An Adaptive Role for BDNF Val66Met Polymorphism in Motor Recovery in Chronic Stroke

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Little is known about the influence of genetic diversity on stroke recovery. One exception is the polymorphism in brain derived neurotrophic factor (BDNF), a critical neurotrophin for brain repair and plasticity. Humans have a high-frequency single nucleotide polymorphism (SNP) in the prodomain of the *BDNF* gene. Previous studies show that the BDNF Val66Met variant negatively affects motor learning and severity of acute stroke. To investigate the impact of this common BDNF SNP on stroke recovery, we used a mouse model that contains the human BDNF Val66Met variant in both alleles (BDNF^{M/M}). Male BDNF^{+/+} and BDNF^{M/M} littermates received sham or transient middle cerebral artery occlusion. We assessed motor function regularly for 6 months after stroke and then performed anatomical analyses. Despite reported negative association of the SNP with motor learning and acute deficits, we unexpectedly found that BDNF^{M/M} mice displayed significantly enhanced motor/kinematic performance in the chronic phase of motor recovery, especially in ipsilesional hindlimb. The enhanced recovery was associated with significant increases in striatum volume, dendritic arbor, and elevated excitatory synaptic markers in the contralesional striatum. Transient inactivation of the contralateral striatum during recovery transiently abolished the enhanced function. This study showed an unexpected benefit of the BDNF Val66Met carriers for functional recovery, involving structural and molecular plasticity in the nonstroked hemisphere. Clinically, this study suggests a role for BDNF genotype in predicting stroke recovery and identifies a novel systems-level mechanism for enhanced motor recovery.

Key words: BDNF; cerebral ischemia; gait; motor function; striatum; Val66Met polymorphism

Introduction

Stroke remains a leading cause of long-term disability in the United States. Although adaptive neuroplasticity and remodeling occur for an extended period after stroke, most patients have incomplete recovery (Carmichael, 2006; Nudo, 2007). While the natural history of spontaneous stroke recovery and imaging studies in humans have provided some understanding in recovery (Cramer, 2008), the biological determinants of endogenous recovery after stroke remain to be elucidated.

Brain-derived neurotrophic factor (BDNF) promotes neuronal survival, differentiation, synaptic plasticity, and angiogenesis in normal and ischemic tissue (Chao, 2003; Kermani et al., 2005; Wagner et al., 2005). A common single nucleotide polymorphism (SNP) in the human *BDNF* gene leads to a substitution of a valine

to methionine at codon 66 (Val66Met). The BDNF SNP occurs only in humans, with a prevalence ranging from 20 to 30% in Caucasians up to 70% in the Asian population (Egan et al., 2003; He et al., 2007). Clinical studies suggested that the Met allele negatively impacts stroke recovery among survivors of subarachnoid hemorrhage (Siironen et al., 2007; Vilkkii et al., 2008; Mirowska-Guzel et al., 2012). Additionally, mice with the SNP exhibit angiogenic and behavioral deficits in an acute phase (Qin et al., 2011). It was, however, the Met allele that promoted the recovery of executive function in individuals with traumatic brain injury (Krueger et al., 2011), indicating a more complex role of the SNP, possibly depending on the injury context and outcome parameters.

Knock-in mice homozygous for the Met allele (BDNF^{M/M}) display similar phenotypic hallmarks as humans who carry the SNP: smaller hippocampus, memory deficits, and increased anxiety-related behavior (Sklar et al., 2002; Egan et al., 2003; Sen et al., 2003; Chen et al., 2006). Moreover, the neurons cultured from these mice showed reduced regulated BDNF secretion but normal constitutive secretion (Chen et al., 2006). Regulated BDNF secretion modulates synaptic plasticity (Poo, 2001; Kline et al., 2010). Specifically, it regulates the maturation of inhibitory synapses, which fosters a network-level adaptation for homeostasis between excitation and inhibition (Seil and Drake-Baumann, 2000; Hong et al., 2008).

Ischemic stroke resulting from occlusion of the proximal middle cerebral artery (MCA) primarily affects the striatum, a subcortical structure that exhibits activity-dependent plasticity and

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is important for controlling movement and motor learning (Dang et al., 2006; Kreitzer and Malenka, 2008; Bateup et al., 2010). Several studies have reported the impact of this BDNF SNP on cognitive function. The high occurrence of the SNP in humans, however, raises the intriguing possibility that the Met allele may confer selective advantages through noncognitive function. To address the impact of the SNP on functional recovery in chronic stroke, we assessed long-term motor/gait functions, which are relevant to human recovery, in mice carrying the SNP and asked whether the functional recovery is coupled with structural and morphological plasticity. Here we report that the BDNF^{M/M} mice display unexpected enhancement of motor function during stroke recovery and that the enhancement is associated with structural and synaptic plasticity in the contralesional striatum.

Materials and Methods

Animals. The Institutional Animal Care and Use Committee of Weill Medical College of Cornell University approved the use of animals and procedures performed. A total of 187 BDNF^{+/+} and BDNF^{M/M} male mice were used for the study. To prevent genetic drifting and to maintain the phenotype of the mice, the BDNF SNP mice have been backcrossed to C57Bl/6 every 2 years. The mice used in this study are 10× and 11× backcrossed. We used BDNF^{+/+} and BDNF^{M/M} littermates from respective 10× or 11× heterozygote mating to match genetic background within a given experiment for the controlled preclinical studies. The genotyping procedure has been described (Chen et al., 2006). A maximum of five mice were housed in a cage at the institute's animal facility with 12 h light/dark cycle. Food and water were provided *ad libitum* in their cage. Due to the nature of a long-term study, mice were given a code (either tattoo or ear tag) at the beginning of the study. Surgeons who performed sham or middle cerebral artery occlusion (MCAO) procedure and other individuals who performed pretraining and postbehavior testing were blinded to the animals' genotype. The code was revealed after data were collected.

Chronic stroke recovery model. Male BDNF^{+/+} and BDNF^{M/M} mice at 3–4 months of age were randomly assigned to undergo sham or transient MCAO by an intraluminal method as described previously (Cho et al., 2005; Qin et al., 2011). Mice were anesthetized briefly with isoflurane (1.5–2.0%) with a mixture of oxygen and nitrogen (30/70%). A fiber optic probe connected to a laser-Doppler flowmeter (Periflux System 5010, Perimed) was glued to the right parietal bone (2 mm posterior and 5 mm lateral to bregma) for continuous monitoring of cerebral blood flow (CBF) before, during, and after MCAO. Sham surgery consisted of exposure of the right carotid artery at its bifurcation, but without occlusion. The surgical site was exposed for 40 min to balance the anesthesia time with the MCAO group. Postsurgical care was the same in the two groups. The proximal MCA was transiently occluded using a 6-0 Teflon-coated black monofilament surgical suture (Doccol) for 30 min. The transient MCAO perturbs blood flow in the striatum, thalamus, and the cortex with the striatum as the structure most affected by the occlusion. The relatively short duration of 30 min MCAO used in this study produced sizable infarction in the MCA territory ~35 mm³ (~20% ipsilesional hemisphere) at 3 d poststroke (Qin et al., 2011), which was accompanied by acute motor and gait impairment with good recovery. Core body temperature was kept at 37 ± 0.5°C during the entire procedure. Mice were then placed in a recovery cage, where the body temperatures were maintained at 37 ± 0.5°C until the animal regained consciousness and resumed activity. The mice were then returned to their home cages where they were previously housed together. Animals that exhibited both CBF reduction of >80% during MCAO and CBF recovery of >80% by 10 min after reperfusion were included in the study. Moxifloxacin (100 mg/kg) was daily administered subcutaneously for 3 d to reduce acute poststroke peripheral infections (Meisel et al., 2004). In addition, saline was subcutaneously administered daily and hydrogel (ClearH₂O) was given to prevent dehydration during the first week of the poststroke period. Mice typically started to regain body weight around day 5–6 and continued to recover from stroke.

Behavior assessment. Rotarod performance and gait analysis were conducted during a light phase of the cycle as previously described (Qin et al., 2011). The rotarod tests the ability of mice to stay on top of a rotating rod. A rotarod device (Med Associates) was set to accelerate from 4 to 40 rpm over the course of 5 min. The animals were pretrained for 1 week, and the performance recorded on the last day before ischemia was used for preischemic baseline for each animal. Poststroke testing was performed during acute (days), subacute (weeks), and recovery (up to 6 months) phases following stroke. The latency to fall from the rod (performance) was averaged from five trials. Initial poststroke rotarod testing was done on day 6.

Gait changes were assessed using a Noldus Catwalk XT gait analysis system (Noldus Information Technology), which is designed to test important gait functions that are relevant to specific disease conditions (Hamers et al., 2006; Neumann et al., 2009). The CatWalk measures kinematics of gait while the mouse walks from one end of a flat surface to another. Footprints were identified and digitally recorded when the light changes with paw placement on the glass-walking surface. We preselected several gait parameters that are biologically meaningful and relevant to stroke recovery (Qin et al., 2011). These include walk speed and interlimb coordination to gauge overall gait function. For detecting asymmetry, we determined paw contact area, intensity of paw pressure, stride length, and swing speed in four limbs: left front (LF), right front (RF), left hind (LH), and right hind (RH) paw. Mice were pretrained for 2 weeks to cross an illuminated glass walkway three consecutive times, a necessary step to obtain quantifiable digital signals. The average recording on the last 2 d before ischemia was used for preischemic baseline for each animal. Behavioral data were presented as percentage of preischemic baseline (mean ± SEM) to account for interanimal variability.

Tissue preparation. Brains were excised, frozen, and sectioned using a cryostat (Leica). Because infarct typically spans ~6 mm rostrocaudally starting from +2.8 mm from bregma and extending to −3.8 mm, we used an unbiased stereological sampling strategy to collect tissue in the entire region that included infarct. Tissue sections were collected serially at 600 μm intervals for the analysis of infarct volume and subregion volume analysis. Tissue between the intervals was sectioned and cut in half and collected for each hemisphere to determine mRNA levels. Separated cohorts of mice were perfusion-fixed with 4% paraformaldehyde. The brain was postfixed, left in a 30% sucrose solution overnight, and sectioned at a 30 μm thickness using a microtome for immunohistochemistry.

Subregion volume estimation. Using Axiovision software (Carl Zeiss), the entire volume of both hemispheres was measured at 4× objective magnification. From a total of 13 serially collected coronal sections at 600 μm intervals, the following morphological criteria were used to determine the boundary of cortex, striatum, and hippocampus according to the literature (Xie et al., 2010; Zhang et al., 2010). The cortex (+2.8 to −3.8 mm from bregma) was defined by the corpus callosum and external capsule, with the ventral boundary by the rhinal fissure. The superior, lateral, medial, and ventral boundaries of the striatum (+1.7 to −2.0 mm) were defined by the corpus callosum, external capsule, lateral ventricle/corpus callosum, and anterior commissure respectively. The nucleus accumbens was excluded from calculations. The external capsule, alveus of hippocampus, and white matter were used as boundary landmarks for the hippocampus (−0.9 to −3.8 mm). The subregional volume was obtained by integrating volume from each sectional area times distance (600 μm). The value was presented as percentage of contralesional hemispheric volume.

Golgi staining/tracing. Golgi impregnation was done using a FD Rapid GolgiStain method (Chen et al., 2006). Brains were embedded in a 3% agarose solution, blocked, cut using a vibratome (150 μm sections), and mounted onto 0.3% gelatin-coated slides. Slides were then dehydrated through graded ethanols, cleared with Histoclear (3 × 5 min), and coverslipped with DPX mounting medium. Slides containing the Golgi-impregnated brain sections were coded before quantitative analysis. Neurons with isolated cell body, untruncated dendrites, and homogeneous impregnation were randomly chosen from the dorsal striatum and hippocampal dentate gyrus. For each group, 25 medium spiny neurons and 12 hippocampal dentate gyrus neurons were selected. Neurons

were traced under 40× magnification using Neurolucida software. The morphological traits of cells (cell body perimeter, cell body area, Sholl analysis, and fractal dimension analysis) were analyzed using a Neuroexplorer software. The code was revealed after the analysis was completed.

Real-time reverse transcription-PCR for gene expression. Vesicular glutamate transporter 1 and 2 (VGLUT1, VGLUT2) and vesicular GABA transporter (VGAT) mRNA were quantified with real-time quantitative reverse transcription-PCR using fluorescent TaqMan technology as previously described (Kim et al., 2008). PCR primers and probes specific for VGLUT1, VGLUT2, VGAT, and β -actin were obtained as TaqMan pre-developed assay reagents for gene expression (Applied Biosystems). β -Actin was used as an internal control for normalization of samples. The PCR was performed using FastStart Universal Probe Master (Roche) and Applied Biosystems 7500 Fast Real-Time PCR system, according to the instructions of the manufacturer. Reactions were performed in 20 μ l total volume and incubated at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

Immunohistochemistry. For DAB staining, endogenous peroxidase activity was first depleted by incubation with hydrogen peroxide. After blocking in 1% bovine serum albumin (Sigma-Aldrich), sections were incubated overnight with primary antibodies of VGLUT1 and VGLUT2 (1:1000; Millipore Bioscience Research Reagents) and PSD95 (1:500; Invitrogen), followed by Vectastain Elite ABC kit (Vector Labs). For fluorescence, sections incubated with primary antibodies were washed with PBS, followed by incubation with secondary antibodies conjugated with Alexa Fluor 488 or 594 (1:200; Invitrogen) for 1 h at room temperature, and examined under a fluorescent microscope or laser scanning confocal microscope (Carl Zeiss). The number of VGLUT1-immunopositive and VGLUT2-immunopositive dots was counted in two sections at the level of striatum (+1.2 and +0.6 mm from bregma) per animal. We randomly selected nine framed areas (0.1 mm² each) in the contralateral striatum. The number of dots within each frame was counted and the total number of immunopositive dots per frame was averaged and divided by area to obtain density per animal.

Stereotaxic injections. Mice placed in a stereotaxic frame received intracerebral injections of saline or muscimol (0.2 μ g/ μ l, 1 μ l) in the contralateral dorsolateral striatum (0.62 mm anterior, 2 mm lateral, 4 mm ventral from bregma). The solution was delivered at a fixed rate of 82 nl/min using a microinject system. Behavioral testing was performed by individuals who were blind to the genotype and treatment.

Statistics. All data are expressed as mean $\pm$ SEM. The comparisons of anatomical, morphological, and biological data between the two groups were made using the Student's *t* test. For behavioral testing, sample size (minimum *n* = 10/group) was calculated based on predicting detectable differences to reach power of 0.80 at a significance level of <0.05, assuming a 33% difference in mean and a 25% SD at the 95% confidence level. Statistical analyses were performed using two-way ANOVA with Bonferroni's *post hoc* comparisons for (1) genotype and stroke effects or (2) genotype and treatment effects in Prism 6.0 software. The level of significance was set at *p* < 0.05.

Results

Enhanced locomotion and kinematic recovery in BDNF^{M/M} mice

A total of 187 male BDNF^{+/+} and BDNF^{M/M} littermates (3–4 month of age) were used for the study. With preventative antibacterial treatment (Meisel et al., 2004) and intensive posts ischemic care for 7 d, the mortality rate among mice with successful MCAO was 18% (12 of 65) in BDNF^{+/+} and 28% (14 of 49) in BDNF^{M/M} mice. There was no mortality in sham-operated mice (Table 1).

As reported previously (Chen et al., 2006), BDNF^{M/M} mice were heavier than BDNF^{+/+} mice at the time of sham surgery or MCA occlusion (Table 1). In a subset of BDNF^{+/+} and BDNF^{M/M} mice, body weight was measured before and after stroke. In mice receiving the sham procedure, BDNF^{M/M} mice gained their body weights significantly faster than BDNF^{+/+}

Table 1. Body weight, CBF, and breakdown of the number of mice in the study

	Sham		Stroke	
	BDNF ^{+/+}	BDNF ^{M/M}	BDNF ^{+/+}	BDNF ^{M/M}
Total number of animals	31	20	72	64
Body weight (g)	26.07 $\pm$ 0.29	27.53 $\pm$ 0.70*	27.57 $\pm$ 0.36	30.19 $\pm$ 0.80**
Failed MCAO	—	—	7	15
Successful MCAO	—	—	65	49
CBF reduction (%)	—	—	85.47 $\pm$ 0.82	82.91 $\pm$ 1.33
CBF reperfusion (%)	—	—	129.0 $\pm$ 6.03	131.2 $\pm$ 7.58
Death in postprocedure	0	0	12	14
Animals entered in study	31	20	53	35

Values are expressed as mean $\pm$ SEM. **p* < 0.05, ***p* < 0.01 versus BDNF^{+/+}.

Table 2. Longitudinal body weight measurement

	Sham		Stroke	
	BDNF ^{+/+}	BDNF ^{M/M}	BDNF ^{+/+}	BDNF ^{M/M}
Prior to stroke	26.5 $\pm$ 1.4	29.5 $\pm$ 2.3	28.1 $\pm$ 2.6	31.7 $\pm$ 4.2*
3 d	25.9 $\pm$ 1.3	28.5 $\pm$ 2.8	22.2 $\pm$ 2.1	25.5 $\pm$ 4.2
5 d	25.7 $\pm$ 1.2	28.5 $\pm$ 2.5	20.9 $\pm$ 2.1	24.3 $\pm$ 4.0
1 week	25.8 $\pm$ 1.3	28.5 $\pm$ 2.4	21.4 $\pm$ 2.4	24.4 $\pm$ 3.9
2 weeks	26.1 $\pm$ 1.3	30.1 $\pm$ 2.9	25.0 $\pm$ 1.9	27.5 $\pm$ 2.3
1 month	26.4 $\pm$ 1.9	32.1 $\pm$ 3.3***	25.9 $\pm$ 2.0	29.3 $\pm$ 3.1
2 months	29.1 $\pm$ 2.1	36.3 $\pm$ 5.2***	27.7 $\pm$ 2.1	31.4 $\pm$ 4.0
4 months	33.2 $\pm$ 3.4	45.0 $\pm$ 7.4***	30.1 $\pm$ 2.6	35.5 $\pm$ 5.7***
6 months	35.1 $\pm$ 3.7	48.7 $\pm$ 7.4***	30.5 $\pm$ 2.7	38.2 $\pm$ 6.9***

Values are expressed as mean $\pm$ SEM (grams). *N* = 12–18 for sham and 18–22 for stroke groups. Two-way ANOVA with *post hoc* Bonferroni's tests. **p* < 0.05, ****p* < 0.001 versus BDNF^{+/+}.

mice at 2, 4, and 6 months (Table 2). However, the overall weight changes in poststroke mice were similar between the genotypes. Compared with preischemic baseline, body weights were similarly reduced at 5 d postischemia but returned to their baseline at ~4 months in both genotypes (Table 2).

We assessed motor/gait function using a priori chosen behavior parameters in BDNF^{+/+} and BDNF^{M/M} mice during acute and recovery phases following stroke. Stroke-induced percentage changes were presented against their own preischemia baseline to account for interanimal variability. Both BDNF^{+/+}-sham and BDNF^{M/M}-sham mice showed a similar age-related decline in rotarod performance (Fig. 1A). Following stroke, BDNF^{+/+} mice showed progressive impairment in rotarod performance during 6 months of recovery. As previously reported (Qin et al., 2011), BDNF^{M/M}-stroke mice displayed a greater acute impairment. However, the performance was markedly improved by 2 weeks. The Met-induced performance crossovers, typically occurring at 2–4 weeks poststroke, was followed by sustained enhancement during a recovery phase, reaching similar levels of performance as sham-operated mice at 6 months (Fig. 1A). This longitudinal rotarod performance demonstrates that the BDNF SNP caused greater impairment in an acute phase, accelerated recovery during a subacute period, and protection from age-related decline during a chronic recovery phase.

The restoration of walking is a high priority for people who have suffered a stroke (Harris and Eng, 2004). Thus, we selected parameters to assess speed and coordination of gait. There was no age-related decline in walk speed in sham-operated BDNF^{+/+} and BDNF^{M/M} mice (Fig. 1B). Walk speed was impaired in BDNF^{+/+}-stroke mice at 1 week, returned to prestroke baseline at 2 weeks, and started to decline at 2 months. The sustained deficit in BDNF^{+/+}-stroke mice during this late recovery phase reflected a stroke-induced behavioral deficit, as sham mice showed no age-related impairment at this time. In contrast, BDNF^{M/M}-stroke mice maintained their walk speed similar to

sham-operated mice throughout the post-ischemic period and were significantly better than BDNF^{+/+}-stroke mice at 1 and 6 months. Regularity index, a degree of interlimb coordination during gait, showed impairment at 1 week in both BDNF^{+/+}-stroke and BDNF^{M/M}-stroke mice (Fig. 1C). Compared with the BDNF^{+/+}-stroke mice, the regularity index of BDNF^{M/M}-stroke mice was higher during 2–6 months of the recovery phase. The data suggest that the Met allele may be associated with overall enhancement of whole-body locomotion and kinematic recovery.

Gait kinematic analysis reveals compensation in BDNF^{M/M} mice

Stroke causes hemiparesis, and we hypothesized that CatWalk gait analysis would reveal impairments in the LF and LH limbs after right MCAO. To test asymmetry, we assessed paw contact area, pressure exerted on the glass plate, stride length, and swing speed in LF, LH, RF, and RH limbs. Both BDNF^{+/+}-sham and BDNF^{M/M}-sham mice showed relatively constant maximum contacting area on the plate (Fig. 2A) or intensity, which is proportional to the weight supported on that paw (Fig. 2B). Following stroke, BDNF^{+/+} BDNF^{M/M} mice showed acute impairment at 1 week in maximum contact area and mean intensity with greater impairments in BDNF^{M/M}-stroke mice. BDNF^{M/M}-stroke mice, however, displayed rapid improvement beyond preischemic baselines, and these overshoots were especially prominent in the ipsilesional RH limb (Fig. 2A,B).

Stride length measures distance between consecutive steps in the same paw. Sham mice, regardless of genotype, displayed relatively constant stride lengths in all four limbs over 6 months (Fig. 2C). Stroke shortened the stride length in both BDNF^{+/+} and BDNF^{M/M} mice, with spontaneous recovery at 2 weeks. Compared with BDNF^{+/+}-stroke mice, BDNF^{M/M}-stroke mice displayed significantly larger stride lengths at 6 months in all four limbs, and from 1 month on in the RH limb (Fig. 2C, right). Swing speed, computed from stride length over swing duration, is a parameter that assesses the temporal relation of gait. Although the parameter was relatively constant in all four limbs during 6 months in sham groups, stroke induced acute slowing in both genotypes (Fig. 2D). BDNF^{+/+} mice showed stroke-induced decline in the late recovery phase, but BDNF^{M/M} mice showed significantly faster swing speed in LF, RF, and RH limbs that was most prominent at later time points (Fig. 2D). Since the mice underwent a unilateral stroke in the right hemisphere, the prominent enhancement in the ipsilesional RH limb suggests an involvement of the contralesional (left) hemisphere in behavioral enhancement in these BDNF^{M/M}-stroke mice.

Larger contralesional striatum in BDNF^{M/M}-stroke mice

To investigate whether the behavioral changes in BDNF^{M/M} mice are associated with anatomical alterations, subregional volume

Figure 1. Enhanced locomotion and kinematic recovery in BDNF^{M/M} mice. Longitudinal behavior test in BDNF^{+/+} and BDNF^{M/M} mice during acute (d, days), subacute (w, weeks), and recovery (m, months) phases following stroke along with age-matched sham-operated mice. **A**, Rotarod performance, latency to fall measured in seconds. **B**, Walk speed, the average speed expressed in distance units per second. **C**, Regularity index, a parameter to gauge the degree of interlimb coordination. All results are presented as percentage of preischemic baseline (% Pre, mean $\pm$ SEM). +/+, BDNF^{+/+} mice; M/M, BDNF^{M/M} mice; $n = 12$ –16/group, two-way ANOVA with *post hoc* Bonferroni's tests * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus preischemic baseline (Pre), # $p < 0.05$, ## $p < 0.01$, +/+ stroke versus M/M stroke.

was measured in the brains of BDNF^{+/+} and BDNF^{M/M} mice at 6 months poststroke. In age-matched sham animals, we found no difference between the genotypes in striatal and cortical volumes (Fig. 3B,C), but did find a previously reported volume reduction in the BDNF^{M/M} hippocampus (Fig. 3D; Chen et al., 2006). Stroke reduced the ipsilesional hemispheric volume in both genotypes. The reduction was mainly due to decreased volume in the striatum (Fig. 3F) and, to a lesser extent, in the cortex and hippocampus (Fig. 3E,G). Estimated infarct size at 6 months, obtained by subtracting the volume of the ipsilesional hemisphere from that of the contralesional hemisphere, was similar between the genotypes (percentage of contralesional volume, +/+ vs M/M, 14.2 ± 2.4 , 15.7 ± 1.8 , $p = 0.62$, $n = 8$ /group), which is consistent with our previous report (Qin et al., 2011). The Met allele-associated behavioral enhancement was therefore unlikely due to the difference in infarct size.

In the contralesional hemisphere, there was a selective increase in striatal volume in the BDNF^{M/M} mice but the increases were not detected in the cortex and hippocampus (Fig. 3E–G). Further comparison between stroke and age-matched sham mice showed that stroke reduced the contralesional striatal volume in BDNF^{+/+} mice (sham vs stroke, $7.92 \pm 0.28\%$ vs $6.87 \pm 0.12\%$, $n = 5$ –8, $p < 0.05$) but not in BDNF^{M/M} mice (sham vs stroke, 8.00 ± 0.27 vs 7.69 ± 0.27 , $n = 5$ –8, ns). This suggests that BDNF^{M/M} mice are either resistant to stroke-induced atrophy or

Figure 2. Enhanced gaits in the ipsilesional hindlimb in BDNF^{M/M} mice. Longitudinal behavior test in BDNF^{+/+} and BDNF^{M/M} mice during 6 months poststroke along with age-matched sham-operated mice. **A**, Maximum contact area, the maximum area of a paw that comes into contact with the glass plate. **B**, Mean intensity, measure of pressure exerted on the glass plate. **C**, Stride length, distance between consecutive steps with the same paw. **D**, Swing speed, speed of the paw during swing. All results are presented as percentage of preischemic baseline (% Pre, mean $\pm$ SEM). +/+, BDNF^{+/+} mice; M/M, BDNF^{M/M} mice. $n = 12$ –16/group, two-way ANOVA with *post hoc* Bonferroni's tests, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus preischemic baseline (Pre), # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, +/+ stroke versus M/M stroke. LF, left front; LH, left hind; RF, right front; RH, right hind limb.

prone to hypertrophy in the contralesional striatum. The selective anatomical changes in the contralesional (left) striatum in BDNF^{M/M} mice raised the intriguing possibility of the involvement of this structure in controlling motor recovery through the ipsilesional (RH) limb.

Morphological changes in striatal neurons in BDNF^{M/M}-stroke mice

To evaluate the cellular basis of the Met allele-induced structural plasticity, we assessed the morphology of medium spiny neurons (MSNs), a major type of neurons, in the contralesional striatum 6 months after stroke (Fig. 4A). MSN sizes, indicated by soma perimeters and areas, were similar between BDNF^{+/+}-sham and BDNF^{M/M}-sham mice. Following stroke, BDNF^{M/M} mice exhibited increased cell body perimeter and area (Fig. 4B,C). While dendritic arbor in MSNs were similar between genotypes in sham mice, those of BDNF^{M/M}-stroke mice were much more complex at 60–120 μ m distance from soma (Fig. 4D). This was accompanied by increased branching that fills neuronal fields (Fig. 4E).

The hippocampus is another structure that showed the Met-associated volume change in control mice (Fig. 3D). We therefore analyzed the morphology of the neurons in the hippocampus to confirm the observed anatomical and cell biological effects of the BDNF SNP. We found that the reduced hippocampal volume in

BDNF^{M/M}-sham mice (Fig. 3D) was accompanied by less dendritic arborization and complexity in the hippocampal neurons compared with BDNF^{+/+}-sham mice (Fig. 4F,G), confirming the anatomical and morphological link. Neurons from the contralesional hippocampus showed no differences in poststroke dendritic arborization and complexity between the genotypes (Fig. 4F,G). Lack of stroke-induced morphological changes in the contralesional hippocampal neurons suggests that the striatum, rather than the hippocampus, is involved in stroke recovery.

Elevated excitatory synaptic markers in the contralesional striatum of BDNF^{M/M} mice

The striatum largely receives excitatory afferents from the cortex and thalamus. Because the transition from inhibition to excitation has been associated with behavior improvement (Dang et al., 2006; Clarkson et al., 2010), we hypothesized that the Met-induced behavioral enhancement is associated with elevated expression of excitatory markers in the contralesional striatum. In sham mice, BDNF^{M/M} mice exhibited higher mRNA levels of the excitatory synapse components VGLUT1 and VGLUT2 compared with wild-type controls (Fig. 5A). mRNA levels of inhibitory synaptic marker VGAT were not different between the genotypes. The selective increase of VGLUT1/2 in the BDNF^{M/M} sham mice suggests that increased expression of excitatory markers

may be an intrinsic feature of BDNF^{M/M} mice. Following stroke, BDNF^{M/M} mice exhibited higher VGLUT1/2 mRNA levels in the contralesional hemisphere while there were no differences in the ipsilesional hemisphere (Fig. 5B). There were no differences in VGAT expression between the genotypes in both hemispheres. The ratio of VGLUT1/2 mRNA expression over VGAT in the contralesional hemisphere was higher in BDNF^{M/M} mice (Fig. 5C). Immunolocalization of VGLUT1 and VGLUT2 proteins in the contralesional striatum showed more abundant and intense VGLUT1 and VGLUT2 immunoreactivity in the contralesional striatum of BDNF^{M/M} mice (Fig. 5D). Confocal images from BDNF^{M/M} mice showed close physical proximity of VGLUT1 and VGLUT2 presynaptic proteins with PSD-95, an excitatory postsynaptic marker, indicating that the expression of VGLUT1 and VGLUT2 occurs at the level of synapse (Fig. 5E).

Greater reliance on the contralesional striatum of BDNF^{M/M} mice for recovery

Pharmacological inactivation is an effective and reversible tool for defining the functional contributions of cortical and subcortical control of movement (Martin and Ghez, 1999). To address the importance of the striatum in motor function, we administered muscimol, a GABA receptor agonist, into the contralesional striatum to transiently inactivate the structure 1 month after stroke, when BDNF^{M/M} mice outperform BDNF^{+/+} mice (Fig. 1). There was no noticeable change from baseline rotarod performance in saline-treated BDNF^{+/+} and BDNF^{M/M} mice (Fig. 6A). In both genotypes, muscimol acutely impaired rotarod performance, which was recovered by 12 h following administration; the severity and duration of the deficits were greater in BDNF^{M/M} mice, with a significant genotype difference at 6 h (Fig. 6A). Muscimol also caused transient impairment of walk speed in both genotypes (Fig. 6B). Similar to rotarod performance, the deficit in walk speed was also larger in BDNF^{M/M} mice. Regularity index was not affected by muscimol in either genotype (Fig. 6C). In individual limb assessment, we found that muscimol treatment minimally affected maximum contact area and mean intensity in all four limbs (data not shown). However, the treatment caused transient impairment in stride length and swing speed in all four limbs, and the deficits were greater in BDNF^{M/M} mice (Fig. 6D,E). The larger and longer deficits in muscimol-treated BDNF^{M/M} mice indicate a greater dependence on the contralesional striatum for functional recovery in Met homozygotes.

Discussion

Structural plasticity and behavioral adaptation occur for an extended period following stroke. This study addressed the specific genetic influence of a human SNP at codon 66 (Val66Met) of the BDNF gene in chronic stroke. Our major finding is that the mice expressing the BDNF Met allele unexpectedly displayed enhanced motor recovery after chronic stroke compared with the genetic background-matched and age-matched mice expressing the wild-type BDNF alleles. The Met allele-associated enhance-

Figure 3. Larger striatal volume in the contralesional hemisphere of BDNF^{M/M}-stroke mice. **A**, A representative Nissl-stained section at the level of striatum. **B–G**, Subregional volumes in age-matched sham (**B–D**) and in 6-month-poststroke (**E–G**) mice were determined from serially collected sections at a 600 μ m interval for the cortex (**B, E**, +2.8 mm from bregma to –3.8 mm), striatum (**C, F**, +1.7 mm from bregma to –2.0 mm), and hippocampus (**D, G**, –0.9 mm from bregma to –3.8 mm). All results are presented as percentage (%) of contralesional hemisphere volume (mean $\pm$ SEM). BDNF^{+/+} (+/+) and BDNF^{M/M} (M/M) mice. Two-way ANOVA with *post hoc* Bonferroni's tests, * p < 0.05, +/+ versus M/M mice. n = 5 (sham) and 8 (stroke)/group, # p < 0.05, ## p < 0.01, ### p < 0.001, versus corresponding contralesional hemisphere. Contra, Contralesional; Ipsi, ipsilesional; Ctx, cortex; St, striatum.

ment selectively involves the contralesional striatum, which showed structural and synaptic plasticity after stroke not observed in other regions. This was accompanied by the enhanced gait in ipsilesional hindlimb and overall improvement of motor and kinematic recovery. Despite the predicted negative influence of the Met allele in motor system function and experience-dependent plasticity (Egan et al., 2003; Kleim et al., 2006; McHughen et al., 2010), this study highlights a selective advantage to carrying the common human BDNF SNP in motor recovery in chronic stroke. While the severity of tissue damage influences the extent of injury-induced plasticity (Frost et al., 2003), lack of difference in infarct size between the genotypes (Qin et al., 2011) suggests a true genetic influence of BDNF SNP on injury-induced structural plasticity and behavioral adaptation.

Anatomical and structural plasticity in the noninjured hemisphere

Compared to wild-type mice, BDNF^{M/M} mice were previously shown to have anatomical and morphological differences at baseline that were associated with cognitive deficits and anxiety related behavior (Chen et al., 2006). Here we report differences in function that emerged only after injury. An issue regarding the surprising enhancements in motor function in Met homozygotes is whether they are an unexpected consequence of established phenotypic differences or whether they represent a newly defined advantage of carrying two Met alleles postinjury. Our findings suggest that the BDNF^{M/M} genotype confers a functional benefit involving the contralesional striatum. While ischemic stroke at the proximal MCA largely affects the ipsilesional striatum, clinical studies suggested an involvement of the noninjured hemisphere in behavioral improvement in human patients with subcortical stroke (Calautti et al., 2001; Gerloff et al., 2006). Congruent with these reports, we found selective increase in striatal

Figure 4. Morphological changes in striatal medium spiny neurons (MSNs) in BDNF^{M/M}-stroke mice. **A–E**, Neurons were obtained from the contralesional hemisphere in BDNF^{+/+} (+/+) and BDNF^{M/M} (M/M) mice 6 months after stroke and in age-matched sham mice. Morphological analyses of MSNs in the striatum. **A**, Representative tracing of Golgi-stained MSNs in the contralesional striatum. **B–E**, Assessment of striatal MSN size by determining perimeter of cell soma (**B**), area of MSN soma (**C**), Sholl analysis to determine dendritic arbor (**D**), and fractal dimension for complexity index (**E**). **F, G**, Morphological analyses of hippocampal neurons for dendritic arbor (**F**) and fractal dimension for complexity index (**G**). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, BDNF^{+/+}-stroke versus BDNF^{M/M}-stroke mice. $n = 5$ – 6 mice/group (4–5 neurons from each mouse, total 25 neurons).

volume in the hemisphere contralesional to stroke (Fig. 3) and enhanced neuronal dendritic arbors and complexity in the neurons from this subregion (Fig. 4). Thus, the plasticity in the contralesional striatum may drive the enhancement of functional recovery in the mice carrying the Met allele.

Adaptive role of the Met allele

Synaptic balance between excitation and inhibition is critical for processing sensory information, motor activity, and cognitive function (Brown et al., 1996; Cline, 2005). Structural and synaptic adaptation following striatal injury is likely to occur through complex basal ganglia–cortical circuits. Primary inputs to the striatum are excitatory afferents from the cortex and thalamus, which then project inhibitory efferents to the globus pallidus (GP) and substantia nigra pars reticulata (SNr) involving direct and indirect pathways. The pathways project back to the thalamus and cortex to complete the striato-thalamic-cortical circuits (Kreitzer and Malenka, 2008; Kozorovitskiy et al., 2012). The net

effect of the direct and indirect pathways is respective facilitation and inhibition of movement. Thus, a potential shift to excitation in the BDNF^{M/M} striatum, probably resulting from disinhibition of inhibitory inputs to the thalamus through GP and SNr, may accentuate excitatory cortical afferents to the striatum. The inferred excitation may be particularly relevant to the mechanism of reorganization and behavioral improvement. Mice lacking the NMDAR1 subunit in the striatum showed disrupted motor learning and synaptic plasticity (Dang et al., 2006). In stroke, reduced excessive GABAergic tonic inhibition (disinhibition, thereby net result of excitation) or activation of AMPA receptors promoted functional recovery (Clarkson et al., 2010, 2011). Additionally, reduction of the inhibitory markers in the perilesional area is necessary for reorganization and mediates stroke recovery (Zeiler et al., 2013). A shift of network balance to excitation may be an intrinsic feature of the Met allele because BDNF^{M/M}-sham mice also exhibited increased excitatory synaptic markers in the brain (Fig. 5A). In light of the finding that excitation is function-

Figure 5. Enhanced excitatory synaptic markers in the contralesional striatum of BDNF^{M/M} mice. **A**, Gene expression of excitatory (VGLUT1 and VGLUT2) and inhibitory (VGAT) synaptic marker in age-matched sham BDNF^{+/+} (+/+) and BDNF^{M/M} (M/M) mice. **B**, Gene expression of excitatory and inhibitory synaptic markers in the contralesional (contra) and ipsilesional (ipsi) hemispheres 6 months postischemia. **C**, Ratios of excitatory over inhibitory marker expression in the contralesional hemisphere of 6-month-postischemic BDNF SNP mice. $n = 5$ (sham) and 8 (stroke)/group. **D**, VGLUT1 and VGLUT2 protein expression in the contralesional striatum and quantification. Densities were presented as number of immunopositive dots per framed area (0.1 mm^2), $n = 4$ –5 mice/groups. **E**, Confocal images from the contralesional striatum of 6-month-poststroke M/M mice showing close proximity of VGLUT1/2 with PSD-95, an excitatory postsynaptic marker. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, +/+ versus M/M mice. Scale bar, $10 \mu\text{m}$.

Figure 6. Greater reliance on the contralesional striatum of BDNF^{M/M} mice for recovery. Assessment of acute motor/gait function following vehicle or muscimol injection in the contralesional striatum of 1-month-postischemic BDNF^{+/+} (+/+) and BDNF^{M/M} (M/M) mice. **A**, Rotarod performance, latency to fall, measured in seconds. **B**, Walk speed, the average speed expressed in distance units per second. **C**, Regularity index, parameter to gauge the degree of interlimb coordination. **D**, Stride length, distance between consecutive steps with the same paw. **E**, Swing speed, speed of the paw during swing. The behavior before treatment was used as a baseline. All results are presented as percentage of preischemic baseline (% Pre, mean $\pm$ SEM). $n = 10$ /group. Two-way ANOVA with *post hoc* Bonferroni's tests, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ muscimol versus vehicle; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ +/+ versus M/M. LF, left front; LH, left hind; RF, right front; RH, right hind limb.

ally beneficial (Clarkson et al., 2010), the working model is that Met-induced excitation bias in the contralesional striatum drives recovery in the chronic phase of stroke recovery in Met homozygotes through a compensatory effect on the contralesional hindlimb. The larger reversible impairment of motor and gait functions in Met homozygotes by muscimol suggests a potential Met-induced shift to excitation and the importance of the contralesional striatum on behavioral adaptation in chronic stroke.

Despite histological evidence that the lesion in the MCAO model is restricted to the side of occlusion, gait analysis revealed acute gait impairments in the limbs of both sides (Fig. 2). Similarly, the bilateral deficits were also evident upon unilateral inactivation of the contralesional striatum by muscimol (Fig. 6). Dozens of studies have documented this phenomenon in both rodents and humans (Pandian and Arya, 2013; Darling et al., 2011; Schaefer et al., 2009). Following stroke, patients often experience loss in strength and speed of movement in the ipsilesional arm. Similarly, acute impairments in the ipsilesional forelimb were described in rodents, indicating that the ipsilesional side was not “nonaffected” but rather “less affected.” While the mechanism that accounts for the acute bilateral deficits is not clear, underlying events may involve plastic changes in the intact hemisphere and disturbed interhemispheric connectivity due to the ongoing infarction in the affected hemisphere (Gonzalez et al., 2004; van Meer et al., 2010).

Clinical relevance

Limitations of the present findings include the undetermined relevance of improving motor function of unaffected limbs in quadruped rodents to humans. However, the organization of the lumbar central pattern generator between rodents and humans is comparable, and these circuits are controlled by similar supraspinal mechanisms (Rossignol, 2006; Gerasimenko et al., 2008). Thus, this study could provide insights into the genetic component of human locomotor/kinematic recovery following stroke. An additional caveat is that BDNF^{M/M} mice often overshoot beyond preischemic baselines. The functional significance of this compensatory plasticity is unclear as it could reflect adaptive or maladaptive responses. However, the association between the overshoots and better, faster, and larger gait throughout the recovery phase suggests they are adaptive compensations necessary for regaining motor control.

Interruption of blood flow in MCA is a common cause of cerebral infarction involving basal ganglia. The size of the basal ganglia in the rodent brain, as a proportion of total brain volume, is approximately twice that of the human basal ganglia (Swanson, 1995). Therefore, a 20% subcortical infarct in the rodents may ~10% subcortical infarct in human when anatomical proportions between the species are considered. Because infarct size in lacunar stroke, a common type of stroke in human with pure motor hemiparesis with good recovery (Bamford et al., 1987; Longstreth et al., 1998; Bejot et al., 2008), typically ranges from 4.5 to 14% of a hemisphere (Carmichael, 2005), the infarct size in the current mouse stroke model would be relevant to human subcortical stroke.

In summary, the key mechanisms stipulated here include the BDNF SNP-induced behavioral changes at the system level resulting from compensation in the nonstroked side, structural changes in the noninjured hemisphere, and cellular changes associated with structural/synaptic plasticity. The mechanistic insights into the differences between known BDNF polymorphisms would be valuable in predicting the course of stroke recovery based on an individual's BDNF SNP. Additionally, the Met allele-

induced biphasic profile, greater acute deficits followed by enhanced recovery, provides potential windows for manipulating synaptic balance in chronic stroke. Strategies aimed at reducing acute excitation in Met carriers and/or enhancing delayed excitation for the subjects with the Val allele may provide an individual's BDNF SNP-based therapy. Testing the concept of Met allele-induced plasticity and behavior adaptation in stroke models that affect other areas of the brain is warranted.

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