Neural stem cells improve cognition via BDNF in a transgenic model of Alzheimer disease

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Neural stem cell (NSC) transplantation represents an unexplored approach for treating neurodegenerative disorders associated with cognitive decline such as Alzheimer disease (AD). Here, we used aged triple transgenic mice (3xTg-AD) that express pathogenic forms of amyloid precursor protein, presenilin, and tau to investigate the effect of neural stem cell transplantation on AD-related neuropathology and cognitive dysfunction. Interestingly, despite widespread and established Aß plaque and neurofibrillary tangle pathology, hippocampal neural stem cell transplantation rescues the spatial learning and memory deficits in aged 3xTg-AD mice. Remarkably, cognitive function is improved without altering Aß or tau pathology. Instead, the mechanism underlying the improved cognition involves a robust enhancement of hippocampal synaptic density, mediated by brain-derived neurotrophic factor (BDNF). Gain-of-function studies show that recombinant BDNF mimics the beneficial effects of NSC transplantation. Furthermore, loss-of-function studies show that depletion of NSC-derived BDNF fails to improve cognition or restore hippocampal synaptic density. Taken together, our findings demonstrate that neural stem cells can ameliorate complex behavioral deficits associated with widespread Alzheimer disease pathology via BDNF.

beta-amyloid | neurotrophin | synapse | tau | memory

For many neurological disorders, therapies are largely palliative and based on small molecule designs. However, studies have begun to examine stem cell-based therapies as a novel strategy to treat disorders such as Parkinson disease, ischemia, and spinal cord injury (1–3). Notably, each of these disorders primarily affects a single neuronal subtype and/or a restricted anatomical region. In contrast, Alzheimer disease (AD), the most prevalent age-related neurodegenerative disorder, is characterized by multiple pathologies that afflict several neuronal subtypes across multiple brain regions. Although stem cells have been suggested as a potential therapy for AD, to date, this approach has not been directly tested in transgenic AD models. Consequently, it is critical to obtain preclinical evidence to determine whether neural stem cell (NSC) transplantation offers symptomatic or disease-modifying effects for AD.

AD is characterized by the accumulation of 2 hallmark lesions, Aβ plaques and neurofibrillary tangles (NFTs), which are accompanied by gliosis and widespread neuronal and synaptic loss, causing progressive loss of memory and cognitive function. Here, we used 3xTg-AD mice, a model that recapitulates many of the salient features of AD (4, 5), to directly test the efficacy of NSC transplantation on AD-related cognitive and neuropathological outcomes.

Results

GFP-Neural Stem Cells Self-Renew and Are Multipotent. We first characterized the NSCs used in this study in vitro to determine if they are capable of self-renewal and multipotent; giving rise to all 3 neural lineages (neurons, astrocytes, and oligodendrocytes). Immunohistochemical analysis of undifferentiated NSCs revealed coexpression of GFP with sox-2 and nestin, well-established markers of multipotency (supporting information (SI) Fig. S1). In contrast, removal of mitogen and neuronal differentiation, induces expression of neuronal, and glial markers (Fig. S1). Thus, the GFP-expressing cells used represent multipotent, self-renewing neural stem cells.

Transplanted NSCs Rescue Cognitive Deficits. To determine whether NSC transplantation improves cognition in mice with well-established plaque and tangle pathology and behavioral deficits, we stereotactically delivered 100,000 murine NSCs to both hippocampi of 18-month-old 3xTg-AD (n = 18) and age-matched nonTg mice (n = 10, Fig. 1 *A–D*). As a control, additional age-matched 3xTg-AD (n = 9) and nonTg mice (n = 10) were injected with an equivalent volume of vehicle. To facilitate the identification of engrafted cells, we used NSCs derived from haplotype-matched GFP-expressing transgenic mice (6). Previous studies showed these GFP-NSCs engraft well into the murine brain, yielding neurons, astrocytes, and oligodendrocytes (6, 7). One month after NSC delivery, mice were habituated, trained, and tested on 2 hippocampal-dependent behavioral tasks: Morris water maze (MWM) and context-dependent novel object recognition.

As expected, vehicle-injected 3xTg-AD mice demonstrated significant impairments in the MWM relative to age-matched vehicle-injected nonTg mice (Fig. 1 *E–G*). In contrast, injection of NSCs into 3xTg-AD mice rescued the learning and memory impairments, as indicated by significantly shorter latencies during both MWM acquisition and probe trial testing (Fig. 1 *E–F*). Likewise, NSC-injected 3xTg-AD mice crossed the former location of the platform during probe trial testing, almost twice as often as vehicle-injected transgenic mice, demonstrating a strong memory for the platform's former location (Fig. 1*G*). Aged nonTg mice do not exhibit memory impairments; consequently, NSC delivery did not alter nonTg performance.

As a second test of hippocampal-dependent memory, we used context-dependent novel object recognition, a task that takes advantage of the innate preference mice have to recognize when previously familiar objects are placed into a novel context. Mice were exposed to 2 identical objects in a specific context (round cage), followed by exposure to a different pair of identical objects within a different context (square cage). Twenty-four hours later, mice were tested for their ability to recognize an object that had now been placed out-of-context. Unimpaired mice typically spend 70–80% of their time exploring an out-of-context object, whereas mice with hippocampal memory deficits perform at chance levels (50%). As expected, vehicle-injected 3xTg-AD mice were impaired, spend-

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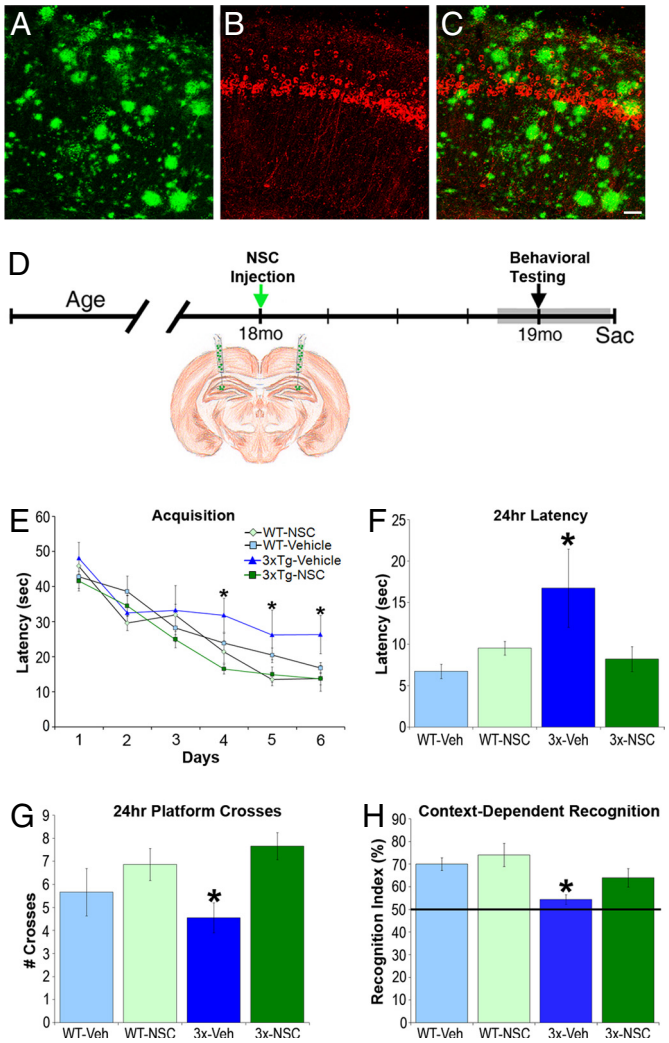

Fig. 1. Neural stem cell transplantation improves AD-related cognitive dysfunction. Eighteen-month-old 3xTg-AD mice exhibit robust plaques (*A* and *C*; green) and tangles (*B*; red) within the hippocampus. (*D*) 100,000 GFP-NSCs or vehicle control were stereotactically injected. Four weeks later, learning and memory was tested. (E) MWM training revealed that all groups learn the task. However, NSC-injected 3xTg-AD mice exhibit significantly shorter escape latencies on days 4–6 of training vs. vehicle-injected transgenics (ANOVA, $P < 0.04$, FPLSD $P < 0.029$). (F) In probe trial testing, NSC-injected 3xTg-AD mice also achieve significantly shorter latencies than vehicle-injected 3xTg-AD mice and perform equivalent to nonTg controls (ANOVA, $P = 0.042$, FPLSD $P = 0.010$). (G) Likewise, NSC-injected 3xTg-AD mice cross the former platform location more often than control-injected transgenics (ANOVA, $P = 0.014$, FPLSD $P = 0.002$). (H) Context-dependent object recognition testing reveals that vehicle-injected 3xTg-AD mice are impaired, spending an equivalent amount of time exploring both objects. In contrast, NSC-injected 3xTg-AD mice exhibit a partial but significant recovery in this task (ANOVA, $P = 0.0047$, FPLSD $P = 0.041$, vs. vehicle-injected 3xTg-AD mice). Data presented as mean ± SEM. (Scale bar, 45 μm.)

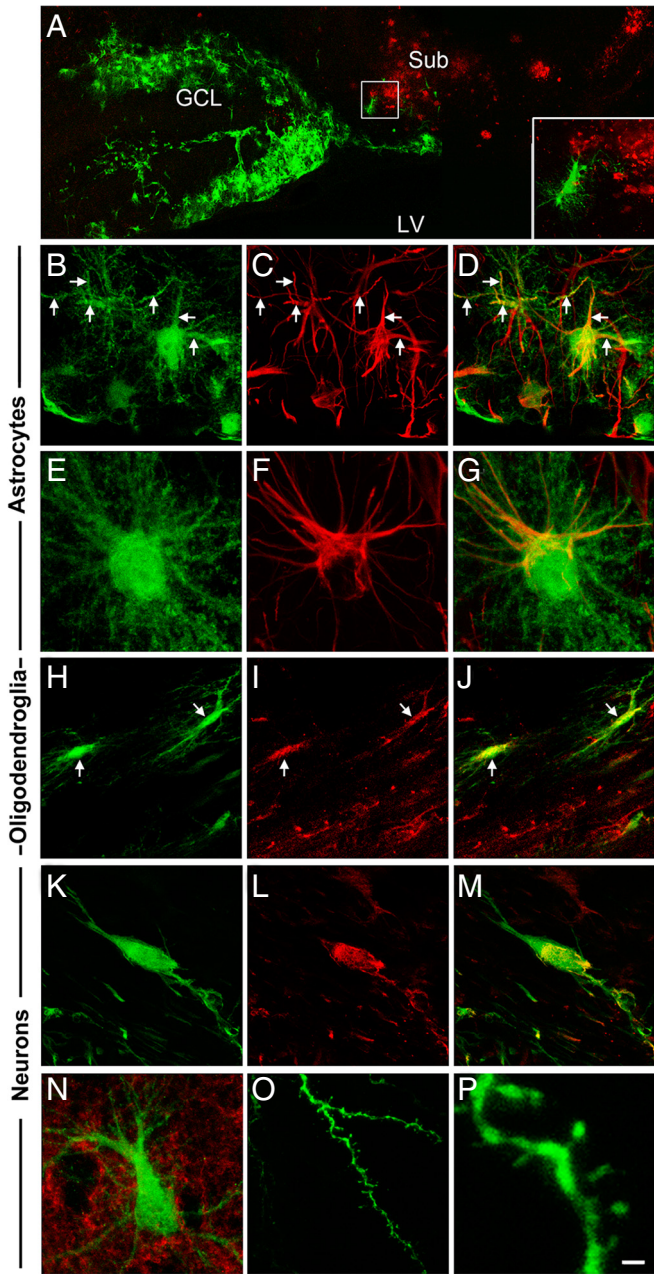

Fig. 2. Engrafted neural stem cells differentiate into neurons, astrocytes, and oligodendrocytes. (*A*) GFP-expressing NSCs (green) migrated from their hippocampal injection site and engrafted predominately within 2 major regions, either surrounding the granule cell layer (GCL) of the dentate gyrus or within white matter tracts including the fimbria fornix and corpus callosum. Confocal microscopy revealed only limited chemotaxis of NSCs toward Aβ plaques (red; *A Inset*). LV: lateral ventricle, Sub: subiculum. Engrafted NSCs differentiated into all 3 lineages. (*B–G*) The majority of NSCs (39.4%) differentiated into astrocytes, coexpressing GFAP (red). (*H–J*) Within white matter tracts, NSCs often exhibited oligodendroglial morphology and 26.4% coexpressed the oligodendroglial marker (GalC; red). (*K–M*) Far fewer NSCs adopted a neuronal fate (5.8%) as evidenced by expression of doublecortin (red). (N) neuronally-differentiated NSCs with pyramidal cell morphology surrounded by presynaptic terminals (synaptophysin, red), or exhibiting dendritic spine architecture (*O–P*) were also occasionally observed. (Scale bars, 80 μm in *A*, 12 μm in *B–D*, 5 μm in *E–G* and *N*, 15 μm in *H–J*, 7 μm in *K–M*, 1.5 μm in *O* and 200 nm in *P*.)

ing only 54% of their time exploring the out-of-context object (Fig. 1*H*). In contrast, NSC injection significantly improved the behavioral deficit in 3xTg-AD mice, (Fig. 1*H*). Thus, using 2 independent behavioral paradigms, we show that transplantation of NSCs in the brains of aged 3xTg-AD mice with advanced AD-related neuropathology rescues learning and memory deficits.

NSCs Differentiate and Show Limited Chemotaxis Toward Aβ Plaques. To begin to examine the mechanism by which NSC transplantation ameliorates cognitive impairment, we examined the migration and differentiation of engrafted cells. Confocal microscopy demonstrated that 5 weeks post injection, engrafted NSCs had differentiated into all 3 lineages: neurons, astrocytes, and oligodendrocytes (Fig. 2). Quantification revealed no significant differences in the

differentiation potential of NSCs injected into 3xTg-AD versus nonTg mice (Table S1). Similar to others (8), the majority of NSCs differentiated into astrocytes, with 39.4% ± 2.8 of GFP+ cells coexpressing glial-fibrillary acidic protein (GFAP) (Fig. 2 *B–G*). Notably, GFAP immunoreactivity localizes to the primary processes and soma of astrocytes, whereas GFP expression labeled the numerous fine processes of these cells. Approximately 26.4% ± 2.0 of GFP+ cells adopted an oligodendroglial fate, evidenced by coexpression of galactocerebroside (GalC) (Fig. 2 *H–J*). In contrast, only a small proportion of NSCs adopted a neuronal fate (5.8% ± 2.3), coexpressing the early neuronal marker doublecortin (red, Fig. 2 *K–M*). GFP cells that exhibited a pyramidal neuronal morphology and GFP-labeled dendritic spines were also occasionally observed (Fig. 2 *N–P*), although we cannot be certain that these labeled pyramidal cells were not the result of cell fusion rather than NSC-derived neurogenesis.

NSCs migrate toward areas of brain injury and gliosis (9) and aged 3xTg-AD mice exhibit significant gliosis in association with plaques. Hence, we examined whether NSCs were associated with Aß plaques. Interestingly, we observed only limited migration to or association with plaques, suggesting that plaques fail to elicit significant secretion of NSC-attracting chemokines (Fig. 2*A Inset*). NSCs did, however, show migration toward 2 other specific brain microenvironments. NSCs expressing oligodendroglial markers were frequently observed within white matter tracts adjacent to the hippocampus. Likewise, NSCs expressing astroglial or neuronal markers were commonly observed surrounding the outer edge of the dentate gyrus granule cell layer (Fig. 2*A*). Interestingly, endogenous adult neurogenesis occurs within the subgranular zone of the dentate gyrus (10), suggesting this brain region may also provide a favorable niche for transplanted NSCs.

Aβ and tau Pathology Are Not Altered by NSC Transplantation. Aβ-lowering treatments consistently rescue cognitive deficits in many AD transgenic models (4, 11, 12). More recently, we and others found that paradigms that diminish soluble tau can also ameliorate cognitive decline (13–15). In contrast, very few treatments restore cognition in AD models without attenuating at least one of these pathologies. To determine whether NSC delivery modulated either pathology, we performed extensive biochemical and histological analysis. Quantitative analysis of both soluble and insoluble levels of Aβ40 and Aβ42 by ELISA revealed no differences after NSC transplantation (Fig. 3 *A* and *B*). Soluble Aß oligomers are strongly implicated in AD-related cognitive dysfunction (16), but examination of oligomer levels using a conformational-sensitive antibody (A11) revealed no differences between groups (17) (Fig. 3 *C* and *H*). We also assessed plaque pathology using immunofluorescent density and dot blot analyses with the fibrillar conformational-sensitive antibody OC (18), again no differences between NSC- and vehicle-injected mice were detected, in line with the ELISA quantification of insoluble Aβ (Fig. 3 *D–F* and *H*). Likewise, APP, insulin-degrading enzyme (IDE), a major Aβ-degrading protease, total human tau, and phospho-tau (ser396/404) showed no differences in steady-state levels (Fig. 3 *G–H*).

Somatodendritic mislocalization of tau is an important component of AD-related pathology; consequently, we assessed phospho-tau (ser199/202) distribution within hippocampal CA1 neurons by confocal microscopy and optical densitometry. However, no differences in the level or distribution of phospho-tau were detected within the pyramidal cell layer, stratum radiatum, or molecular layer of CA1 (Fig. 3 *I–K*). Thus, NSC transplantation rescues cognitive performance in 3xTg-AD mice without altering Aβ or tau pathology.

NSC-Induced Cognitive Improvement Is Accompanied by Increased Hippocampal Synaptic Density and Elevated BDNF. In AD, cognitive dysfunction correlates best, not with Aβ or tau pathology, but rather with hippocampal synaptic density (19). Growing evidence also

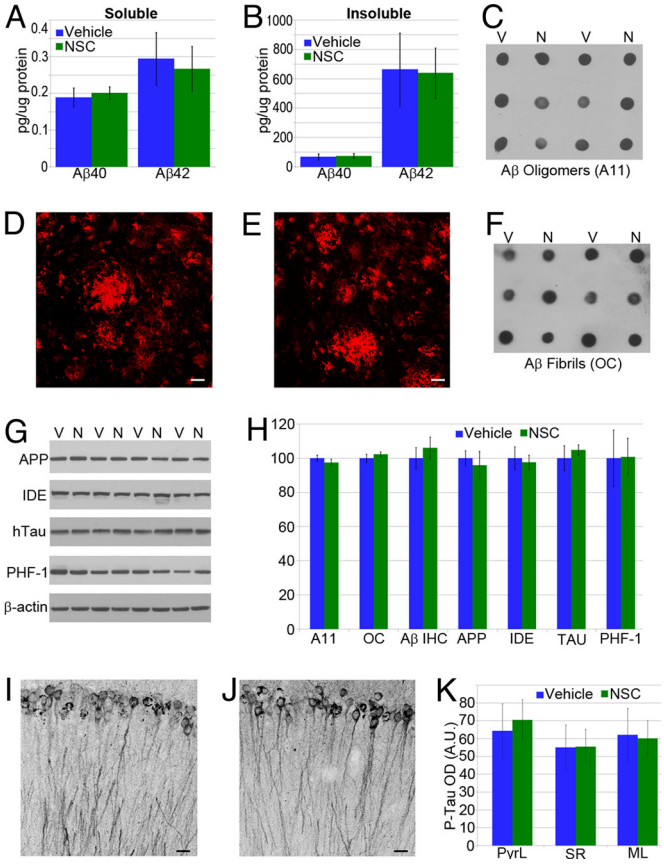

Fig. 3. Aβ and tau pathology is not altered by neural stem cell transplantation. Biochemical and histological analyses reveal no differences between vehicle- and NSC-injected 3xTg-AD mice in Aβ and tau pathology. ELISA for soluble (*A*) and insoluble (*B*) levels of Aβ40 and Aβ42 reveal no differences in brain Aβ load (ANOVA $P = 0.69$, mean ± SEM). (*C*) Likewise, dot-blot analysis with the oligomer-specific antibody A11 revealed no changes in soluble oligomers ($P = 0.36$). (*D–E*) Immunofluorescent microscopy demonstrated no differences in plaque load within the hippocampus of vehicle-injected (*D*) versus NSC-injected (*E*) mice, quantified in (*H*) ($P = 0.52$). (*F*) Dot-blot analysis with the fibril-specific antibody OC also revealed no changes ($P = 0.40$). (*G*). Immunoblot analysis of APP, IDE, total human tau, and PHF-1 phosphorylated tau (S396/S404) also revealed no differences between groups ($P > 0.56$). Quantification in (*H*) is normalized to control levels ± SEM. (*G–I*) Immunofluorescent assessment of phosphorylated tau (S199/S202) within CA1 pyramidal neurons also revealed no differences in the somatodendritic localization of tau between vehicle- (*I*) and NSC- (*J*) injected mice; quantification in (*K*). Vehicle and NSC-injected samples denoted by V and N respectively. Pyr: CA1 pyramidal cell layer, SR: stratum radiatum. (Scale bars, 30 μm in *D–E*, 25 μm in *I–J*.)

suggests that soluble Aβ oligomers impair cognition and long-term potentiation by binding to and altering synaptic shape, composition, and density (16, 20). Given that NSC transplantation had no apparent effect on either Aβ or tau, yet rescued cognitive function, we hypothesized that NSCs might compensate for the toxic effects of oligomers on synaptic connectivity. Hence, we examined synaptic density in the stratum radiatum of CA1 of the hippocampus. CA1 pyramidal neurons exhibit extensive tangle pathology in AD and in 3xTg-AD mice, and projections onto CA1 dendrites within the stratum radiatum play an important role in memory. To quantify synaptic density, we used a well-established method previously used in landmark studies examining both human AD cases and APP transgenic mice (19, 21). The presynaptic protein synaptophysin was immunofluorescently labeled and analyzed by Z-stack confocal microscopy followed by optical densitometry. Comparison of NSC- versus vehicle-injected 3xTg-AD mice revealed a highly significant 67% increase in synaptic density in the NSC-injected mice (Fig. 4

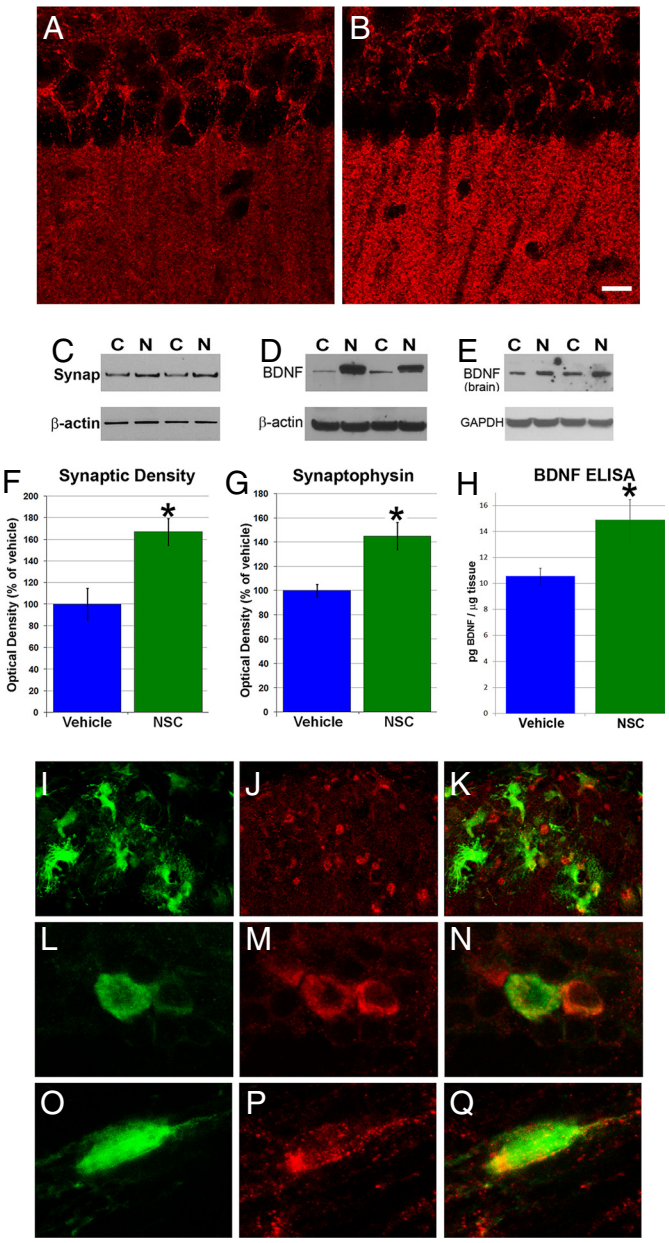

Fig. 4. Neural stem cells increase synaptic density and produce BDNF. (*A–C*) Confocal optical densitometry revealed that compared to vehicle-injected 3xTg-AD mice (*A*), NSC-injected 3xTg-AD mice (*B*) exhibit a 67% increase in synaptophysin immunoreactivity within the stratum radiatum of CA1 (red puncta, quantified in *F*, $P = 0.0028$). (*C*) Immunoblot analysis of vehicle-injected (C) and NSC-injected 3xTg-AD mice (N) also revealed a significant 45% increase in synaptophysin following NSC transplantation (quantified in G, $P = 0.01$). (*D*) Comparison of NSC-derived (N) and control neuronal cell line (C) cultures revealed a high level of BDNF expression within NSCs. (*E*) In vivo analysis by Western blot and further quantification by ELISA (*H*) demonstrated a significant elevation of BDNF in NSC-injected mice (N) versus control-injected mice (C) ($P = 0.047$). (*I*) Confocal microscopy for GFP-NSCs (*F*) and pro-BDNF (*J*) demonstrate that engrafted NSCs often coexpress pro-BDNF (*K*). Expression of pro-BDNF within adjacent non-GFP-expressing endogenous cells was also observed (*K*, red only). (*L–Q*) Colocalization between GFP NSCs (green) and total BDNF was also observed. Data in *F–H* is normalized to control levels and shown as mean ± SEM. (Scale bars, 10 μm in *A* and *B*, 18 μm in *I–K*, 3 μm in *L–Q*.)

A–B, quantified in *F*). Western blot analysis confirmed these findings, revealing a 45% increase in synaptophysin protein levels in NSC-injected mice (Fig. 4*C*, quantified in *G*). Thus, this difference provides a structural basis for the observed improvement in cognition in the NSC-injected 3xTg-AD mice.

A primary mechanism by which synaptic connectivity is altered involves expression and responsiveness to neurotrophins. Within the adult hippocampus, BDNF in particular, plays a central role in the synaptic remodeling associated with memory (22). Previous studies showed that NSCs can express high levels of BDNF in vitro (23), therefore we next examined whether altered BDNF expression might underlie the observed synaptic and cognitive effects of NSC transplantation.

Immunoblot analysis revealed high BDNF level in NSC cultures versus a control neuronal cell line (Fig. 4*D*). Hence, we examined whether engrafted NSCs produced high levels of BDNF 5 weeks after transplantation, using 3 approaches. Firstly, we examined BDNF levels in NSC-injected versus vehicle-injected brains by immunoblotting (Fig. 4*E*). We also quantified BDNF levels by sandwich ELISA, revealing a significant elevation within NSC-injected mice (Fig. 4*H*). Lastly, we used double-label confocal microscopy and detected NSCs that continued to express BDNF 5 weeks after transplantation (Fig. 4 *I–Q*). These data suggested that NSC-induced elevation of BDNF might mediate the improved cognitive performance in 3xTg-AD mice by enhancing hippocampal synaptic connectivity. However, to more fully examine this putative mechanism, we performed 3 additional critical experiments.

To further examine the effects of NSC-derived BDNF in vitro, we used a microfluidic cell culture device that greatly facilitates axonal-specific experimentation and analysis (24). Using this device, we determined whether conditioned media derived from differentiated NSCs promote axonal outgrowth, and if so, whether BDNF mediates this effect. After 7 days, primary neurons were exposed to 1 of 3 treatments: control conditioned media, NSC-conditioned media, or NSC-conditioned media in which BDNF had been removed by immunoprecipitation (Fig. S2). Quantification of axonal outgrowth demonstrated that NSC-derived conditioned media markedly increased axonal growth and density versus control treatments. In contrast, BDNF-preabsorbed NSC conditioned media exhibited little or no growth-promoting activity, instead producing morphological changes consistent with axonal degeneration. Thus, NSC-derived BDNF greatly enhances axonal outgrowth in vitro.

To further examine the mechanism of NSC-induced cognitive recovery in vivo, we asked whether BDNF alone was sufficient to elicit similar behavioral improvements by delivering recombinant BDNF (0.25 μg) or vehicle into the hippocampi of aged 3xTg-AD mice ($n = 8$). Five days after surgery, mice were trained and tested on MWM. Acute BDNF injection did not significantly affect task acquisition (Fig. S3*A*). However, in probe-trial testing, animals that received BDNF performed significantly better than vehicle-injected controls, crossing the former platform location almost twice as frequently as controls ($P = 0.02$, Fig. S3*C*). Thus, BDNF alone is sufficient to improve memory performance. To determine whether the mechanism of behavioral recovery induced by BDNF was similar to that observed for NSCs, we analyzed synaptic density, revealing a significant increase within the hippocampus (Fig. S3 *D–F*).

BDNF Is Essential for NSC-Induced Cognitive Rescue. To determine whether BDNF is required for NSC-induced cognitive rescue, we used lentiviral delivery of shRNA to stably knockdown BDNF expression in NSCs and then transplanted these cells into 3xTg-AD mice. Pilot experiments identified an optimal shRNA construct from among 5 constructs targeting the coding region of murine BDNF. NSCs were transduced with the shRNA construct, stably selected, and BDNF expression was examined by Western blot, revealing a 78% reduction in BDNF versus control NSCs (Fig. 5*A*). Next, we delivered BDNF shRNA NSCs into the hippocampus of 18-month-old 3xTg-AD mice ($n = 9$). An additional set of age-matched 3xTg-AD mice ($n = 10$) were injected with control NSCs and mice were also compared to age and sex-matched vehicle-

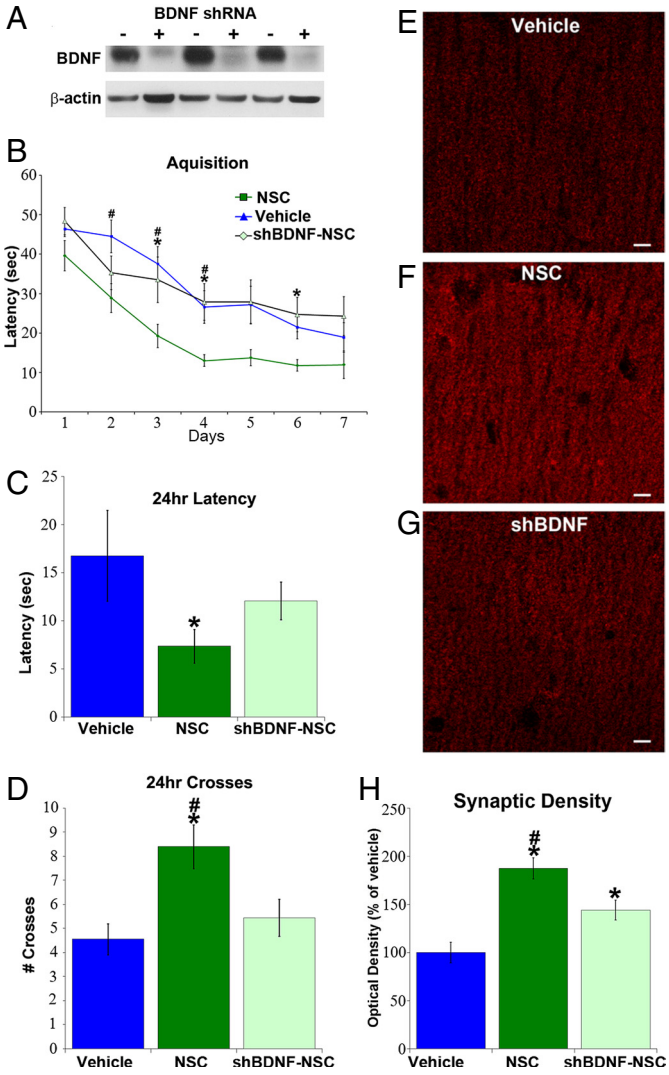

Fig. 5. BDNF is necessary for NSC-induced cognitive rescue and increased synaptic density. (*A*) NSC BDNF expression was reduced 78% by shRNA knockdown ($P = 0.0006$). Eighteen-month-old 3xTg-AD mice were injected with BDNF-shRNA NSCs or control NSCs and 1 month later tested in the MWM. (*B*) Control NSCs improved cognition leading to shorter escape latencies on days 3–6 of training (ANOVA $P = 0.027$, FPLSD $P < 0.03$). In contrast, BDNF-shRNA NSC-injected mice showed no improvement and were impaired vs. control NSC-injected animals (FPLSD $P < 0.013$). In probe trial testing (*C*) control NSC-injected animals found the former platform location significantly faster than vehicle-injected mice (FPLSD $P = 0.037$), whereas BDNF-shRNA NSC-injected mice performed at an intermediate level that was not significantly different from vehicle-injected mice (FPLSD $P = 0.29$). (*D*) Likewise, control NSC-injected mice crossed the former platform location significantly more than the other 2 groups (ANOVA $P = 0.0049$, FPLSD $P < 0.0136$). To determine whether NSC-derived BDNF also mediates the observed changes in synaptic density, synaptophysin was quantified. Vehicle-injected animals (*E*) exhibited significantly less immunoreactive puncta than control NSC-injected animals (*F*, ANOVA, $P < 0.0001$, FPLSD $P = 0.0001$). In contrast, BDNF-shRNA NSC-injected animals exhibited an intermediate level of synaptic density (*G*), significantly greater than vehicle-injected mice ($P = 0.014$) but also less than control NSC-injected mice ($P = 0.0093$). (*H*) Significance vs. vehicle-injected mice denoted by *, vs. BDNF-shRNA NSC-injected mice denoted by #. (Scale bar, 10 μm.)

injected controls ($n = 9$). One month after surgery, mice were tested in the MWM and then killed.

These experiments clearly demonstrate that BDNF knockdown within NSCs abolishes the cognitive benefits of NSC delivery (Fig. 5 *B–D*). During MWM acquisition, control NSC-injected 3xTg-AD mice performed significantly better than vehicle-injected transgenic mice. In contrast, BDNF-shRNA NSC-injected mice showed no improvement versus vehicle-injected controls and performed significantly worse than control NSC-injected animals, exhibiting longer escape latencies on days 2–4 of training (Fig. 5*B*). Probe trial testing also clearly indicates that BDNF is necessary for NSC-induced improvements in memory (Fig. 5 *C–D*). Control NSCs-injected animals found the former platform location significantly faster than vehicle-injected mice, whereas BDNF-shRNA NSC-injected animals performed at an intermediate level, not significantly different from vehicle-injected mice. Likewise, control NSC-injected mice crossed the former location of the platform significantly more times than both vehicle-injected and BDNF-shRNA NSC-injected animals (Fig. 5*D*).

Taken together, these data clearly demonstrate that NSC-derived BDNF is necessary for the cognitive benefits induced by NSC transplantation. To determine whether NSC-derived BDNF is also necessary for the observed elevation of hippocampal synaptic density, we analyzed synaptophysin within CA1 stratum radiatum. Vehicle-injected animals exhibited significantly less synaptophysin immunoreactivity than control-NSC injected animals (Fig. 5 *E* and *F*). In contrast, BDNF-shRNA NSC-injected animals exhibited an intermediate level of synaptic density (Fig. 5*G*), which was significantly greater than vehicle-injected mice but also significantly less than control NSC-injected mice (Fig. 5*H*). Thus, BDNF shRNA experiments clearly demonstrate that NSC-derived BDNF is necessary for the cognitive improvement and increased synaptic density observed in NSC-treated 3xTg-AD mice.

Discussion

Here we report that NSC-injection rescues the cognitive phenotype in transgenic mice that exhibit advanced AD-related pathology. Interestingly, the beneficial effects of NSCs on cognition are not mediated by alteration of either Aβ or tau pathology. Instead, NSC-derived cells elevate hippocampal BDNF, leading to increased synaptic density and restoring hippocampal-dependent cognition. Both gain-of-function and loss-of-function experiments highlight the critical role of BDNF in the mechanism of recovery.

The incidence of AD is projected to almost quadruple by mid-century (25), yet currently approved therapies offer only marginal benefits. Interestingly, imaging studies suggest that plaques and tangles accumulate for years or even decades before AD is clinically diagnosed (26). Hence, treatments that enhance or improve cognition, especially in the presence of well-established plaque and tangle pathology are urgently needed. Stem cells are being actively studied for their potential to replace dead or diseased cells (27). However, experimental therapies that rely on this approach are thought to require the precise differentiation of a single cellular phenotype, a challenging task. In the case of AD, multiple neuronal subtypes are affected within several brain regions. Perhaps for this reason, no studies have yet explored the potential utility of stem cell-based therapies for AD.

Recent studies, however, demonstrate that stem cell-based approaches can provide beneficial effects via alternative mechanisms, providing what is often referred to as a "bystander effect" (28). For example, NSC transplantation can improve function by providing missing or defective enzymes or modulating inflammation (8). Alternatively, NSCs can preserve endogenous neuronal function by providing trophic support (29).

Our findings clearly demonstrate that NSCs improve cognition in 3xTg-AD mice via a bystander-like mechanism. Transplanted NSCs differentiate into neurons, astrocytes, and oligodendrocytes but have no effect on Aβ and tau pathology. Instead, NSC transplantation produces a marked increase in hippocampal synaptic density that is mediated at least in part via increased BDNF. To examine this mechanism, we used several complementary approaches. NSC-injected mice exhibit significantly higher levels of BDNF and increased hippocampal synaptic density and microfluidic experi-

ments demonstrate that NSC-derived BDNF enhances axonal outgrowth. To determine whether BDNF is itself sufficient to mediate cognitive recovery, we delivered recombinant BDNF to the hippocampus of aged 3xTg-AD mice. These gain-of-function experiments revealed a significant improvement in memory and synaptophysin levels that closely mimicked the effects of NSC transplantation. Lastly, we used a genetic approach to dramatically reduce BDNF expression in NSCs and determine the precise role of NSC-derived BDNF in cognitive recovery. BDNF knockdown in transplanted NSCs not only failed to improve cognition, but also diminished the effect of transplantation on synaptic density. Taken together, these multiple lines of evidence demonstrate that NSC transplantation improves cognition in large part by elevating BDNF expression and enhancing endogenous synaptic connectivity. However, it is likely that other NSC-derived factors or effects may also play a more minor role in the observed cognitive and synaptic recovery.

Interestingly, a growing body of work suggests that disruptions in BDNF may play a critical role in the etiology of AD. BDNF is decreased within the brains, serum, and CSF of patients with mild cognitive impairment and AD and can even correlate with mini-mental state examination scores (30, 31). Aβ oligomer treatment also decreases BDNF mRNA expression (32). Thus, reduced BDNF is clearly implicated in AD.

Our finding that NSC transplantation improves cognition in 18-month-old 3xTg-AD mice is particularly exciting as this is 1 of only 2 treatments that have been shown to restore learning and memory in transgenic mice that exhibit well-established Aβ and NFT pathologies, with immunotherapy being the only other treatment (13). It is also particularly notable that NSC transplantation was effective without concomitantly reducing Aß or tau pathology, an effect that has not previously been demonstrated in this and many other models of AD. Taken together our findings indicate the potent effect neurotrophins have on brain function despite rampant pathology and suggest that the further development of cell-based therapies or other methods that modulate neurotrophin levels could provide a viable approach to treat AD (33).

Materials and Methods

Mice, NSC Transplantation, and Behavior. All animal procedures were performed in accordance with National Institutes of Health and University of California guidelines. 3xTg-AD mice have previously been characterized and are maintained on a hybrid C57BL6/129 background (4, 5) (see *SI Methods*). Both C57Bl6 and 129 background strains have the identical MHC haplotype (H-2b) as the NSCs used. NSCs were harvested from postnatal day 1 GFP transgenic mice (C57/Bl6, gift of Dr. M. Young) as described (6), grown as adherent monolayers, and transplanted at passage 14. Before transplantation cells were trypsinized, washed, triturated, and filtered through a 70 μm mesh. NSCs were then counted and resuspended 50,000 cells/μl in vehicle (1x HBSS w/ 20 ng/ml hEGF). Stereotactic delivery of NSCs followed previously described methods (7) and used coordinates relative to Bregma of: AP: -2.06, ML: ± 1.75, DV: -1.75 (see *SI Methods*). Behavioral studies examined hippocampal-dependent learning and memory using Morris water maze and novel object recognition tasks following standard protocols (4) (see *SI Methods*).

BDNF shRNA Knockdown. Lentiviral particles were produced from 5 shRNA plasmid clones (Sigma Mission NM_007540) by cotransfecting 293FT cells with lentiviral packaging mix (Invitrogen). Twenty-four hours later, media was replaced with serum-free media and 1 day later conditioned media collected, diluted 1:1 in NSC media, and applied to NSCs overnight. Transduced NSCs were then selected with puromycin (1 μg/ml). Five stable lines were generated and compared and optimal knockdown was achieved with Sigma cat#TRC0000065384 (sequence:CCGGGCAGTATTTCTACGAGACCAACTCGAGT TGGTCTCGTAGAAATACTGCTTTTTG). BDNF shRNA-NSCs were then prepared and transplanted as detailed above.

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