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Activated CX3CL1/Smad2 signals prevent neuronal loss and Alzheimer’s tau pathology-mediated cognitive dysfunction

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Abstract

Neurofibrillary tangles likely cause neurodegeneration in Alzheimer's disease (AD). We demonstrate that the CX3CL1 C-terminal domain can upregulate neurogenesis, which may ameliorate neurodegeneration. Here we generated transgenic (Tg-CX3CL1) mice by overexpressing CX3CL1 in neurons. Tg-CX3CL1 mice exhibit enhanced neurogenesis in both subgranular and subventricular zones. This enhanced neurogenesis correlates well with elevated expression of TGFβ2, TGFβ3 and activation of their downstream signaling molecule Smad2. Intriguingly, the enhanced adult neurogenesis was mitigated when Smad2 expression was deleted in neurons, supporting a role for the CX3CL1-TGFβ2/3-Smad2 pathway in the control of adult neurogenesis. When Tg-CX3CL1 mice were crossed with Alzheimer's PS19 mice, which overexpress a tau P301S mutation and exhibit age-dependent neurofibrillary tangles and neurodegeneration, overexpressed CX3CL1 in both male and female mice was sufficient to rescue the neurodegeneration, increase survival time, and improve cognitive function. Hence, we provide in vivo evidence that CX3CL1 is a strong activator of adult neurogenesis, reduces neuronal loss, and improves cognitive function in Alzheimer's disease.

Significance Statement: This study will be the first to demonstrate the enhanced neurogenesis by overexpressed CX3CL1 is mitigated by disruption of Smad2 signaling and independent of its interaction with CX3CR1. Overexpression of CX3CL1 lengthens the lifespan of PS19 tau mice by
enhancing adult neurogenesis while having minimal effect on tau pathology.

Enhancing neuronal CX3CL1, mainly the C-terminal fragment, is a therapeutic strategy for blocking or reversing neuronal loss in Alzheimer’s or related neurodegenerative disease patients.
Introduction

CX3CL1 is a membrane-anchored signaling chemokine (Bazan et al., 1997; Pan et al., 1997), recognized to exert its signaling function through interaction with its sole cognate receptor CX3CR1 (Imai et al., 1997). CX3CL1 is shed near the transmembrane domain to release the C-XXX-C motif containing N-terminal domain for the binding to CX3CR1. In the brain, CX3CL1 is mainly and constitutively expressed by neurons, while CX3CR1 is predominantly expressed by microglia (Hatori et al., 2002). This neuron-glia cross-talk has been shown to regulate or modulate important functions such as microglial migration and activation, neuronal protection or impairment depending on normal or diseased conditions (Limatola and Ransohoff, 2014; Paolicelli et al., 2014; Lauro et al., 2015). Recently, the short intracellular domain of CX3CL1 has been shown to mediate a back-signaling function independent of CX3CR1; Tg-CX3CL1-ct mice, which overexpress only this C-terminal domain, exhibit enhanced neurogenesis (Fan et al., 2019).

The role of CX3CL1 in Alzheimer’s disease (AD) pathogenesis remains to be understood (Guedes et al., 2018). AD is characterized by amyloid deposition due to excessive accumulation of β-amyloid peptides (Aβ), neurofibrillary tangles due to hyperphosphorylation of tau protein, neuronal loss and synaptic dysfunction in the brain (Braak and Braak, 1997). Altered CX3CL1/CX3CR1 signaling activity likely regulates pathological changes in various ways. Deficiency in CX3CR1 may reduce amyloid deposition in AD mouse models overexpressing mutant APP or APP/PS1 (Lee et al., 2010) and prevents
neuronal loss in a 3XTg AD mouse model overexpressing mutant APP/PS1/tau (Fuhrmann et al., 2010). Mice deficient in CX3CL1, the ligand of CX3CR1, were also shown to have reduced Aβ deposition (Lee et al., 2014). Others noted that decreasing CX3CL1/CX3CR1 signaling activity exacerbated plaque-independent cognitive deficits in APP-overexpressing AD mouse models (Cho et al., 2011); deficiency in CX3CR1 was found to increase tau hyperphosphorylation (Bhaskar et al., 2010; Cho et al., 2011; Lee et al., 2014) and to increase neurodegeneration (Cardona et al., 2006). Overexpressed soluble CX3CL1 via adenoviral transformation in a Tg4510 model of tauopathy reduces tau pathology and prevents neurodegeneration, but shows no effect on amyloid deposition in mice overexpressing mutant APP and PS1 (Nash et al., 2013). The postulated mechanisms include the CX3CR1-mediated microglial migration and activation, and altered release of pro-inflammatory cytokines such as IL-1β and TNFα (Fuhrmann et al., 2010; Lee et al., 2014). Intriguingly, overexpressing only CX3CL1-ct in 5xFAD mice can reduce amyloid deposition and neuronal loss (Fan et al., 2019), which is independent of CX3CR1 function. Hence, the N- and C-terminal fragments of CX3CL1 can impact AD pathogenesis by acting on different pathways.

To further understand the in vivo roles of CX3CL1, we generated transgenic Tg-CX3CL1 mice, which overexpress CX3CL1 under the control of a prion promoter, and examined the in vivo roles of overexpressed neuronal CX3CL1. We observed overexpressed neuronal CX3CL1 did not obviously elicit changes in inflammatory responses in Tg-CX3CL1 mice based on the
morphology of microglia in comparison to wild-type (WT) littermates. Instead, Tg-CX3CL1 mice exhibited enhanced neurogenesis in both subventricular (SVZ) and subgranular (SGZ) zones. TGFβ2/3 expression was elevated and phosphorylation of Smad2 was increased in Tg-CX3CL1 mouse brains, similar to mice overexpressing only the neuronal CX3CL1 C-terminal fragment (Fan et al., 2019). If the smad2 gene was ablated in forebrain neurons by conditional deletion, enhanced neurogenesis in Tg-CX3CL1 was mitigated. Importantly, enhanced expression of CX3CL1 in Alzheimer’s transgenic PS19 mice caused a reduction in neurodegeneration, improved cognitive function and increased lifespan. Together, our results reveal that increased neurogenesis by more neuronal CX3CL1 is sufficient to reverse neuronal loss in AD.
Materials and Methods

Generation of Tg-CX3CL1 mice

Tg-CX3CL1 was generated by insertion of HA-tagged CX3CL1 human cDNA between exon 2 and exon 3 of mouse prion protein gene DNA at two unique Xho I sites in the Mo-Prp plasmid vector, and prion promoter predominantly drives expression of transgene in neurons (Borchelt et al., 1997). The pair of primers for the transgene used for PCR-based genotyping was 5'-ACCTGTAGCTTTGC-3' and 5'-TTCAGACGGAGCAT-3'. Mouse RTN3-specific primers (5'-CACAGGTAGAAATGGCCAAGA-3' and 5'-CAGCTTGAATGACAGACTTATAGACT-3') were included in the PCR to identify mouse sequence. The identified and selected founder line was crossed with C57BL6/J mice for at least 6 generations to obtain relative pure genetic background. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Lerner Research Institute in compliance with the guidelines established by the Public Health Service Guide for the Care and Use of Laboratory Animals. For animal studies as discussed in each figure, both male and female mice were used and usually in relatively balanced numbers (see summary in Table 1).

Western blotting and antibodies

Total proteins were extracted from freshly dissected mouse tissue using modified RIPA buffer. Equal amounts of protein (30 or 50 μg) were resolved on a NuPAGE Bis-Tris gel (Invitrogen, Carlsbad, CA) and transferred onto a nitrocellulose membrane (Invitrogen). Routinely, at least two mice from each genotype group
were used for western blot analysis. Antibodies used for western are: p-Smad2 (#3104, RRID: AB_390732), Smad2/3 (#8685;RRID: AB_10889933), p-Smad1 (9553s; RRID: AB_2107775), Smad1 (#6944; RRID: AB_10860070), p-tau-PHF13 (#9632s; RRID: AB_2266237), Cell Signaling Technology, Danvers, MA, USA); Calretinin (SC-11644; RRID:AB_634545), TGFβ1 (SC-146; RRID:AB_632486), TGFβ2 (SC-90; RRID:AB_2303237), TGFβ3 (SC-82; RRID:AB_2202303), SNAP25 (SC-20038; RRID:AB_628264) (Santa Cruz Biotechnology Inc., Santa Cruz, CA); Synaptophysin mouse antibody (S5768, RRID:AB_477523, Sigma-Aldrich); p-Tau AT8 (MN1020; RRID:AB_223647, Thermo Fisher Scientific); Tau-PHF (AT-180, Thr 231, MN1040, RRID:AB_223649, Thermo Fisher Scientific) actin (A5441; RRID: AB_476744) (Sigma-Aldrich, St. Louis, MO); BrdU (ab6326; RRID:AB_305426, Abcam, Cambridge, MA); NeuN (MAB 377; RRID: AB_2298772, EMD Millipore, Billerica, MA); HA (#1867423; RRID: AB_10094468) (Roche, Indianapolis, IN); Iba1 (Wako Chemicals, AB_839504, 1:500); SMI22 (Covance, AB_2313859, 1:1000). HRP-conjugated secondary antibodies were used and visualized using enhanced chemiluminescence (Thermo Fisher Scientific, Waltham, MA).

**Immunohistochemical staining**

Immunohistochemical staining experiments were performed according to standard methods as previously described (Hu et al., 2018). Routinely, half mouse brains were surgically removed, fixed in 4% paraformaldehyde for 12 h, and immersed in 20% sucrose overnight at 4°C. Brains were sagittally sectioned (16μm thick) on a freezing microtome (Microm GmbH, Walldorf, Germany).
Sections were permeabilized with 0.3% Triton X–100 for 30 min. After being rinsed in phosphate-buffered saline (PBS) three times to remove detergent, the sections were heated by microwave in 0.05 M citrate–buffered saline (pH 6.0) for 5 min, blocked with 5% normal goat serum, and incubated with individual primary antibodies at the following dilutions: AT8 or AT180 (1:1000), Iba1 (1:500), SMI22 (1:1000), and synaptophysin (1:400). After washing with PBS three times, sections were incubated with secondary antibodies conjugated with Alexa fluor 488 or Alexa fluor 568 (Molecular Probes).

**Neurogenesis assays**

Neurogenesis was assessed by BrdU labeling experiments as described previously (Hu et al., 2013). Briefly, mice were injected intraperitoneal with 50 mg/kg of BrdU (Sigma, B9285) at P21 twice at 12 hour intervals. The animals were then sacrificed 24 hours after the first injection of BrdU. Brain samples were fixed in 4% PFA at 4°C overnight and then transferred into 30% sucrose in phosphate-buffered saline (PBS) for 2 days. Sections were pretreated with serial 1M and 2M HCl, stained with primary antibody BrdU, and then were processed by using VECTASTAIN-ABC Kits (Vector Laboratories, Burlingame, CA) and SigmaFAST DAB with metal enhancer tablets (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s instructions. After color development, the sections were dehydrated by incubation for 3 min in each of 70, 95, and 100% ethanol, followed by 5 min in two changes of xylenes and mounted using Permount. Labeled cells were quantified by stereology. Every third section (of a
total of 30 sections) was counted under a 100x objective and the sum was multiplied by 3 to estimate the total number of BrdU-positive cells in the region. Cells were counted if they were in or touching the SGZ, and cells were excluded if they were more than two cell diameters from the GCL.

To analyze neural differentiation, 15 mg/kg of BrdU was injected once daily for 5 days beginning at P11. The animals were sacrificed after an additional 21 or 28 days as specified in the text, and brain sections were examined with double labeling by using primary antibodies to BrdU (1:100) and NeuN (1:1000).

Y-maze and novel object recognition tests
Both behavioral tests were routinely performed in the lab and all procedures were previously described in detail (Luo et al., 2014). Animals at the age of eight months were used in this study and the number of animals is specified in each figure. Y-maze tests were conducted first and object recognition tests were conducted a week later.

Contextual Fear Conditioning Test
Contextual fear conditioning test was conducted routinely in the Yan lab (Luo et al., 2014; Hu et al., 2018). The first day involves testing mice for the conditioning by placing them in the chamber (Med Associates, St. Albans, VT) for 3 min (phase A) before the stimulating sound at 2800 Hz and 85 dB for 30 sec (phase B, conditioning stimulus). The last 2 sec was given a 0.7 mA continuous foot shock (phase C, unconditioned stimulus). Phases B and C were repeated once after mice rested for 30 sec in the chamber. The second day was to test mice for their contextual memory in the same chamber for 3 min without either sound or
foot shock. On the third day, mice were tested for their tone memory in a different chamber environment with the sound, but no foot shock. Fear memory of the mice was measured as the percentage of freezing, which was defined as the percentage of time completely lacking movement, except for respiration, in intervals of 5 sec.

Statistical analysis

Quantitative data are presented as means ± SEM. All experiments were independently repeated at least three times. Statistical analyses were conducted using Prism 6 software (GraphPad Software). Statistical comparisons between groups were analyzed for significance by one-way analysis of variance (ANOVA) with Tukey’s post hoc test and Student’s t test. In the case of multiple variances, two way Anova test with post-hoc Bonferroni and three way Anova test with Tukey’s multiple comparisons were also performed. Significant P values are denoted by the use of asterisks in the text and figures (*P < 0.05, **P < 0.01, ***P < 0.001). Error bars in each case represent standard error of the mean.
RESULTS

Transgenic mice overexpressing CX3CL1 show enhanced neurogenesis

To explore the in vivo role of neuronal CX3CL1, we generated transgenic Tg-CX3CL1 mice, which were engineered to overexpress CX3CL1 transgene, including the heavily glycosylated mucin-like stalk of fractalkine (Fong et al., 2000), in neurons of broad brain regions by the murine prion promoter (Figure 1A). After pronuclear microinjection of the linearized CX3CL1 transgene, which contains a C-terminal HA tag, thirteen independent founder lines of mice were detected to express different levels of CX3CL1 and four lines were chosen for further characterizations. In this study, the line with approximately 3-fold greater expression of CX3CL1 than WT was used (Figure 1B). As expected, the CX3CL1-HA transgene, detected by an antibody to HA, was predominantly expressed in neurons and was not readily detectable in glia at young and older ages (Figure 1C).

Tg-CX3CL1 mice exhibited no obvious growth defects or reduction in viability. Although the CX3CL1/CX3CR1 pathway has been suggested to modulate microglial migration and activation, enhanced expression of CX3CL1 did not visibly alter either microglia numbers or morphology in young Tg-CX3CL1 mouse brains compared to WT littermates (examples of 2- and 6-month-old samples are shown in Figure 1-1). Although functional assays or molecular changes remained to be established, marginal activation of microglia might be present in older Tg-CX3CL1 mouse brains (examples of 11- and 20-month-old samples are shown in Figure 1-1). For astrocytes, no obvious astrocytosis was
visibly detected in various stages (early developments in Figure 1-2A and example of an 11-month-old brain is shown in Figure 1-2B). Instead, overexpression of CX3CL1 was found to induce neurogenesis, as a visible increase in the thickness of dentate granule cell layers was noted in many Tg-CX3CL1 brains (examples in Figure 1C).

To validate increased neurogenesis, we performed a BrdU pulse labeling experiment by intraperitoneal injection of BrdU at postnatal day 21 (P21). After a 12 hr pulse, we examined the total number of BrdU-positive cells by immunohistochemistry. It is clear that the total numbers of BrdU+-cells were greater in the Tg-CX3CL1 dentate gyrus (DG) when compared to WT littermate controls (Figure 2A). For further validation, we conducted stereological quantification and showed that BrdU+-cells in Tg-CX3CL1 subgranular zone (SGZ) were increased by approximately 22.2% compared to that in WT (Figure 2B; 9475.2±912.4 cells per Tg-CX3CL1 DG vs. 7464.7±594.7 per WT DG, n=5 animals, P<0.001). To analyze neural differentiation, 15 mg/kg of BrdU was injected once daily for 5 days beginning at P11 and sacrificed after growing for an additional 4 weeks. Increased neurogenesis was also evident in P45 Tg-CX3CL1 SGZ (Figure 2C). Further quantification validated this increase (Figure 2D).

We also compared BrdU pulse-labeled dividing cells in 8-month-old brains, as neurogenesis was much less active compared to that in early developmental stages. Unlike neurogenesis during early development, significantly fewer BrdU+ cells were detectable in WT SGZ (Figure 2E) and even fewer BrdU+ cells were
found in WT SVZ (Figure 2F), consistent with the fact that adult neurogenesis mainly occurs in the SGZ (Goncalves et al., 2016). Remarkably, increases in BrdU+ cells in Tg-CX3CL1 SGZ and SVZ were again evident, showing more active neurogenesis in both neurogenic niches of Tg-CX3CL1 brains (Figure 2E-F). Therefore, we demonstrate that enhanced expression of neuronal CX3CL1 induces neurogenesis in the adult mouse brain.

**Overexpressed CX3CL1 activates TGFβ-Smad signaling**

We recently showed that membrane-bound CX3CL1 is sequentially cleaved by α-/β- and γ-secretases to release the intracellular domain (CX3CL1-ICD), which can translocate into the nucleus to activate gene expression. Mice overexpressing only CX3CL1-ct leads to increased expression of TGFβ2 and TGFβ3 and the activation of the TGFβ-Smad signaling pathway (Fan et al. 2019).

Herein, our western blot analyses using hippocampal lysates from Tg-CX3CL1 mice again showed higher protein levels of TGFβ3 (Figure 3A). Although overall protein levels of TGFβ2 were less readily detected, quantification showed a significant increase in the Tg-CX3CL1 hippocampus (Figure 3B). Their increased expressions correlated with activation of the downstream molecule Smad2 as levels of phosphorylated Smad2 (p-Smad2) were also clearly increased in Tg-CX3CL1 (Figure 3A-B). Noticeably, p-Smad1 and total Smad1 were not significantly different. Hence, in the brain, Smad2 is markedly activated by overexpressed CX3CL1 in neurons, consistent with that in Tg-CX3CL1-ct mice.
Down-regulation of Smad2 signaling blocks CX3CL1-mediated neurogenesis

Smad2-deficient mice were previously shown to be embryonic lethal (Nomura and Li, 1998; Weinstein et al., 1998) and therefore Smad2^{fl/fl} mice with loxP sites flanking exon 2 of Smad2 were later produced (Ju et al., 2006). To determine the contribution of Smad2 to neurogenesis, we bred Tg-CX3CL1 mice with Smad2 conditional deletion mice (https://www.jax.org/strain/022074). We chose CaMKIIα-cre transgenic mice (T29-1, https://www.jax.org/strain/005359) to express cre recombinase and delete Smad2 in forebrain neurons in CX3CL1/Smad2^{fl/fl} mice. Our western blot analyses showed the expressed cre recombinase effectively deleted Smad2 in CX3CL1/Smad2^{fl/fl} and CX3CL1/Smad2^{fl/+} mouse neurons as total Smad2 and p-Smad2 levels were correspondingly reduced (Figure 4A). Tg-CX3CL1 with homozygous deletion of Smad2 showed dramatic reduction in Smad2 expression while heterozygosity of Smad2 resulted in less reduction in Smad2 and p-Smad2 compared to littermate controls, which expressed no cre in Smad2^{fl/fl} mice.

We then compared neurogenesis using the same approach as described above, and found that deletion of Smad2 in P21 Samd2^{fl/fl} mice by cre recombinase in the forebrain significantly decreased neurogenesis in their SGZ regions when compared to non-floxed littermate controls (referred as WT, Figure 4B). While enhanced neurogenesis was consistently shown in Tg-CX3CL1 SGZ, this enhanced neurogenesis was significantly reduced in CX3CL1/Smad2^{fl/fl} mouse SGZ. Further quantification confirmed this significant reduction (Figure...
Overexpressed CX3CL1 in AD Tau mouse models reverses neuronal loss

Since overexpressed neuronal CX3CL1 enhances neurogenesis, we asked whether this enhancement could reverse neurodegeneration in AD mouse models. It has been reported that PS19 transgenic mice (P301S Tg mice), expressing the P301S mutant form of human microtubule-associated protein tau driven by the Prp promoter, showed a significant loss of neurons in the hippocampus and ventricular dilatation (brain atrophy) starting between eight to nine months of age (Yoshiyama et al., 2007). Yoshiyama et al. further reported a phenotype of hunched back and paralysis, followed by inability to intake food by 10 months in this model, with approximately 80% of the animals dying by 12 months of age.

We therefore compared PS19 transgenic mice with those expressing CX3CL1 transgene (Tg-CX3CL1/PS19 mice) in a longitudinal study. We noted that expression of tau transgene was not significantly altered by overexpressed CX3CL1 in CX3CL1/PS19 mice, nor were the levels of AT8-positive or PHF-13-positive tau based on western analyses (Figure 5A; example of 8-month-old...
samples; bar graphs in 5B). Smad2 phosphorylation in PS19 transgenic mice was lower compared to WT littermates, but was reversed to higher in Tg-CX3CL1/PS19 mice, although not as high as that in Tg-CX3CL1 mice. We also noted that PS19 mice showed a significant reduction in synaptophysin and SNAP25 (Figure 5A), two critical proteins for synaptic strength. CX3CL1 overexpression markedly reversed this reduction.

Mature neurons in 8-month-old mice were labeled with NeuN antibody. At this age, PS19 transgenic mice had already exhibited significant loss of granule cells in the dentate gyrus compared to WT littermates, while this loss was reversed by overexpressed CX3CL1 (Figure 5C). The loss of neurons in CA regions was more evident in 11-month-old PS19 transgenic mice (Figure 5D; quantification shown in 5E), consistent with this prior report (Yoshiyama et al., 2007). The most dramatic loss of neurons was seen in cortical regions, as the density of cortical neurons was clearly sparse (Figure 5F; quantification shown in 5G). Our data showed that increased expression of CX3CL1 was able to reverse this neuronal loss in the two examined age groups.

Overexpressed CX3CL1 in AD mouse models increases lifespan and enhances learning and memory

The lifespan of PS19 transgenic mice have been previously shown to be shortened (Yoshiyama et al., 2007), and we found no PS19 transgenic mice were able to survive beyond 12 months of age in our experiments. The occurrence of paralysis in PS19 transgenic cohorts was seen at an average of 321±23 days
(Figure 6A-B), consistent with the original report (Yoshiyama et al., 2007). Remarkably, a majority of Tg-CX3CL1/PS19 mice survived beyond 12 months with an average of 397±41 days. We also noted that 3 out of 15 of these mice (20%) lived up to the age of 18 months (all mice were sacrificed at this age for biochemical analyses). Tg-CX3CL1 mice had a normal lifespan compared to WT littermates, although it is not examined whether they would have a longer lifespan.

Enhanced neurogenesis in Tg-CX3CL1 mouse brains correspondingly increased synaptic density labeled by synaptophysin (Figure 6C), consistent with the changes in synaptophysin protein levels shown in Figure 5A. On the contrary, synaptic density in PS19 transgenic mouse brains was reduced dramatically, and this reduction was reversed by overexpressed CX3CL1 (Figure 6C).

Improved cognitive functions by overexpressed CX3CL1

To examine the functional changes arising from increased synaptic density by CX3CL1 expression, we conducted learning and memory tests. We first conducted a Y-maze spontaneous alternation test, which measures spatial working memory. While all four genotypes of mice (WT, Tau-transgenic, CX3CL1-transgenic, and CX3CL1/PS19 double transgenic) at the age of 8 months showed similar numbers of total entrances in the three arms, PS19 tau transgenic mice displayed significantly less spontaneous alternations (Figure 7A; n=12, *P<0.01, two-way ANOVA). Mice overexpressing CX3CL1 showed a slight increase in total entrances, but no obvious enhancement in working memory.
despite increased neurogenesis at this age (n=12, *P<0.05, one-way ANOVA).

When CX3CL1 was overexpressed in PS19 Tau transgenic background, reduced working memory was rescued, likely related to reduced neuronal loss (n=12, *P<0.05, two-way ANOVA).

We then conducted a novel object recognition test, which is used to test for recognition memory (Bevins and Besheer, 2006; Antunes and Biala, 2012). This test measures exploring time spent with a novel object compared to a familiar object. While all four genotypes of mice spent a similar total time exploring objects, PS19 transgenic mice showed no discrimination between novel vs familiar objects (Figure 7B). This impairment was remarkably rescued by the overexpression of CX3CL1 in Tg-CX3CL1/PS19 mice (n=12, ***P<0.001, two way Anova test).

We also conducted a contextual fear conditioning test according to the previously published procedure (Luo et al., 2014). PS19 mice clearly exhibited impaired learning and memory when they were placed back into the conditioned chamber on day 2, with only 18.24 ± 3.948% (N=10) freezing time compared to 41.10 ± 4.935% in WT mice (N=11, Figure 7C). This impaired conditioned fear memory was ameliorated when CX3CL1 was overexpressed in Tg-CX3CL1/PS19 mice (42.77 ± 7.204% in 12 Tg-CX3CL1/PS19 mice vs 48.75 ± 5.553% in 13 Tg-CX3CL1 mice; *P<0.05, three way Anova test).
CX3CL1 is a type I transmembrane cytokine; its N-terminal domain contains a C-XXX-C motif, which is capable of binding to CX3CR1 to exert signaling transduction between neurons and microglia. Under normal conditions, CX3CL1 is mainly expressed by neurons and at a low level by astrocytes, but not microglia; CX3CR1 is predominantly expressed by microglia (Hatori et al., 2002; Hughes et al., 2002; Hulshof et al., 2003). In our initial study of mice overexpressing neuronal CX3CL1 (Tg-CX3CL1), we found that changes of microglia were not readily visible in young Tg-CX3CL1 mouse brains (Figure 1-1). Further studies are required to confirm potential molecular and functional changes in microglia when CX3CL1 is overexpressed. It will be more informative when mice overexpressing only C-terminal CX3CL1 will be examined in parallel.

Activation of microglia is evident in PS19 transgenic mice, clearly arising from abnormal tau phosphorylation (see microglia morphology in Figure 5C-D). It appears microglial activation in Tg-CX3CL1/PS19 mice was comparable to that in PS19. This is consistent with the fact that total levels of AT8 or PHF13 on the western blot were not visibly altered by overexpressed CX3CL1. Confocal examination of brain sections with antibody AT8 also showed that strong AT8-immunity of neurons in PS19 transgenic and Tg-CX3CL1/PS19 mouse brains were comparable and tau aggregations were also noted in Tg-CX3CL1/PS19 brains (Figure 5-1); non-specific background such as blood vessels was detected in WT brains and Tg-CX3CL1. Intriguingly, one prior study expressing soluble CX3CL1 via adenoviral transformation in a Tg4510 model of tauopathy was
shown to reduce tau pathology (Nash 2013), and CX3CR1 deficiency impair
microglia to internalize tau aggregates (Bolos et al., 2017). Glia cells contribute
to the reduction of AD pathology including removal of tau aggregates (Leyns and
Holtzman, 2017; Wang et al., 2018; Vogels et al., 2019). Although enhanced
expression of neuronal CX3CL1 in the PS19 transgenic background has no
obvious effect on reducing tau pathology, it prevents or reduces
neurodegeneration. It should be noted that multiple effects existed in our study,
which may differ from other two studies including the differences of mouse
models and full-length vs soluble form of CX3CL1 as well as the C-terminal back
signaling (Fan et al., 2019).

Enhanced neurogenesis in Tg-CX3CL1 mice begins during early
development and lasts throughout their lifespan, consistent with that seen in Tg-
CX3CL1-ct mice. This enhanced neurogenesis occurs not only in the SGZ,
which is the predominant region for adult neurogenesis, but also in the SVZ of
the lateral ventricle. A prior study by infusive expression of soluble CX3CL1 was
also found to enhance hippocampal neurogenesis, and this effect was blocked by
infusion of neutralizing antibody against CX3CR1 (Bachstetter et al., 2011). It
also suggests that loss of CX3CR1 function impairs hippocampal neurogenesis.
In this study, we cannot exclude the contribution of overexpressed CX3CL1 in
Tg-CX3CL1 mice to enhanced neurogenesis through the CX3CL1-CX3CR1
effect, but we have focused our attention on the role of CX3CL1-ct back signaling,
mainly because Tg-CX3CL1-ct mice have similarly enhanced neurogenesis in
SGZ and SVZ (Fan et al., 2019).
We demonstrate that mice expressing CX3CL1-ct exhibit increased expression of TGFβ2 and TGFβ3, but not TGFβ1. TGF-β2/3 evokes its physiological functions through binding to two different receptor serine/threonine kinases, type I and type II TGF-β receptors (Shi and Massague, 2003). Binding of TGF-β2/3 to the receptor, which is a tetrameric complex, will trigger recruitment of the anchor protein SARA (Smad anchor for receptor activation) and subsequently increase phosphorylation of the cytoplasmic signal transducers Smad2 and Smad3. Phosphorylated Smad2 and Smad3 will translocate into the nucleus, where they regulate gene expression. We showed that mice overexpressing either CX3CL1 (Figure 3 in this study) or CX3CL1-ct (in Fan et al., 2019) similarly elevate expression of TGFβ2 and TGFβ3 as well as activation of Smad2 and Smad3, indicating that this effect is CX3CR1-independent.

Smad2 and Smad3 appear to control gene expression in a combination of homo- and heterodimeric states, and they display a differential role in embryonic development. Complete deletion of the Smad2 gene in mice causes embryonic lethality due to failure to establish an anterior-posterior axis of gastrulation and mesoderm formation (Weinstein et al., 2000), while mice deficient in the Smad3 gene are viable and can survive for several months (Datto et al., 1999; Yang et al., 1999), indicating the existence of differential roles in vivo. To determine whether Smad2 has a role in neurogenesis, we obtained a mouse model with conditional deletion of Smad2 (Liu et al., 2004). Mice with Smad2 deleted from their forebrain neurons showed a small reduction in adult neurogenesis in our study, and this reduction could reverse the enhanced
neurogenesis in Tg-CX3CL1 (Figure 4), indicating that TGFβ2/3-Smad2 pathway contributes to the enhanced neurogenesis by CX3CL1 ICD.

The observed neurogenesis in Tg-CX3CL1 mice prompted us to test whether enhanced adult neurogenesis would be beneficial for reversing neuronal loss in neurodegenerative diseases such as Alzheimer’s disease. A recent study shows exacerbated cognitive deficits in AD by disrupted adult neurogenesis (Hollands et al., 2017). We chose PS19 mice for this purpose as this mouse model displays neuronal loss in broad brain regions and the degrees of neuronal loss and brain atrophy correlate with severely reduced survival time, with few PS19 mice living beyond 12 month of age (Yoshiyama et al., 2007). As demonstrated in Figure 6A, overexpressed CX3CL1 in the PS19 mouse model significantly increased the lifespan. Most Tg-CX3CL1/PS19 mice, except two, lived beyond 12 months of age with three eventually sacrificed at the age of 18-months for analyses. This increase can likely be attributed to the reversal of neuronal loss, as newborn neurons may replenish degenerated neurons to enhance synaptic connectivity. We noted that synaptic density was significantly greater in the Tg-CX3CL1/PS19 than in PS19 hippocampal region (Figure 6B). Consequently, cognitive function, measured by performances on Y-maze, open field and contextual fear conditioning tests, was significantly improved in Tg-CX3CL1/PS19 compared to PS19 mice.

In summary, we demonstrate a role of CX3CL1 in promoting adult neurogenesis in broad brain regions (SGZ and SVZ), through activating the TGFβ-Smad2 signaling pathway. This effect is attributable to the C-terminal
fragment, which can induce expression of TGFβ2/3 -Smad2 pathway. Since hippocampal neurogenesis persists in the aged and diseased human brain (Tobin et al., 2019), enhancing neuronal CX3CL1, mainly the C-terminal fragment, may well be a viable therapeutic strategy for blocking or reversing neuronal loss in treating patients of Alzheimer’s or related neurodegenerative diseases.

Acknowledgements

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**Figure 1: Generation of transgenic mice overexpressing CX3CL1.** (A) Schematic illustration of the construct used for expressing CX3CL1 transgene driven by the prion promoter. Human CX3CL1 cDNA (373 amino acids) contains one copy of an HA tag at its C-terminal end. (B) Tg-CX3CL1 mice express about three-fold more CX3CL1 compared to wild-type (WT) mice. (C) CX3CL1 transgene is expressed by neurons. As shown in the example of 11-month-old Tg-CX3CL1 brains, CX3CL1-HA is co-localized with NeuN, and this co-localization was clearer in the enlarged images. Tg-CX3CL1 mice cause no visible morphological changes in microglia (Figure 1-1) and astrocytes (Figure 1-2). An antibody specific to the HA tag was used for staining the transgene. Scale bar is 30 μm.

**Figure 2: Enhanced neurogenesis following overexpression of CX3CL1.** (A) Postnatal day 21 (P21) mice were BrdU-pulsed labeled for 24 hrs and fixed brain sections were labeled by antibody to BrdU. BrdU-positive cells were significantly more in Tg-CX3CL1 SGZ compared to wild-type controls. (B) Stereological quantification confirmed greater numbers of BrdU-positive dividing cells in Tg-CX3CL1 SGZ. N=5 pairs of mice (**P<0.01, student t-test). (C) Mice at P45 were pulsed-labeled by BrdU and examined by confocal staining with antibody to BrdU (red) and NeuN (green) for mature neurons. (D) BrdU-positive cells were counted on one out of every ten continuous sections and the number reflected an average per section. N=5 pairs of mice (*P<0.05). (E-F) Eight-month-old mice were similarly BrdU-pulse labeled and examined by immunohistochemical
staining with antibody to BrdU. Visibly more BrdU-positive cells were found in both Tg-CX3CL1 SGZ (E) and SVZ (F). Scale bar is 30 μm.

**Figure 3: Overexpressed CX3CL1 induces TGFβ2/3-Smad2 signaling.** (A) Mouse hippocampal lysates (4-month old) were examined by western analyses, and the antibodies used in the assay were specified. (B) Bar graphs show normalized comparisons to the loading control (actin). N=3 independent experiments (*P<0.05, **P<0.01, student t-test).

**Figure 4: Deletion of Smad2 reverses enhanced neurogenesis in Tg-CX3CL1 mice.** (A) Deletion of Smad2 in mice was achieved by breeding Smad2fl/fl mice with CamK2a-Cre mice. Hippocampal and cortical protein lysates from the indicated genotypes of mice were examined by Smad2 and phosphorylated Smad2 antibodies by western blotting. Reduced expression of Smad2 was seen in CX3CL1/Smad2fl/+ mice and near ablation was observed in CX3CL1/Smad2fl/fl mice. Loading controls are reflected by non-specific reacted bands and actin antibody. (B) P21 mice were pulse-labeled by BrdU and fixed brain sections were examined by immunohistochemical staining with anti-BrdU antibody. Scale bar is 30 μm. (C) Quantification was conducted to confirm significance in neurogenesis among genotypes of mice (N=3 animals, **P<0.05, two way Anova test with post-hoc Bonferroni).
Figure 5: Overexpressed neuronal CX3CL1 rescues neuronal loss in PS19 mice. (A) PS19 mice expressing mutant tau (P301S) in neurons. Western blot analyses from hippocampal lysates showed significantly elevated expression of total tau, and this elevation was maintained in Tg-CX3CL1/PS19 mice. Phosphorylated tau, detected by AT8 or PF13 antibodies, showed similar hyperphosphorylation in PS19 and Tg-CX3CL1/PS19 brains. Moreover, AT8-positive punctas remained in Tg-CX3CL1/PS19 brains (Figure 5-1). CX3CL1 levels were lowered in PS19 brains, but were elevated due to CX3CL1 overexpression validated by two antibodies. P-Smad2 and synaptophysin were lower in PS19 mice, and this reduction was reversed by overexpression of CX3CL1. (B) Bar graphs showed relative levels of AT8-positive tau/tau, PHF13-positive tau/tau and p-Smad2/Smad2 (N=2 in each group, three experiments, *P<0.05, ***P<0.001, two way Anova test with post-hoc Bonferroni). (C) Loss of neurons in CA and dentate gyrus was visible in PS19 mice, and this reduction was partially rescued. (D) Enlarged view of CA1 showing dramatic loss of neurons labeled by NeuN (green) in PS19 mice. Activation of microglia, labeled by Iba1 antibody, was evident in PS19 mice and also in T-CX3CL1/PS19 mice. (F) Loss of neurons was visible in the cortical region of PS19 mice, and this reduction was significantly reversed after overexpression of CX3CL1 in Tg-CX3CL1/PS19 mice. Scale bar is 60 μm. (E, G) Quantification of neurons in images of hippocampal (E) and cortical regions (G) as specified (N=3 slices each, *P<0.05, ***P<0.001, two way Anova test with post-hoc Bonferroni).
Figure 6: Increased survival times and synaptic densities in Tg-CX3CL1/PS19 mice. (A-B) PS19 mice were not able to survival beyond 12 months, and this survival time was significantly increased by overexpression of CX3CL1 in Tg-CX3CL1/PS19 mice. However, none of the Tg-CX3CL1/PS19 mice lived to 18 months of age as reflected in Kaplan-Meier curve (N=12 in the PS19 and 14 in the Tg-CX3CL1/PS19 mice group, ***P<0.001, student t-test). Both WT and Tg-CX3CL1 mice have normal lifespans and easily live beyond 2 years of age. (C) Confocal staining was performed to examine synaptic density labeled by synaptophysin in the hippocampus, and PS1 mice showed severely reduced synaptic density. Scale bar is 30 μm.

Figure 7: Increased learning and memory in Tg-CX3CL1/PS19 mice. (A) A Y-maze test was conducted to assess spatial learning memory, and PS1 mice showed a clear reduction in spontaneous alternations, while this reduction was reversed in Tg-CX3CL1/PS19 mice. Total arm entrances on the Y-maze did not differ among the different genotypes of mice. (B) A novel object recognition test was conducted to assess recognition memory. PS19 mice showed no discrimination between novel and familiar objects on the test, and this impairment was reversed by overexpression of CX3CL1 in Tg-CX3CL1/PS19 mice. Each dot represents one animal. (C) A contextual fear conditioning test was performed to assess hippocampus-dependent memory in a fear conditioning chamber. No significant differences in the percentage of freeze time during the day 1 conditioning test among different genotypes of mice. Percentage of total freeze
time of mice on Day 2 was compared to reflect contextual learning ability. PS19 mice exhibited significantly decreased freezing time, suggesting impairment in memory, while mice expressing CX3CL1 exhibited reversal of this impairment. The test of tone-mediated cue memory on Day 3 was also reduced in PS19, although not reaching statistical significance (*P<0.05, **P<0.01, ***P<0.001, N=10 in WT and PS19, 11 in CX3CL1 and 13 in CX3/PS19 groups, two way Anova test with post-hoc Bonferroni for Y-maze experiment, and three way Anova test with Tukey's multiple comparisons for novel object recognition and contextual fear conditioning experiments).

Extended Figure Legends

Figure 1-1: Tg-CX3CL1 mice show no obvious changes in microglia. Fixed brain sections from different age groups of Tg-CX3CL1 mice and their littermates were reacted with antibody Iba1 to label microglia. From 2-, 6-, 11- and 22-month-old samples, no obvious increase in the total number of microglia was observed, and no ramification of microglia or morphological changes were seen in Tg-CX3CL1 brains, indicating no microglial proliferation or activation was caused by overexpression of neuronal CX3CL1. Scale bar is 30 μm.

Figure 1-2: No obvious changes in astrocytosis or astrogenesis are induced by neuronal CX3CL1 overexpression. (A) Fixed brain samples were stained with Smi22 antibody for GFAP to label astrocytes. No significant
increase in the numbers of astrocytes or morphological changes of astrocytes were noted in samples from postnatal day 11 (P11), P20, or P30. Hence, overexpression of CX3CL1 does not promote astrogenesis or cause astrocytosis.

(B) Confocal staining of fixed brain samples by SMI22 (green) for GFAP, expressed by astrocytes, showed no obvious changes in proliferation or morphological changes in astrocytes caused by overexpression of CX3CL1, labeled by HA antibody in red. **Scale bar is 30 μm**

**Figure 5-1: No alteration in tau pathology results from overexpression of CX3CL1.** Fixed brain sections were labeled with AT8 antibody for phosphorylated tau and HA antibody for CX3CL1 transgene. It is clear that overexpressing CX3CL1 in Tg-CX3CL1/PS19 mice did not significantly alter the tau pathology. **Scale bar is 30 μm.**
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Figure A: The hCX3CL1-HA transgene in TG-CX3CL1 mice.

Figure B: Western blot analysis of CX3CL1 and Actin in WT and CX3CL1 hippocampus. Relative protein level to control: WT vs. CX3CL1.

Figure C: Immunofluorescence staining of NeuN (green), HA (red), and Merged images. 11M.
Fan et al. Figure 3
A

B

C

**Figure 4**

**A**

Western blot analysis showing P-Smad2, Smad2, and Actin levels in Hippocampus and Cortex tissues from wild type (WT), CX3CL1/Smad2^+/−, CX3CL1/Smad2^−/−, WT, CX3CL1/Smad2^+/−, and CX3CL1/Smad2^−/− mice.

**B**

Immunohistochemical staining for P21 in WT, Tg-CX3CL1, Smad2^+/−, and Tg-CX3CL1/Smad2^−/− mice. Scale bar: 30μm.

**C**

Bar graph showing the number of BrdU^+ cells per section for WT, CX3CL1, Smad2^+/−, and Tg-CX3CL1/Smad2^−/− mice. **p < 0.01**.
A

Onset of Paralysis (days)

PS19  CX3CL1/PS19

***

B

Percent survival

Months

PS19  CX3CL1/PS19

C

WT  CX3CL1  PS19  CX3CL1/PS19

HA

11M

Synaptophysin

Merge

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A

Total Entrances

WT  PS19  CX3CL1  CX3CL1/PS19

B

% of Total Time Spent

WT  PS19  CX3CL1  CX3CL1/PS19

Familiar  Novel

C

Spontaneous Alternations (%)

WT  PS19  CX3CL1  CX3CL1/PS19

Freeze (% Time)

Conditioning  Contrast  Cue

Fan et al. Figure7
Table 1: Mice used for different experiments are summarized.

| Figure | Technique | Age of animals       |
|--------|-----------|----------------------|
| 1C     | IHC       | P30 and 11 months    |
| 2A-B   | 2 pulses of BrdU every 12 hours followed by IHC | P21          |
| 2C-D   | BrdU daily for 5 days followed by 4 weeks then IHC | P11 to P45   |
| 2E-F   | 2 pulses of BrdU every 12 hours followed by IHC | 8 months     |
| 3A     | Brain lysates | P30                |
| 4A     | Brain lysates | P30                |
| 4B     | 2 pulses of BrdU every 12 hours followed by IHC | P21          |
| 5A     | Brain lysates | 7 months          |
| 5B     | IHC       | 8 months            |
| 5C-D   | IHC       | 11 months           |
| 6A     | Survival curves | Up to 18 months |
| 6B     | IHC       | 11 months           |
| 7A-C   | Behavioral assay | 8 months          |
| S1     | IHC       | 2, 6, 11, and 20 months |
| S2     | IHC       | P11, P20, P30, and 11 months |

Abbreviation: IHC, immunohistochemistry; P, postnatal day.