Background and Purpose—Stroke is the leading cause of long-term disability in the United States, yet no drugs are available that are proven to improve recovery. Brain-derived neurotrophic factor stimulates neurogenesis and plasticity, processes that are implicated in stroke recovery. It binds to both the tropomyosin-related kinase B and p75 neurotrophin receptors. However, brain-derived neurotrophic factor is not a feasible therapeutic agent, and no small molecule exists that can reproduce its binding to both receptors. We tested the hypothesis that a small molecule (LM22A-4) that selectively targets tropomyosin-related kinase B would promote neurogenesis and functional recovery after stroke.

Methods—Four-month-old mice were trained on motor tasks before stroke. After stroke, functional test results were used to randomize mice into 2 equally, and severely, impaired groups. Beginning 3 days after stroke, mice received LM22A-4 or saline vehicle daily for 10 weeks.

Results—LM22A-4 treatment significantly improved limb swing speed and accelerated the return to normal gait accuracy after stroke. LM22A-4 treatment also doubled both the number of new mature neurons and immature neurons adjacent to the stroke. Drug-induced differences were not observed in angiogenesis, dendritic arborization, axonal sprouting, glial scar formation, or neuroinflammation.

Conclusions—A small molecule agonist of tropomyosin-related kinase B improves functional recovery from stroke and increases neurogenesis when administered beginning 3 days after stroke. These findings provide proof-of-concept that targeting of tropomyosin-related kinase B alone is capable of promoting one or more mechanisms relevant to stroke recovery. LM22A-4 or its derivatives might therefore serve as “pro-recovery” therapeutic agents for stroke. (Stroke. 2012;43:1918-1924.)

Key Words: neurotrophin • small molecule • stroke recovery
stroke. LM22A-4, a small molecule ligand designed to mimic the loop II domain of BDNF, binds selectively and specifically to TrkB. It acts as a partial agonist, phosphorylating TrkB and activating multiple downstream targets in vivo and in vitro. As expected from a partial agonist, LM22A-4’s effects are qualitatively and quantitatively different than the native BDNF protein.

We found that LM22A-4 begun 3 days after hypoxic–ischemic stroke significantly improved gait in recovering mice. We observed improvement in limb swing speed on digital gait analysis, and LM22A-4 accelerated the return to normal gait accuracy on ladder testing. LM22A-4 also doubled neurogenesis in areas adjacent to the stroke, specifically the penumbral cortex and dorsolateral striatum.

This study demonstrates for the first time that a TrkB-specific small molecule ligand is capable of improving functional recovery when treatment is initiated in a clinically relevant time window, several days after stroke. These results provide proof-of-concept evidence that activation of TrkB alone is a potential therapeautic approach for accelerating stroke recovery in patients who have had a stroke.

Methods

Mice

All use of animals was according to protocols approved by the Stanford Institutional Animal Care and Use Committee and conducted according to the National Institutes of Health Guide for Care and Use of Animals. Three hundred twenty-one male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME, 000664) were 4 months old at the beginning of each experiment.

Drug Administration

LM22A-4 (N,N',N'-tris [2-hydroxyethyl]-1,3,5-benzene tricarboxamide) was synthesized by Ricerca Biosciences (Concord, OH) and characterized by liquid chromatography, mass spectroscopy, and bioactivity as described. Intranasal dosing is described in the online-only Data Supplement. Bromodeoxyuridine (BrdU) was dissolved in saline at 5 mg/mL and dosed at 50 mg/kg/day intraperitoneally for 6 days.

Behavior

We measured gait using 3 tests, a horizontal ladder test, automated analysis of spontaneous gait (stride length and swing speed; Noldus Catwalk), and Rotorod (San Diego Instruments). Details of postoperative care, stratification, and behavioral tests are provided in the online-only Data Supplement Methods.

Stroke Models

Details of hypoxic–ischemic stroke procedures are described in the online-only Data Supplement Methods and histological outcomes in online-only data Supplement Figure I. Stroke mice underwent right carotid occlusion followed by hypoxia; sham mice underwent neck dissection without carotid occlusion or hypoxia. Mice were eliminated from cohorts if they died before Day 3 or if they did not exhibit a large functional deficit (approximately 20% were in each group). Studied mice all had >17% error on the horizontal ladder on Day 1 and scored <220 seconds average in 4 Rotorod trials on Day 2. In addition, a blinded observer eliminated 1 mouse from each stroke group because the stroke location was well posterior to the motor cortex. Mortality was the same in saline- and LM22A-4-treated mice (2 in each after assignment to groups and before dosing, 3 in each after dosing started). Distal middle cerebral artery occlusion stroke was induced as previously described for axonal sprouting studies.

Figure 1. LM22A-4, given intranasally at 0.22 mg/kg daily for 7 days, increases subventricular zone neurogenesis in uninjured adult mice. A, Immunohistochemistry for BrdU+ in the subventricular zone (SVZ). Bar, 100 μm. B, Total BrdU+ cells in the SVZ. *P<0.01, analysis of variance. C, Immunostaining for BrdU, Dcx, and NeuN. Bar, 50 μm. D, Total number of new neuroblasts in the SVZ (BrdU+/Dcx+ cells), n=5 per group; *P<0.05, Student t test; graphs, mean±SEM. BrdU indicates bromodeoxyuridine; Dcx, doublecortin; NeuN, neuronal nuclei.

Tissue preparation, Western blots, mass spectroscopy, hemisphere size quantification, immunohistochemistry, stereology, and measurement of dendritic arborization and axonal sprouting after stroke are described in the online-only Data Supplement Methods.

Statistics

Prism and JMP statistical software were used for data analysis with α set at P<0.05 for all tests. All graphs display mean±SEM.

Results

LM22A-4 Promotes Neurogenesis in the Uninjured Brain

BDNF may promote neurogenesis by increasing survival of immature neurons. To determine whether LM22A-4 also increases neurogenesis, we administered 0.022 or 0.22 mg/kg LM22A-4 or saline vehicle for 6 days to uninjured mice and BrdU on Days 1 to 6 and euthanized them on Day 7. The number of BrdU+ cells in the subventricular zone increased 50% after the 0.22 mg/kg LM22A-4 dose (Figure 1A–B). The lower dose of LM22A-4 produced a 20% increase that did not reach statistical significance. LM22A-4 treatment did not alter the percent of BrdU+ cells that coimmunostained for doublecortin on confocal images (83% versus 78%, in saline versus 0.22 mg/kg LM22A-4). Overall, LM22A-4 resulted in a 36% increase in doublecortin+/BrdU+ cells in the subventricular zone (Figure 1C–D). A dosage of 0.22 mg/kg was used for subsequent studies.

LM22A-4 Gets Into the Brain and Increases TrkB Phosphorylation After Stroke

Hemibrains ipsilateral to stroke were harvested from 7 mice treated with LM22A-4 from Day 3 to 10 after hypoxic–ischemic stroke.
Chemic stroke euthanized 1 hour after the last dose. We found a concentration of LM22A-4 of 5.5 ± 2.6 nmol/L by liquid chromatography/mass spectroscopy. To examine whether LM22A-4 would continue to phosphorylate TrkB in brain tissue after daily treatment, we administered LM22A-4 or saline from Days 3 to 10 after hypoxic–ischemic stroke, ran homogenates on Western blots, and probed for phosphorylated and total full-length TrkB (Figure 2). We found a small but significant (1.2-fold) increase in the ratio of phosphorylated: total TrkB with no change in total TrkB.

LM22A-4 Treatment Beginning 3 Days After Stroke Improves Gait

To test if LM22A-4 would improve functional recovery after a completed stroke, we began drug treatment Day 3 after hypoxic–ischemic stroke. Mice were trained as shown (Figure 3A) before stroke or sham surgery. Tests that correspond to stroke size in our hands, ladder testing on Day 1 after stroke and Rotorod testing on Day 2 (online-only Data Supplement Figure II), were used to stratify stroked mice into severely impaired and functionally equivalent groups (Figure 3B–C).

Groups were then randomly assigned to receive LM22A-4 or saline. Mortality was not different after stratification and did not alter the equivalence of the functional impairment in saline- versus LM22A-4-treated stroke groups. LM22A-4 treatment did not affect stroke size (Figure 3D). This suggests that differences observed in motor function are due to enhancement of neurological recovery rather than a primary neuroprotective effect.

With respect to gait accuracy on a horizontal ladder, mice treated with LM22A-4 recovered faster than saline-treated mice (Figure 4A–B). This beneficial effect of LM22A-4 was significant for both measures (percent correct steps, \( P = 0.0349 \); absolute foot faults \( P = 0.0289 \), repeated-measures analysis of variance). Saline-treated mice recovered to normal, so LM22A-4 treatment accelerated the course of this spontaneous recovery. In automated gait analysis, left forelimb stride length during spontaneous walking showed a trend toward improvement in the LM22A-4-treated stroked animals (\( P = 0.099 \), repeated-measures analysis of variance; Figure 4C). Swing speed was 50% of baseline in the stroked, saline-treated group and did not recover, whereas LM22A-4 treatment induced recovery to baseline (repeated-measures analysis of variance).

Figure 2. LM22A-4 phosphorylates TrkB in the stroked brain. A, Representative images for phosphorylated (pTrkB-Y817) and total full-length TrkB (TrkB-FL) in striatal homogenates from 5 mice in each treatment group. B, Quantification of TrkB phosphorylation (phosphorylated: total full-length TrkB), \( n = 10 \) to 11 per group; * \( P < 0.05 \), Student t test; graph, mean ± SEM. TrkB indicates tropomyosin-related kinase B.

Figure 3. Experimental protocol and stratification. A, Experimental design. Stratification into equally impaired groups was based on the results of (B) the ladder test on Day 1 and (C) Rotorod on Day 2. D, Hemisphere volume ipsilateral to stroke 72 days after stroke. \( n = 10 \) in stroked groups and 6 in sham. Graphs, mean ± SEM.
analysis of variance, \( P = 0.0332 \); Figure 4D). Rotorod function did not recover and was not different between LM22A-4- and saline-treated stroked mice (data not shown). We observed no significant differences between sham mice treated with saline versus LM22A-4 in any of these functional tests (data not shown). The same experimental paradigm, repeated in a smaller group of mice for 6 weeks, also showed significant improvements in ladder testing and automated gait analysis but no difference in Rotorod (data not shown).

**LM22A-4 Treatment Increases Neurogenesis After Stroke**

Neurogenesis is implicated in recovery from stroke\(^1\)\(^2\) and increased by BDNF.\(^3\) To measure neurogenesis, we administered BrdU on Days 3 to 8 (Figure 3A). Unbiased stereology was used to quantify the number of BrdU+ cells in the penumbral cortex and dorsolateral striatum, both adjacent to the stroke. Both also receive input from the infarcted motor cortex. For comparison, BrdU+ cells in the ventral striatum were quantified, because it is not adjacent to the stroke and does not receive input from infarcted cortical regions.\(^1\)\(^3\) None of these regions contained significant numbers of BrdU+ cells in sham mice (data not shown). After stroke, all 3 regions contained higher numbers of BrdU+ cells, which was not significantly affected by LM22A-4 (Figure 5).

LM22A-4 treatment approximately doubled the percent of BrdU+ cells that colocalized with the mature neuronal marker neuronal nuclei in regions adjacent to the stroke (cortex and dorsolateral striatum) but had no effect in the ventral striatum (Figure 5). This resulted in an approximately 2-fold increase in neurogenesis. Thus, LM22A-4-treatment augmented neurogenesis primarily in regions adjacent to the stroke and increased production and/or survival of mature neurons at both 6 and 10 weeks after stroke. We also immunostained for the immature neuronal marker doublecortin. There was a significant increase in area covered by doublecortin+ cells at both 6- and 10-week time points, implying that LM22A-4 treatment continues to promote neurogenesis even many weeks after stroke (Figure 6).

**LM22A-4 Does Not Affect Angiogenesis, Neuroinflammation, Glial Scar Formation, Dendritic Arborization, or Axonal Sprouting After Stroke**

There are multiple other potential mechanisms by which a TrkB agonist could improve recovery. We examined and found no difference in angiogenesis (online-only Data Supplement Figure III), neuroinflammation and glial scar formation (online-only Data Supplement Figure IV), or dendritic arborization (online-only Data Supplement Figure V). Because the size and complexity of hypoxic–ischemic stroke makes it difficult to assess axonal sprouting, we examined axonal sprouting in the distal middle cerebral artery occlusion stroke model instead and found no significant differences at 3 weeks (online-only Data Supplement Figure VI).

**Discussion**

The key finding of this study is that stimulation of TrkB signaling with a small molecule partial agonist, beginning 3 days after stroke, can improve functional recovery. Our experimental paradigm was chosen to minimize the potential effects of LM22A-4 on acute neuronal death\(^9\) and to model both disabling stroke and a delayed treatment timeframe that would be clinically relevant to the majority of patients with stroke. This work adds substantially to what is known about
the TrkB ligand LM22A-4. We previously found that LM22A-4 is selective for TrkB and has neuroprotective properties in vitro and that it improved motor learning when it was given to rats beginning at the time of controlled cortical impact. However, administering LM22A-4 at the time of injury meant that improvements in motor learning could be due to decreased neuronal death rather than an enhancement of plasticity and might not translate to regaining motor function after a stroke-induced fixed deficit.

In contrast, in the current study, we trained mice before stroke to evaluate functional recovery rather than learning and administered LM22A-4 beginning 3 days after stroke to minimize any neuroprotective effects. We also studied outcomes that could potentially be altered by TrkB signaling: neurogenesis, angiogenesis, weight change, axonal sprouting, inflammation, and glial scarring. We found LM22A-4 produced dramatic effects on neurogenesis but had no effect on the other processes. Extensive behavioral training may have

Figure 5. LM22A-4 treatment beginning Day 3 after stroke approximately doubled the number of new mature neurons present in the dorsolateral striatum and penumbral cortex at 6 and 10 weeks after stroke. A, Representative immunostaining for BrdU and NeuN demonstrating a double-positive cell (arrow). B, Drawing of a representative coronal section with the regions that were quantified noted. Black horizontal lines, stroke core; blue, dorsolateral striatum; magenta, ventral striatum; green, penumbral cortex. C–D, Stereological estimation of total BrdU+, percent BrdU+/NeuN+, and total BrdU+/NeuN+ cells in affected (dorsolateral) and unaffected (ventral) regions of the striatum and penumbral cortex at 6 and 10 weeks after stroke. n=6 to 7 and 10 per group for 6- and 10-week treatments; graphs, means±SEM; *P<0.05, **P<0.01, ***P<0.001, Student t test; bar, 20 μm. BrdU indicates bromodeoxyuridine; NeuN, neuronal nuclei.

Figure 6. Doublecortin (Dcx) immunostaining reveals more new neurons in the hemisphere ipsilateral to the stroke in mice treated with the TrkB ligand LM22A-4. Dcx+ immunostaining from mice treated with saline or LM22A-4 and euthanized either 6 (A) or 10 weeks (B) after stroke. Graphs, quantification of area covered by Dcx immunostaining. n=6 to 7 and 10 per group for 6- and 10-week treatments; graph; mean±SEM; **P<0.01, Student t test; bar, 200 μm. TrkB indicates tropomyosin-related kinase B.
had a proneurogenic effect. However, because control mice received identical training, the observed increase in neurogenesis occurred on top of this background. LM22A-4 also increased neurogenesis in the absence of stroke and training (Figure 1).

We chose hypoxic–ischemic stroke because it is a high-throughput model that allows behavioral testing to be performed on large groups of mice in parallel, minimizing the effects of day-to-day variability. It results in a significant neurological injury similar to that seen in disabling human stroke. Hypoxia causes thrombosis on the side of unilateral common carotid occlusion, resulting in injury to the ipsilateral cortex, hippocampus, and striatum but sparing the contralateral hemisphere, which exhibits normal perfusion during hypoxia.14,15

With this study, LM22A-4 joins a short list of small molecules that can promote recovery from stroke when given days afterward. A GABAA antagonist (L655,708) and the ampakine CX1837 improve recovery when administered 3 and 5 days after stroke.16,17 The α-1 receptor agonist SA4503 and niaspan improve recovery when administered 2 days and 24 hours after a stroke, respectively.18,19 Both niaspan and CX1837 may act at least partially by stimulating BDNF production or downstream pathways,17,20 and α-1 receptor stimulation potentiates BDNF effects.21 LM22A-4 specifically binds and activates the BDNF receptor TrkB,9 and we find that this alone is sufficient to promote recovery.

The limitations of our study are that we have only studied 1 age (young adult), 1 gender (male), and 1 stroke model. Also, we examined axonal sprouting in mice with smaller, focal, strokes and a shorter treatment. Our results do not exclude a drug effect on axonal sprouting with either longer treatment or in a different stroke model. Older animals, sexes, additional stroke models, and other time windows will be important to evaluate in the future.

In patients with stroke, walking speed is critical in determining disability and independence; it defines who will be able to ambulate independently.22 The largest effect of LM22A-4 in our study was on limb swing speed. In saline-treated mice, this measure was reduced by stroke to nearly 50% of normal and did not recover. In contrast, limb swing speed in LM22A-4–treated mice recovered to a degree that it was statistically indistinguishable from limb swing speed in mice without stroke (Figure 4D). LM22A-4’s effects on gait may be due to an effect on striatal medium spiny neurons, which coordinate movement and are sensitive to the neurotrophic effects of BDNF. BDNF is normally delivered to these neurons in the dorsolateral striatum through inputs from the primary motor cortex,23 which is injured in this stroke model, and pharmacological TrkB activation may compensate for this loss. Increases in TrkB phosphorylation in striatal homogenates after 7 days of LM22A-4 treatment are consistent with this hypothesis (Figure 2). This suggests that LM22A-4 or another TrkB ligand will facilitate recovery from stroke and may reduce or prevent disability in patients after potentially disabling strokes.

Two types of cellular events have been implicated in functional recovery after stroke, neurogenesis and neuronal plasticity. BDNF may promote both by binding its 2 receptors, TrkB and p75 neurotrophin receptors. We tested here whether LM22A-4, as a small molecule ligand selective for the TrkB receptor, would be able to induce these effects. Neurogenesis occurs in the weeks and months after stroke and may augment damaged neuronal networks.24–26 BDNF may increase neurogenesis by increasing survival of new neurons,2,3,27 perhaps by strengthening new synapses. Plasticity likely contributes to functional recovery through rewiring of neuronal circuits, as reviewed recently.12,28,29 BDNF and TrkB are critical for activity-dependent plasticity and long-term potentiation.1,30 In contrast, activation of p75 neurotrophin receptors, BDNF’s low-affinity receptor, may inhibit axonal outgrowth and promote neuronal death.31,32 A selective TrkB ligand such as LM22A-4, which does not activate p75 neurotrophin receptors, may be more beneficial after stroke than BDNF. Our results show that LM22A-4 promotes neurogenesis, but we found no evidence for effects on axonal sprouting, a reflection of plasticity. This is consistent with data that BDNF signaling does not increase post-stroke cortical sprouting.17 Further study of sprouting patterns and synaptic structure and function in stroke models in which functional recovery can be quantified16,17 will be needed to conclusively determine if LM22A-4 has an effect on post-stroke plasticity.

Stimulation of TrkB signaling may have significant advantages as a treatment strategy in stroke. It will likely have positive effects at both early and late time points. Because LM22A-4 is a small molecule, it, or 1 of its derivatives, has the potential to be inexpensive and universally available to patients with stroke in a way that would not be practical for stem cell or even recombinant protein therapies. Finally, relative BDNF deficiency occurs with aging in association with decreased functional recovery,33 suggesting the possibility that TrkB pathway stimulation could improve recovery particularly in aged patients, who are disproportionately affected by stroke.

Acknowledgments
We thank Lijun Xu, Vivian Nguyen, and Danielle Simmons for surgical assistance; and Mehrdad Shamloo and Angelo Encarnacion of the Stanford Behavioral and Functional Neurosciences Laboratory for help with behavioral testing.

Sources of Funding
These studies were supported by National Institute of Neurological Disorders and Stroke KO8 NS050304 (Dr Buckwalter), the Stanford Stroke Center (Dr Buckwalter), Taube Philanthropies (Dr Longo), the Koret Foundation (Dr Longo), the Jean Perkins Foundation (Dr Longo), and the Department of Veteran’s Affairs (Dr Massa).

Disclosures
Dr Longo is a founder of PharmatrophiX, a company focused on the development of small molecule ligands for neurotrophin receptors.

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Stroke. 2012;43:1918-1924; originally published online April 24, 2012; doi: 10.1161/STROKEAHA.111.641878

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