses. In marked contrast, mBDNF showed no colocalization with synaptophysin (Figure 8D). Failure to localize at synapses may contribute to the *in vivo* phenotypes associated with the val66met polymorphism.

## Discussion

This study examines the role of BDNF in hippocampal function and episodic memory in humans. Our strategy was to study the functional consequences of the only reported common, single nucleotide polymorphism that alters amino acid sequence in the pro-region of the human BDNF gene. We have employed several *in vivo* approaches, including cognitive testing, fMRI, and <sup>1</sup>H-MRSI to demonstrate the impact of this polymorphism on human brain function. We have also investigated



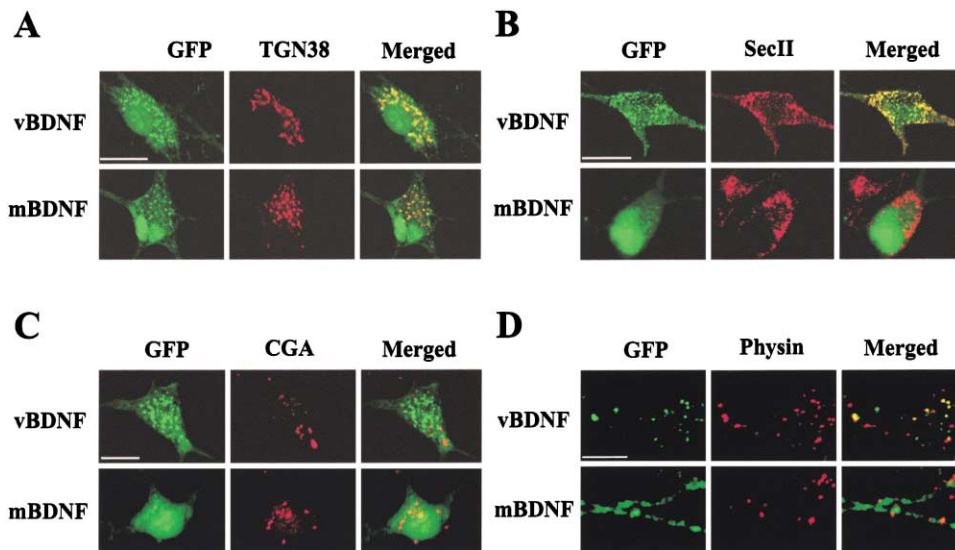


Figure 8. Subcellular Localization of val BDNF and met BDNF in Hippocampal Neurons

Neurons infected with either vBDNF-GFP or mBDNF-GFP (green) were fixed and stained with various antibodies (red). The cell body region of pyramidal neurons is shown in (A), (B), and (C). Scale bar equals 10  $\mu$ m.

(A) Golgi marker TGN38. Note that some vBDNF-GFP and mBDNF-GFP signals (yellow on Merged) are colocalized with TGN38.

(B) Secretory vesicle marker secretogranin II (SecII). Note the colocalization of vBDNF-GFP, but not mBDNF-GFP with SecII.

(C) Large dense core marker chromogranin A (CGA). Both vBDNF and mBDNF fail to colocalize with CGA.

(D) Synapse marker synaptophysin. Neuronal processes of a pyramidal cell are shown. Note that vBDNF punctates often colocalize with synaptophysin while mBDNF does not colocalize at all.

potential molecular and cellular mechanisms underlying the biological effects of this polymorphism, using virus-mediated transfection of val BDNF or met BDNF into cultured hippocampal neurons. The *in vivo* experiments provide converging evidence that BDNF plays a role in human verbal episodic memory as well as hippocampal neuronal integrity and physiological activation. The *in vitro* data suggest that the val66met substitution impacts activity-dependent secretion of BDNF. These results are important for several reasons. First, they demonstrate the feasibility of studying, *in vivo*, the effects of specific genes on hippocampal biology and memory. Second, they show that variation within the normal range of human verbal episodic memory involves BDNF signaling, suggesting that the basic molecular mechanisms of LTP and spatial memory in lower species have been conserved in the more highly evolved verbal episodic memory of humans. Third, the results implicate a specific genetic polymorphism as a factor in altered human hippocampal function, which is therefore likely to have an impact on susceptibility to or expression of illnesses that involve hippocampal neuronal integrity. Finally, the present data also reveal the importance of the pro-region of the BDNF polypeptide, particular val66 and perhaps the nearby sequence, in intracellular trafficking and secretion of BDNF.

Cognitive aspects of human brain function have been largely inaccessible to direct molecular experimental approaches. Limited data have come from pharmacological challenge studies (Mattay et al., 1996), but this approach has been constrained by the relative paucity of drugs and their regional and molecular nonspecificity, compared with the number of genes likely involved in cognition. More recently, genetic studies have identified

polymorphisms related to neurological disorders that produce severe cognitive deficits, but their relevance for normal human cognition is unclear (de Geus et al., 2001). One exception is the ApoE gene, a variant of which (ApoE4) increases risk for Alzheimer's disease (Roses, 1998) and may exert a negative effect on cognition and physiology even in unaffected subjects (Bookheimer et al., 2000).

The current study has several unique features. First, we focus on the normal range of cognition in a cohort including healthy, middle aged subjects. Second, a specific polymorphism in a promising candidate gene is selected and examined using multiple clinical neurobiological methods. The results provide converging evidence for a specific genetic effect on hippocampal neurochemistry, physiology, and cognition. Third, cellular and molecular biological approaches are used to address the underlying mechanisms. This combined approach provides direct *in vivo* data that the molecular mechanisms related to activity dependent BDNF secretion and signaling, such as LTP and synaptic plasticity, may underlie the human ability to form memories. This approach is probably generalizable to many other genes and cognitive processes. It is also worth emphasizing that these results demonstrate the remarkable power of fMRI methods to detect specific genetic effects with substantially lower numbers of subjects than are required for more complex human phenotypic measures (see also Egan et al., 2001b; Hariri et al., 2002).

Our results also speak to a current debate about the genetics of human intelligence. Specific cognitive abilities are partially correlated, and the extent of this correlation is referred to as "g", synonymous with IQ. Much genetic epidemiological data suggest that the genetic

determinants of different cognitive abilities largely overlap with each other and with IQ. In other words, genes influence specific cognitive traits by virtue of their “top down” effects on *g* (Plomin, 1999). The alternative “bottom up” view that genes contribute to IQ primarily through their unique and more direct effects on specific cognitive modules has found little support. The present data are consistent with the latter formulation. We did not detect an effect of BDNF on IQ or any other cognitive ability (although we did not examine some other types of memory, such as priming, skill and habit acquisition, and fear conditioning). A recent study of the role of the COMT gene on working memory and prefrontal physiology, but not IQ, provides further evidence for this view (Egan et al., 2001b). While COMT and BDNF may ultimately be shown to exert small effects on IQ, our current data suggest that their effects are somewhat specific and mediated through “modular” cognitive elements.

### Cellular Processing of BDNF

An unusual aspect of BDNF as a neurotrophic molecule is its secretion via both constitutive and regulated pathways (Goodman et al., 1996; Mowla et al., 1999). Activity-dependent secretion of BDNF makes it an attractive candidate for mediating various forms of synaptic plasticity, such as hippocampal LTP (Katz and Shatz, 1996). BDNF secreted in response to transient or sustained neuronal activity is likely to participate in acute modulation of synaptic transmission or long-term regulation of synaptic structures and functions, respectively. It is therefore critical to understand the molecular mechanisms that control activity-dependent BDNF secretion. We show that depolarization-dependent secretion of BDNF in hippocampal neurons is significantly impaired when val66 in the proBDNF sequence is replaced with a met. This might be due to the inability of met BDNF to be sorted from Golgi to appropriate secretory granules. Thus, our study identifies the pro-region of BDNF, particularly the val66 and adjacent regions, as an important molecular determinant for intracellular sorting and activity-dependent secretion of this molecule. Finally, using double-staining techniques, we demonstrated val BDNF-containing secretory granules are colocalized with synaptophysin, a marker for synapses. In contrast, met BDNF aggregates are accumulated in the cell body and rarely colocalize with synaptophysin. This result suggests that even if it can be secreted in small amounts near the cell body through the constitutive pathway, the met BDNF protein cannot be secreted at synapses. This may hamper local and synapse-specific regulation by BDNF, leading to impairments in hippocampal function and episodic memory in humans. Of course, the proposed cellular mechanism is based on transfection of artificial constructs alone and the effects of val66met on *in vivo* hippocampal BDNF targeting/secretion remain uncertain. Thus, our conclusion that these defects contribute to impairments in human hippocampal activity and memory must be viewed as tentative.

### Clinical Caveats and Implications

Several caveats about our clinical data are worth emphasizing. First, the magnitude of the effects of BDNF genotype on *in vivo* measures of hippocampal function

is small. This is expected, given the likely polygenic genetic architecture of these functions. However, the convergence of data using three *in vivo* methods and the replication of fMRI findings in two independent cohorts argues strongly that the BDNF effect is at the level of hippocampal function. Similarly, studies of heterozygote BDNF knockout rodents, who presumably have intermediate BDNF levels, demonstrate clear physiological (Korte et al., 1995) and behavioral (Lyons et al., 1999) abnormalities, suggesting that secretion levels are critical. A second caveat applies to genetic studies in general whereby spurious results can arise when ethnic groups are mixed (“population stratification”). Because we see identical effects in one ethnic group alone (European Americans) and because similar effects are seen in both the normal controls and our schizophrenia families, stratification is unlikely to explain our results (Wacholder et al., 2000). Third, genetic association *per se* cannot exclude a potential effect of another nearby polymorphism in linkage disequilibrium with val66met. We resequenced the BDNF coding region in 16 subjects (32 chromosomes) and scanned for other polymorphisms in 66 subjects. Only one rare synonymous change was observed, suggesting linkage disequilibrium to a nearby locus does not account for these results. The demonstration that the met allele is both associated with relatively poorer human hippocampal function and impaired intracellular trafficking and regulated secretion argues strongly that the effects in humans are due specifically to the met allele. Finally, the BDNF val66met polymorphism may have important implications for clinical medicine. While we did not find evidence that BDNF was associated with increased risk for schizophrenia, the met allele may affect other human illnesses. Impaired memory and hippocampal function is present in many human disorders, including Alzheimer’s disease (Morris et al., 2001), head trauma, various psychiatric conditions, and even normal aging. Because the distribution of hippocampal function in met/met subjects is shifted downward, such patients may suffer greater functional impairment.

In conclusion, we have demonstrated that an amino acid altering polymorphism at codon 66 in the BDNF gene affects intracellular distribution, packaging, and release of the BDNF protein *in vitro*. Furthermore, in humans, this polymorphism has significant effects on verbal episodic memory, hippocampal physiological activation, and measures of hippocampal neuronal integrity and synaptic abundance. These convergent data suggest that, similar to animal models, BDNF plays a role in human episodic memory by virtue of its effects on hippocampal neuronal function. Inheritance of the BDNF met allele might be expected to confer susceptibility to or modify the expression of conditions that impact adversely on hippocampal function. Finally, because the relatively deleterious met allele, which has not been observed in lower primates, remains in the human genome, it may confer some compensatory advantage in other biological processes.

### Experimental Procedures

#### Human Subjects

Subjects were recruited from the Clinical Brain Disorders Branch “sibling study” of schizophrenia and included healthy controls,

schizophrenic probands, and their mostly unaffected siblings (age 18–60; ethnicity was 6.3% African American, 89.8% European American, and 3.9% other) (Egan et al., 2000). Applicants were screened for factors that could impact brain function, such as head trauma, etc. Availability of the MRI scanner was the only factor determining whether MRSI and fMRI were performed.

#### Cognitive Testing

Subjects performed an extensive cognitive test battery (Egan et al., 2001a). Two tests dependent on episodic memory included the Wechsler Memory Scale, revised version (WMS-R) and the California Verbal Learning Test (CVLT), standard tests of medial temporal lobe function. To reduce comparisons, WMS-R delayed recall scores were chosen as the primary outcome measure, as these scores were most strongly related to genetic risk for schizophrenia in this cohort. Data from Logical Memory I, CVLT 1–5 summary scores, and long delay free recall scores are also described. IQ and reading comprehension (using the WRAT) were also included to control for general intelligence. Exploratory analyses of other types of memory were performed using the Wisconsin Card Sorting Test (WCST), a test of working memory/executive function (perseverative errors T scores) and verbal fluency for categories, a measure of semantic memory (Egan et al., 2001a).

#### Functional Magnetic Resonance Imaging

We examined hippocampal function using fMRI in two cohorts of healthy right-handed subjects (#1: 8 val/vals and 5 val/mets; #2: 12 val/vals and 5 val/mets, including 5 unrelated healthy subjects from the family cohort). The N-back was used as a cognitive challenge; normal subjects show robust hippocampal deactivation (Callicott et al., 2000). We used a block design, alternating between two back (“2B”); subjects must recall a number [1, 2, 3, or 4] seen two stimuli previously) and a control condition (“0B”); subjects identified the number currently seen) (Callicott et al., 2000). Within each cohort, subjects for each genotype group were matched on N back performance to remove possible variance due to performance. Thus, differences in the fMRI data presumably reflect variation in how the information is processed in brain, not in overt behavior.

For the first cohort, whole brain BOLD fMRI data were collected on a 3 Tesla GE scanner using a GE-EPI RT pulse sequence (GE, Milwaukee, WI). For the second cohort, whole brain BOLD fMRI data were collected at 1.5 Tesla (GE, Milwaukee, WI) using a gradient-echo fast spiral acquisition. All fMRI data were processed as described previously (Callicott et al., 2000). Single subject contrast maps were created by contrasting 2B and 0B using a 1 sample T-test ( $p < 0.05$ , corrected). All individual maps showed activation of the typical WM network.

To determine the effect of BDNF genotype, group data were analyzed in two steps. First, a within genotype group analysis tested for voxels showing significant changes ( $p < 0.05$ , cluster size  $> 8$  voxels, uncorrected) in BOLD signal in the two versus zero back condition for each subject. The use of a cluster size  $> 8$  increases the statistical threshold for significance to  $p < 0.0001$ . These voxels were pooled for each genotype group. Second, pooled voxels were used for between group comparisons. A bounding box created a priori (details on request) was used to select only voxels within the hippocampal ROI (identical to a region of interest approach). Between group comparisons were performed as a second level (random effects) one-way ANOVA within SPM 99 ( $p < 0.05$  uncorrected, cluster size  $[k] > 8$ ) (Figure 2A). Coordinates for statistically significant activation clusters were converted back to the standard space of Talairach and Tournoux and reported as the local maxima.

#### <sup>1</sup>H Magnetic Resonance Spectroscopic Imaging (MRSI)

<sup>1</sup>H-MRSI was performed on a GE-SIGNA 1.5 Tesla MR scanner as in earlier reports (Bertolino et al., 1996). The <sup>1</sup>H-MRSI slices were positioned parallel to the sylvian fissure and encompassing the hippocampal formation as visualized on T1 weighted MRI scans. Each volume element (“voxel”) had nominal dimensions of 7.5 mm  $\times$  7.5 mm  $\times$  15 mm (0.84 mL). To produce metabolite maps, location and integration of the signal strength of N-acetyl-aspartate compounds (NAA), creatine plus phosphocreatine (CRE), and choline-containing compounds (CHO) was automatically computed. Metabolite signals

were reported as ratios of the area under the peaks for NAA/CRE, NAA/CHO, and CHO/CRE. Hippocampal formation (inclusive of amygdala, hippocampus, entorhinal cortex, and parahippocampal gyrus) and other regions of interest (dorsolateral prefrontal cortex, putamen, caudate, cingulate, thalamus, and superior temporal gyrus) were examined (Bertolino et al., 1996).

#### Genetic Analysis

BDNF val66met genotype was determined using the Taqman 5'-exonuclease assay (details available on request). To validate genotyping results, relevant amplicons from 75 subjects were sequenced and were identical to those generated from Taqman. The frequencies of the two BDNF alleles were 0.81 for val and 0.19 for met. Genotype frequencies (val/val = 0.67, val/met = 0.28, met/met = 0.05) were in Hardy Weinberg equilibrium. There were no differences between individual groups in allele or genotype frequencies. In the cohort with schizophrenia, no overtransmission of either allele from parents to affected offspring was seen in a family based transmission disequilibrium analysis. HPLC variant scanning of exons and splice sites in 66 subjects of various val66met genotypes and resequencing of the coding open reading frame in 16 subjects revealed no other sequence variants, except a rare synonymous F63F.

#### Statistical Analyses

Effects of genotype on cognitive measures and NAA were assessed using ANOVA. For analyses that included families, we used mixed effects ANOVA. Effects of demographic factors were examined using ANOVA or regression and included as main effects or covariates in BDNF analyses when significant. To avoid population stratification, all analyses were performed on the entire cohort and subjects from one ethnic group (the largest, European Americans); the latter are reported only if different from the former.

#### Cell Cultures and Neurite Growth Assay

Dissociated cultures of hippocampal neurons were prepared using embryonic day 20 rats (E20, Wistar ST) as described (Kojima et al., 2001). Neurons were maintained in Neurobasal medium (Gibco) containing 0.1% horse serum, 2% B27 supplement (Gibco), and 0.5 mM L-glutamine (Sigma) for 6–8 days. Polyethyleneimine-coated glass-bottomed chambers (Matsunami, surface area, 1.5 cm<sup>2</sup>) were used for imaging, and 12-well culture plates (Coster) were used for BDNF immunoassay, Northern, and Western blots. Stable TrkB-expressing PC12 cells, PC12-pAB1 (Nakatani et al., 1998) were cultured in collagen-coated glass-bottomed chambers in Neurobasal medium containing 2% B27 and 0.5 mM L-glutamine. Cells were treated with conditioned media or 50 ng/ml BDNF for 4 days. Neurite outgrowth was assayed by calculating the percentage of cells bearing neurites longer than the cell body (Kojima et al., 2001).

#### Sindbis Virus Construction and Infection

vBDNF was cloned in pEGFP-N1 plasmid (Clontech) between EcoRI and BamHI sites. The val to met mutation in the met BDNF construct was generated by replacing the BstBI and SapI fragment containing the val66 sequence with a mutant dsDNA cassette (CGAACACAT-top and ATCACGTGTT-bottom strands) containing the met66 sequence. Recombinant sindbis virus was constructed according to the Invitrogen manual. Each BDNF-GFP construct was inserted into the multiple cloning site of sindbis vector pSinRep5. The RNAs of pSinRep5-BDNF-GFP and helper DH (26S) were electroporated into BHK cells. The pseudovirions-containing medium (55DME) was collected 36 hr later. Sindbis virus infection of hippocampal neurons was performed as described (Osten et al., 2000) with minor modifications. The titer of the infection was determined by the percentage of cells expressing GFP fluorescence. Cultured neurons were infected at a titer of 10% for five hours, followed by a 24 hr incubation in B27-containing Neurobasal medium. COS-7 cells were infected with the sindbis virus containing the vBDNF-GFP or mBDNF-GFP gene for 24 hr at a titer of 100%, followed by a 24 hr incubation in DMEM containing L-glutamine (500  $\mu$ M). The conditioned media were collected and concentrated by Centriplus (Millipore).

### Northern and Western Blots

RNAs were prepared from infected hippocampal cultures, using the total RNA isolation reagent ISOGEN (NIPPON GENE), separated on agarose gels, and transferred to a Hybond N<sup>+</sup> nylon membrane (Amersham). The BDNF-GFP transcripts were detected by a GFP cDNA probe (Clontech). Hybridization and detection were carried out using AlkPhos direct labeling system, CDP Star, and Hyperfilm ECL (Amersham).

Cell lysates were prepared from infected hippocampal cultures. Conditioned media from infected COS-7 cells were collected and concentrated. The proteins in the lysates or media were separated on SDS-PAGE, transferred to Immobilon P membrane (Millipore), and probed on Western blot replica with an anti-GFP or an anti-BDNF antibody.

To detect TrkB phosphorylation, we treated the hippocampal cultures for 5 min with various factors. These included the conditioned media collected in 12 hr period from non-infected, vBDNF-GFP infected, or mBDNF-GFP infected COS-7 cells. Recombinant BDNF was used as a positive control and BSA as a negative control. The cells were lysed and processed for Western blot using a polyclonal antibody for phospho-Trks (Tyr490; Cell Signaling) and anti-TrkB-specific monoclonal antibody (kindly provided by Dr. S. Koizumi, Novartis Pharma).

### Immunofluorescence Staining and Imaging

Infected neurons were fixed in 4% paraformaldehyde in PBS [pH 7.4] and permeabilized in 0.2% Triton-X. Cells were incubated with the following primary antibodies at 4°C overnight: TGN38 (monoclonal, 1:500; BD Biosciences), synaptophysin (Boehringer Mannheim), chromogranin A (CGA, DAKO), and secretogranin II (SecII, polyclonal, 1:250; kindly provided by T. Watanabe) and then incubated with the appropriate TRITC-conjugated secondary antibodies (1:300; Jackson Labs) for 1 hr at room temperature. The fluorescence images were captured by either a CoolSNAP CCD camera (Photometrics) mounted on an Olympus RX60 microscope or a Bio-Rad RTS2000 laser confocal microscope. GFP-bearing varicosities within a circle (100 μm centered on cell body) were identified and counted using a quantitation menu of the MetaMorph software (Integrated Morphometric Analysis, or IMA). GFP fluorescent granules in the cell body were first visualized using Z-axis confocal scanning with a pinhole size 0.4 μm. The granules were counted at the middle confocal plane, which has the maximal number of granules.

### Immuno-Enzyme Assays

BDNF-GFP protein was measured using the BDNF Emax Immunoassay System (Promega) (Kojima et al., 2001). To reduce the background immunoreactivity of endogenous BDNF, culture dishes were precoated with anti-GFP monoclonal antibody (0.5 μg/well, JL-8, Clontech), instead of anti-BDNF antibody. Standards and samples were incubated in ELISA plates in duplicate, and data obtained from 4 cultures were averaged. Before experiments, cells were washed 3 times with Krebs Ringer. The conditioned media collected at 37°C in a 2 hr period after washes were used as a measure of constitutive secretion. To determine regulated secretion, cells were washed 3 times with Krebs, followed by a 10 min incubation in Krebs at 37°C. This medium was collected and replaced with Krebs containing high K<sup>+</sup> (80 mM NaCl, 55 mM KCl, and 5 mM CaCl<sub>2</sub>) and the cells were incubated for an additional 10 min at 37°C. Conditioned media before and after high K<sup>+</sup> depolarization were used for measurement of regulated secretion.

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