Impact of genetic variant BDNF (Val66Met) on brain structure and function

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Abstract. A common single-nucleotide polymorphism in the human brain-derived neurotrophic factor (BDNF) gene, a methionine (Met) substitution for valine (Val) at codon 66 (Val66Met), is associated with alterations in brain anatomy and memory, but its relevance to clinical disorders is unclear. We generated a variant BDNF mouse ($BDNF^{Met/Met}$) that reproduces the phenotypic hallmarks in humans with the variant allele. Variant $BDNF_{Met}$ was expressed in brain at normal levels, but its secretion from neurons was defective. In this context, the $BDNF^{Met/Met}$ mouse represents a unique model that directly links altered activity-dependent release of BDNF to a defined set of *in vivo* consequences. Our subsequent analyses of these mice elucidated a phenotype that had not been established in human carriers: increased anxiety. When placed in conflict settings, $BDNF^{Met/Met}$ mice display increased anxiety-related behaviours that were not normalized by the antidepressant, fluoxetine. A genetic variant BDNF may thus play a key role in genetic predispositions to anxiety and depressive disorders.

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Brain-derived neurotrophic factor (BDNF), a molecule known to regulate neuronal survival and plasticity, is widely expressed in the developing and adult mammalian brain (Huang & Reichardt 2001, Chao 2003). In addition to regulating neuronal survival and differentiation, BDNF participates in activity-dependent plasticity processes such as long-term potentiation, learning and memory (Lu 2003a). Recently, a common single nucleotide polymorphism (SNP) in the BDNF

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gene leading to a valine to methionine substitution at position 66 in the prodomain (Val66Met) has been identified and shown to influence human hippocampal volume and memory (Egan et al 2003). This BDNF SNP only exists in human and is found to be also associated with altered susceptibility to a variety of neuropsychiatric disorders (Momose et al 2002, Neves-Pereira et al 2002, Sklar et al 2002, Ventriglia et al 2002, Sen et al 2003). This polymorphism in the BDNF gene represents an important initial example of a role for neurotrophins in behavioural processes in humans. This review will focus on the behavioural consequences of this variant form of BDNF in humans, and compare and contrast these findings with those in newly generated mouse model containing this variant BDNF SNP.

Molecular mechanism underlying variant $BDNF_{Met}$

The actions of BDNF are dictated by two classes of cell surface receptors, the TrkB receptor tyrosine kinase and the p75 neurotrophin receptor ($p75^{NTR}$), a member of the tumour necrosis factor (TNF) receptor superfamily (Chao 2003). BDNF binding to TrkB receptor produces neurotrophic responses through rapid activation of the PI3 kinase, Ras/MAPK and PLCγ pathways, thus influence transcriptional events that has multiple effects on cell cycle, neurite outgrowth and synaptic plasticity (Qui & Green 1991, Traverse et al 1992, Cowley et al 1994, Mazzucchelli et al 2002, Rosenblum et al 2002, Chao et al 2006). Signal transduction through p75 independently gives rise to increase in JNK (c-Jun N-terminal kinase) and NF-κB (nuclear factor κB), thus triggering apoptosis (Roux & Barker 2002). Consistent with the critical role of BDNF in synaptic plasticity, BDNF is synthesized and released in an activity-dependent manner (Lu 2003b). In the mammalian brain, BDNF is synthesized as a precursor called proBDNF, which is proteolytically cleaved to generate mature BDNF. ProBDNF may preferentially bind $p75^{NTR}$, whereas mature BDNF preferentially binds TrkB receptor (Lee et al 2001, Teng et al 2005).

The molecular mechanisms underlying altered $BDNF_{Met}$ function have begun to be studied primarily in *in vitro* cell culture systems. The Met substitution in the prodomain was shown in neurosecretory cells and primary cultured neurons to lead to three trafficking defects: (1) decreased variant BDNF distribution into neuronal dendrites; (2) decreased variant BDNF targeting to secretory granules; and (3) subsequent impairment in regulated secretion (Egan et al 2003, Chen et al 2004, Chen et al 2005). In addition, when expressed together in the same cell, $BDNF_{Met}$ alters the trafficking of wild-type BDNF ($BDNF_{Val}$) through the formation of heterodimers that are less efficiently sorted into the regulated secretory pathway (Chen et al 2004). These initial findings are consistent with previous studies indicating that the prodomain of neurotrophins plays an important role in

regulating their intracellular trafficking to secretory pathways (Suter et al 1991). Together, these *in vitro* studies with $BDNF_{Met}$ point to the presence of a specific trafficking signal in the BDNF prodomain region encompassing the Met substitution that is required for efficient BDNF sorting.

Recently, a trafficking protein, sortilin, was shown to be necessary for the efficient sorting of BDNF to the regulated secretory pathway. Sortilin interacts specifically with BDNF in a region encompassing the Met substitution (Chen et al 2005). Replacement of Met at this position led to decreased interaction of BDNF with this trafficking protein and suggests that decreased protein–protein interaction between BDNF and the trafficking machinery as one plausible molecular model for the secretion defect observed with the variant BDNF. This variant BDNF provides an example of how appropriate trafficking of BDNF may have a significant impact on the physiological responses to neurotrophins.

However, fundamental questions remain as to how these *in vitro* effects relate to the *in vivo* consequences of this SNP in humans. The main caveat to these *in vitro* overexpression studies has been that overexpression of neurotrophins has itself led to altered trafficking fates in cultured neurons (Farhadi et al 2000). As the majority of BDNF is released from the regulated secretory pathway in neurons, impaired regulated secretion of $BDNF_{Met}$ represents a significant decrease in available BDNF.

It also remains possible that there are additional defects in $BDNF_{Met}$ processing that may contribute to the observed deficits, although *in vitro* studies in neurons suggest no defect in $BDNF_{Met}$ processing (Egan et al 2003, Chen et al 2004). It has been reported that tissue plasminogen activator, by activating the extracellular protease plasmin, converts proBDNF to the mature BDNF, and that such conversion is critical for late-phase long-term potentiation in the mouse hippocampus (Pang et al 2004). Given that proBDNF preferentially activates $p75^{NTR}$ over TrkB receptor, it is likely that proteolytic conversation of proBDNF may be implicated in BDNF functioning.

Variant $BDNF_{Met}$ and behavioural impairments

Until recently, no genetic associations had been identified linking neurotrophin genes to human cognitive functioning. Given BDNF's established role in mediating processes related to learning and memory (Korte et al 1995, Patterson et al 1996, Desai et al 1999), this increased susceptibility to cognitive impairment suggests that the variation in BDNF may play a role in the development of neuropsychiatric disorders as well as affect nervous system functioning.

However, in any study attempting to associate a gene with pathology or behavioural variation, it is often unclear how the change in genotype results in the phenotypic change. This is especially difficult when attempting to link a

change in genotype with a discrete change in cognitive functioning. It is possible that the identified genetic variant has a direct effect on cognition, but it is also plausible that the genetic variation mediates an effect through some other downstream functional change, or through the regulation of some other gene. These caveats must be kept in mind during this discussion. In addition, the majority of studies that have been conducted to date have either excluded subjects not of European descent, or have removed other ethnic groups prior to data analysis.

One of the most reliable effects observed in carriers of the Met allele (Val/Met) is a difference in hippocampal morphology. In studies of brain morphometry using structural magnetic resonance imaging (MRI) scans, Val/Met individuals have repeatedly been shown to have a smaller hippocampal volume relative to controls who are homozygous for Val allele (Val/Val) (Pezawas et al 2004, Szeszko et al 2005, Bueller et al 2006, Frodl et al 2007). This difference may be related to the role that BDNF and its receptors play in the development as well as continued plasticity of the brain (Huang & Reichardt 2001, Lu et al 2005). Despite a wealth of information on individuals heterozygous for the Met polymorphism, little information exists for individuals who are homozygous for the Met allele (Met/Met) as this genotype is rare in the general population, comprising only 4% of people in Caucasian populations (Shimizu et al 2004, Gratacos et al 2007). In addition to effects on the hippocampus, studies have also shown decreased volume in the dorsolateral prefrontal cortex, an area associated with planning and higher order cognitive functioning, as well subcortical regions such as the caudate nucleus in carriers of the Met allele (Pezawas et al 2004). Recently it was found that Met allele carriers having smaller temporal and occipital lobar grey matter volumes (Ho et al 2006).

One of the common behavioural phenotypes associated with the variant $BDNF_{Met}$ is impairment of higher cognitive abilities. Individuals with the Val/Met genotype have been shown to perform more poorly than control subjects (Val/Val) on memory tasks that rely heavily on the hippocampus (Egan et al 2003, Hariri et al 2003). On the basis of batteries of neuropsychological tests, carriers of the Met allele (Val/Met and Met/Met) were shown to perform worse on tasks that involved recalling places and events, but did not differ from Val/Val individuals on tasks that have been classically shown to be less hippocampal-dependent, such as word learning and planning tasks (Egan et al 2003, Hariri et al 2003). The pattern of brain activation in Val/Met individuals also significantly differed from that of Val/Val subjects during tasks that rely on the hippocampus. Using functional MRI (fMRI), Hariri et al (2003) showed that during a place recognition task, a task that has been shown to result in strong hippocampal activation (Gabrieli et al 1998, Schacter et al 1999, Schacter & Wagner 1999), individuals with the Val/Met genotype had significantly lower levels of activation compared to Val/Val

individuals during both the encoding and retrieval portions of the task. In a similar functional imaging study, Egan et al (2003) employed a task that required individuals to remember a set of stimuli and recall the stimulus that had been presented two stimuli prior to the current stimulus (N-back task), a paradigm that typically results in suppression of the hippocampus. They found that individuals with the Met/Met genotype did not show the same level of hippocampal suppression as subjects with the Val/Val or Val/Met genotypes. These findings taken together suggest that carriers of the Met allele have a selective impairment in hippocampal-dependent memory.

Anxiety is a common symptom among most psychiatric disorders. Several recent studies have looked at the relationship between $BDNF_{Met}$ and trait anxiety. The results have been conflicting, with the Val allele associated with vulnerability in one study and the Met allele designated as the 'risk' allele in another study (Sen et al 2003, Jiang et al 2005, Lang et al 2005). Inconsistency across genetic studies may be attributable to sampling and measurement issues, genetic heterogeneity due to differential sampling of populations or low frequency of homozygous Met carriers, which may lessen the effect size of any particular association. It may also relate to a failure to take into account relevant gene-by-gene and gene-by-environment interactions. A recent investigation found the association between incident stroke and depression with the strongest association for met/met genotype participants (Kim et al 2007). So this study provides evidence for a gene–environment interaction with respect to the impact of stroke on depression. Another study revealed a significant three-way interaction between BDNF genotype, 5-HTTLPR, and maltreatment history in predicting depression (Kaufman et al 2006). Children with the $BDNF_{Met}$ allele and two short alleles of 5-HTTLPR had the highest depression scores, but the vulnerability associated with these two genotypes was only evident in the maltreated children.

Variant $BDNF_{Met}$ knock-in mouse

Recently, a variant BDNF mouse ($BDNF^{Met/Met}$) that reproduces the phenotypic hallmarks in human with this BDNF SNP was generated (Chen et al 2006). The expression of $BDNF_{Met}$ is regulated by endogenous BDNF promoters in the $BDNF_{Met}$ knock-in mouse, which fully mimic the human $BDNF_{Met}$ polymorphism. The $BDNF_{Met}$ knock-in mice has allowed for assessment of the *in vivo* consequences of $BDNF_{Met}$ not only for biochemical but also anatomical and behavioural measures.

Initial secretion studies were performed on neurons obtained from the $BDNF_{Met}$ mice, and BDNF in the resultant media was measured by enzyme-linked immunosorbent assay (ELISA). There was a significant decrease in activity-dependent secretion of endogenous BDNF from $BDNF^{Met/Met}$ mice (~30% decrease). As the

majority of BDNF is released from the regulated secretory pathway in neurons, impaired regulated secretion from $BDNF^{Met/Met}$ neurons represents a significant decrease in available BDNF.

Subsequent anatomical analyses showed there was a significant decrease in hippocampal volume in both $BDNF^{+/Met}$ and $BDNF^{Met/Met}$ mice. Furthermore, Golgi staining was used to visualize individual dentate gyrus neurons of the hippocampus. At 8 weeks of age, there was no difference in the cell soma area among $BDNF_{Met}$ mice and their littermate controls but a decrease in dendritic arbour complexity in $BDNF_{Met}$ mice. Behavioural studies determined that there was a specific impairment in hippocampal contextual fear conditioning in both $BDNF^{+/Met}$ and $BDNF^{Met/Met}$ mice, whereas there was no difference in cue-dependent fear conditioning. $BDNF_{Met}$ mice thus appear to replicate the two hallmark alterations in hippocampal anatomy and hippocampal-dependent learning as human carriers of the $BDNF_{Met}$ allele. These results suggest that $BDNF_{Met}$ mice are a valid animal model for the human variant BDNF SNP.

Subsequent analyses of these mice elucidated a phenotype that had not been established in human carriers: increased anxiety. When placed in conflict settings, $BDNF^{Met/Met}$ mice display increased anxiety-related behaviours in three separate tests and thus provide a genetic link between BDNF and anxiety. Human genetic association studies have been inconclusive as to the contribution of this SNP to with increased anxiety. Two main differences in the mouse study design probably contributed to discerning this anxiety-related phenotype. First, mice were subjected to conflict tests to elicit the increased anxiety-related behaviour, whereas human studies relied on questionnaires. Second, the anxiety-related phenotype was only present in mice homozygous for the Met allele, which suggested that association studies that focused primarily on humans heterozygous for the Met allele may not detect an association.

The form of anxiety elicited in these $BDNF^{Met/Met}$ mice was not responsive to a common selective serotonin reuptake inhibitor (SSRI). These results suggest that humans with this allele may not have optimal responses to this class of antidepressants. Currently, there are no reliable genetic or non-genetic biomarkers to predict who will respond to an SSRI. The transgenic $BDNF^{Met/Met}$ mouse may serve as a valuable model to identify novel pharmacological approaches to treating anxiety symptoms that underlie many neuropsychiatric disorders.

Conclusions

The human genetic variant BDNF (Val66Met) represents the first example of neurotrophin family member, has been linked to anatomical and behavioural phenotypes in humans. However, its relevance to clinical disorders is unclear. The generation of $BDNF_{Met}$ mouse model to test in a more controlled and precise

manner, than in human studies, the contribution of this variant BDNF SNP to neuropsychiatric disease processes. In all, these findings indicate a new direction in therapeutic strategies to rescue anxiety symptoms in humans with this polymorphic allele. Drug discovery strategies to increase BDNF release from synapses or to prolong the half-life of secreted BDNF may improve therapeutic responses for humans with this common BDNF polymorphism.

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DISCUSSION

Flint: Can I clarify one thing about the ValMet polymorphism in the pro-domain? In the mature animal we have heard that there is no pro-BDNF, and you have said that mature BDNF is only found in the adult. Does this mean that this effect has to be developmental?

Lee: No, I think this effect is potentially in the Golgi apparatus. The pro-domain is likely involved in proper protein folding and trafficking. The consensus is that the cutting might occur at the Golgi or within the secretory granule itself. We don't know where the cleavage event occurs. Perhaps what sortilin is doing is maintaining fidelity of folding. It might be needed for proper folding and sorting to occur, and once it is there, its job is done. If you have a decreased protein–protein interaction with the pro-domain and sortilin, the result could be the 25% loss of efficiency in the amount of protein that gets into the granule.

Flint: From your *in situ* hybridizations it looked like you were only seeing a lot of BDNF of protein in the adult.

Lu: That technique cannot distinguish pro and mature BDNF.

Flint: There must be some relationship here.

Barde: You have 10 times more protein, but immunoassays do not distinguish between pro or mature. I think its all mature BDNF anyway, but there is a 10-fold increase as predicted by the *in situ* hybridization and quantitative mRNA determination.

Lee: Presumably, you would agree that in some form pro-BDNF exists during the rapid biosynthetic phase.

Barde: Of course.

Flint: The trouble I have here with this is that you are reporting anatomical changes in the hippocampus, which in my mind must be developmental at some stage. You are showing that the major expression of this gene is postnatal.

Lu: Expression of a gene does not always equate to expression of a protein.

Akil: The hippocampus keeps developing postnatally.

Lee: I think the question is about whether the expression of BDNF early on is enough to cause changes in hippocampal volume.

Malaspina: People can have decreased hippocampal volume even in their 20s. Many things can measurably reduce hippocampal volume without this being developmental.

Flint: He's dealing with a genetic effect.

Lee: We believe that the hippocampal volume difference is not because of a cell loss. It is most likely going to be the dendritic arborization effect: an increase in complexity of the dendritic arbours. This can occur at any time.

Hen: You took us on a surprising tangent. On the one hand, you tell us that these animals don't respond to fluoxetine. Obviously we want to know what is happening in the hippocampus, not in the olfactory bulb. If neurogenesis has anything to do with their lack of response to fluoxetine, we would expect that fluoxetine does not stimulate neurogenesis in these animals. Fluoxetine not only has effects on proliferation, but also on survival and differentiation. These are the effects I'd expect to be absent in your experiments.

Lee: We'll see.

Bothwell: The Met/Met BDNF primarily affects the regulated secretion, and not so much the constitutive secretion. You suggested that BDNF was coming from support cells. Are these glial cells? They might not have the secretory pathway.

Lee: It is possible. There is also controversy about whether BDNF is secreted in a regulated manner from glial cells, based on Cheryl Dreyfus' work (Jean et al 2006). We are not sure which cells are involved.

Lu: What other cells are in there?

Lee: Type B, but mainly gilal cells. Possibly also endothelial cells.

Castrén: So sortilin is also implicated in the interaction with p75 receptor. What is the relationship between these effects? Is there any competition? Is this pure chance?

Lee: Sortilin probably has a binding module that binds multiple growth factors. It just happened that when Barbara Hempstead started studying pro-NGF, sortilin bound to it. 10% of sortilin is at the cell surface. It is probably doing two things. Very similarly to the mannose-6-phosphate receptor, it is probably doing a Golgi-sorting event. It gets to the cell surface and picks up pro-NGF or neurotensin and

does something else. The only time when high p75 levels and high sortilin levels occur together is during injury, not in normal brain. In the normal brain there isn't much p75 around. Even if the sortilin went up there, it probably wouldn't see any p75.

Castrén: Is the site the same as pro-neurotensin?

Lee: This hasn't been mapped.

Akil: With FGF, there are some forms that are secreted, some that stay cytoplasmic and some that go to the nucleus. Is there a sorting difference, with less going into the secretory pathway if some goes somewhere else?

Lee: We think it is a secretory granule. We have done a colocalization with some secretory granule markers. It is not clear what compartment it goes to, and whether it goes to an additional compartment other than this granule-like compartment.

Akil: Are there allelic variants? Is it a matter of amount where a certain percentage goes to secretory granules and the rest dies?

Lu: Is there is a compensatory increase in constitutive secretion in the Met/Met animals?

Lee: This was a sort of overexpression siRNA experiment, but in the Met/Met animal we don't see a compensatory increase in constitutive secretion.

Akil: It is not decreasing either.

Lee: Exactly. What you have picked up on is that the levels of BDNF in the whole brain are identical. It has gone somewhere.

Akil: That's why I am asking: is it cytoplasmic, but not secretory? Is it going to nucleus? Is it doing some other job?

Lee: I don't know. It is somewhere in suspended animation!

Akil: Is there a correlation between allelic variation and olfactory function in humans?

Lee: We don't know.

Lu: We should probably look at BDNF very differently from most of the growth factors, including FGF. Perhaps 10% of BDNF is like other growth factors, with constitutive secretion. The majority of BDNF (> 80%) is going through regulated secretion.

Raff: Are Met/Met humans anxious?

Lee: A good guidepost to this would be the serotonin transporter story. In order to have major depression you need two copies and a horrific childhood. It could be another gene–environment interaction. It just happened that by virtue of a uniform background and a controlled environment, we were able to tease out this phenotype.

Raff: Do we know if they are narcissistic and anxious?

Castrén: There is a study where neuroticism is actually increased. The problem there is that the alleles vary. The Val allele is a risk for bipolar disorder.

Lee: Margit Burmeister (Sen et al 2003)] said that the Val allele was associated with neuroticism. Robert Lipsky's (Jiang et al 2005) group said that it was the Met allele. Another group in Germany said it was neither (Lang et al 2005).

Malaspina: I have a comment about the olfactory deficits. With bipolar it is interesting that lithium improves olfactory discrimination. Using the available techniques people haven't been able to demonstrate any odour discrimination deficits in depression or anxiety. I am now doing olfactory ERPs in these groups. In schizophrenia there are different levels of severity of the deficit. Does this go against your hypothesis that anxious and depressed patients don't have measurable defects?

Lee: I don't know. This might be a very small effect size: there is always variation in the controls.

Malaspina: Controls do remarkably well on olfactory discrimination using the University of Pennsylvania test. The females in general score measurably better than males. These technologies have not been able to pick it up. There may be something that can rescue that BDNF effect in the anxiety and the depression disorders that is a second defect in schizophrenia.

Sendtner: We have done many experiments to localize BDNF within cells that produce it. We have never found it in the nucleus, or evenly distributed in the cytoplasm: it was always in vesicle -like structures, at least in those cells that we investigated. . Most cells in the CNS that express BDNF also express TrkB. Therefore there must be an intelligent sorting mechanism within the cell such that BDNF doesn't get into the same vesicles as TrkB. You did some interesting experiments with the phospho-TrkB antibody. Did you see evidence for abnormal sorting such that these two kinds of vesicles come together within cells?

Lee: We could do those experiments, but we'd have to get the neurons out of the mice. The phospho-TrkB antibody works with immunocytochemistry and with an epitope tag. Theoretically we should be able to follow both of these.

Akil: Is localization with both allelic variants?

Sendtner: Just wild-type.

Akil: My working hypothesis is that the interesting question is whether this changes with the Met allele variant.

Lee: Exactly.

Hen: There is a complicating factor: in the BDNF heterozygotes, there are profound changes in the serotonergic system. I wouldn't be surprised if you found quite a bit of change in the serotonergic system. You mentioned gene–environment interactions. I'd be very interested in seeing interactions between Val66Met polymorphisms and the serotonin transporter polymorphism.

Lu: This has been done to some degree.

Giedd: There are fMRI studies looking at COMT interactions (Harrison & Weinberger 2005) but I did not find any specifically addressing BDNF and sero-

tonin. There is a paper looking at the interaction between serotonin transporter and BDNF and lithium response but it does not use fMRI (Rybakowski et al 2007).

Lu: Are the Met/Met animals aggressive?

Lee: We don't keep them long enough: if the mice are over 4 months old they get very aggressive. There have been studies with the heterozygote transporter knockout mouse and the heterozygote BDNF mice, and everything got worse.

Sklar: What happens if you give mature BDNF back to these mice? Since this is a constitutive knock-in one does not know specifically what is altered downstream or in development.

Lee: People have shown that serotonin 1A and 2A are altered in the heterozygote knockout mouse. We will do binding studies on this. We were talking to Rudy Jaenisch because he has the conditional BDNF over-expressor. This is the way to go: you could theoretically use a virus and inject for localized over-expression.

Tongiorgi: You mentioned a nice restriction in hippocampal volume which correlates well with the reduction in hippocampal volume in humans. Is there any neuroanatomical change so you can measure pruning of dendrites or myelin deficits?

Lee: We have looked at CA1, CA3 and dentate gyrus neurons, and they are statistically significantly smaller. They are smaller from P7.

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