Archetypal Arg169Cys Mutation in NOTCH3 Does Not Drive the Pathogenesis in Cerebral Autosomal Dominant Arteriopathy With Subcortical Infarcts and Leucoencephalopathy via a Loss-of-Function Mechanism

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Background and Purpose—Cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy, the most common heritable small vessel disease of the brain, is caused by dominant mutations in the NOTCH3 receptor that stereotypically lead to age-dependent Notch3ECD deposition in the vessels. NOTCH3 loss of function has been demonstrated for few mutations. However, whether this finding applies to all mutations and whether a loss-of-function mechanism drives the manifestations of the disease remain yet unknown. This study investigated the in vivo functionality of the Arg169Cys archetypal mutation.

Methods—We used mice with constitutive or conditional reduction of NOTCH3 activity, mice harboring the Arg169Cys mutation at the endogenous Notch3 locus (Notch3Arg170Cys), and mice overexpressing the Arg169Cys NOTCH3 mutant (TgPAC-Notch3R169C) on either a Notch3 wild-type or a null background. NOTCH3 activity was monitored in the brain arteries by measuring the expression of NOTCH3 target genes using real-time polymerase chain reaction. Notch3ECD deposits were assessed by immunohistochemistry. Brain parenchyma was analyzed for vacuolation and myelin debris in the white matter and infarcts.

Results—We identified a subset of genes appropriate to detect NOTCH3 haploinsufficiency in the adult. Expression of these genes was unaltered in Notch3Arg170Cys mice, despite marked Notch3ECD deposits. Elimination of wild-type NOTCH3 did not influence the onset and burden of white matter lesions in 20-month-old TgPAC-Notch3R169C mice, and 20-month-old Notch3-null mice exhibited neither infarct nor white matter changes.

Conclusions—These data provide strong evidence that cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy can develop without impairment of NOTCH3 signaling and argue against a loss of NOTCH3 function as a general driving mechanism for white matter lesions in cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy. (Stroke. 2014;45:842-849.)

Key Words: CADASIL ■ etiology ■ mouse models ■ Notch3 protein, mouse

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL) is an archetypal small vessel disease of the brain caused by dominant mutations in the NOTCH3 receptor. Cardinal vascular lesions include deposition of granular osmiophilic material within the basal lamina of smooth muscle cells (SMCs), progressive SMC loss, and fibrosis of the media. Brain pathology involves white matter degeneration, lacunar infarcts, and, ultimately, brain atrophy.

NOTCH3 receptor is predominantly expressed in SMCs of small arteries and pericytes of brain capillaries. Mouse studies have demonstrated a critical role of NOTCH3 in brain artery morphogenesis and maturation during development. Specifically, mice completely lacking NOTCH3 exhibit major structural and functional arterial defects and are more susceptible to ischemic brain injury. On ligand binding, NOTCH3 undergoes a series of proteolytic cleavages that ultimately enable its intracellular domain to translocate to the nucleus, where it binds to the transcription factor recombining binding protein suppressor of hairless (RBPJ) to activate transcription of genes.

CADASIL mutations stereotypically lead to an uneven number of cysteine residues in 1 of the 34 epidermal growth factor repeats constituting the NOTCH3 extracellular domain (Notch3ECD). This promotes the abnormal accumulation of...
mutant Notch3CD at the plasma membrane of SMCs and in the granular osmiophilic material.\textsuperscript{4–6} Our recent work highlighted that aggregation/accretion of Notch3CD in the brain vessels is an early event, promoting the abnormal recruitment of functionally important extracellular matrix proteins that may ultimately cause multifactorial toxicity.\textsuperscript{5}

Although few mutations located in the ligand-binding domain of NOTCH3 (ie, epidermal growth factor repeats 10–11) unambiguously result in abolished NOTCH3 signaling and function, the lack of appropriate tools to directly assess the consequence of mutations on NOTCH3 transcriptional activity, in vivo, in the brain arteries has frustrated definitive resolution of this issue for the most prevalent mutations. On the one hand, normal surface expression, ligand binding, and signaling have been documented for several CADASIL-associated NOTCH3 mutations, although in vitro. Also, the Arg90Cys mutant receptor was shown to efficiently rescue the arterial defects of Notch3 KO mice.\textsuperscript{7–10} On the other hand, functional characterization of the human Arg1031Cys mutation has fueled proposals that CADASIL mutations may initially behave as wild-type or weak hypomorph alleles and acquire loss-of-function properties with aging.\textsuperscript{11} Hence, notwithstanding the toxic gain-of-function properties of mutant NOTCH3, this raises the following fundamental questions: (1) Do all the CADASIL mutants, in the end, consistently result in reduced NOTCH3 activity in the brain arteries? (2) Does a loss of essential NOTCH3 function contribute to the phenotypic manifestations of CADASIL?

The Arg169Cys mutation is a highly prevalent human mutation associated with a typical CADASIL phenotype.\textsuperscript{12} In this study, we tested the hypothesis that this archetypal mutation, which retains normal surface expression, ligand binding, and signaling in vitro,\textsuperscript{13,14} acquires, with aging, loss-of-function properties that drive the brain manifestations. To achieve this, we used mouse models with reduced NOTCH3-RBPJ activity and mouse models for human NOTCH3 Arg169Cys mutation (corresponding to Arg170Cys and Arg171Cys in mouse and rat NOTCH3, respectively) and conducted molecular analyses of the brain arteries as well as neuropathological studies. Together, the data argue against a critical contribution of a loss of NOTCH3 function in the pathogenesis of CADASIL.

Materials and Methods

Mice

The following lines Rbpjflox/flox (C57BL/6J),\textsuperscript{15} SMMHC-CreERT\textsuperscript{2} (C57BL/6J),\textsuperscript{16} Notch3\textsuperscript{−/−} (C57BL/6),\textsuperscript{11} Notch3\textsuperscript{R169C/+} (129EvSv:50% Swiss),\textsuperscript{14} and Notch3\textsuperscript{R169C/−/−} mice (genetic background overall 50% 129EvSv:50% Swiss)\textsuperscript{14} were described previously.

Mice lacking RBPJ in SMC in the adult were generated by breeding Rbpjflox/flox with SMMHC-CreERT\textsuperscript{2} mice. SMMHC-CreERT\textsuperscript{2} Rbpjflox/flox mice were subjected to intraperitoneal injection of 100 μL of tamoxifen (1 mg/mL; Sigma-Aldrich, Louis, MO) dissolved in sunflower oil at 10 weeks of age for 5 consecutive days. SMMHC-CreERT\textsuperscript{2} Rbpjflox/flox mice injected with oil served as controls. SMMHC-CreERT\textsuperscript{2} mice crossed with Rosa26-Stopfl-LacZ reporter mice\textsuperscript{17} were used to monitor tamoxifen-induced Cre/Lox recombination (Figure IA–IC in the online-only Data Supplement).

To generate TgPAC-Notch3\textsuperscript{R169C, Notch3−/−}, mice, we first crossed TgPAC-Notch3\textsuperscript{R169C} mice with Notch3−/− mice. F1 TgPAC-Notch3\textsuperscript{R169C, Notch3−/−} mice were subsequently mated with Notch3+/− mice for 2 generations to obtain F3 mice. The mice used in the study, TgPAC-Notch3\textsuperscript{R169C, Notch3−/−}, TgPAC-Notch3\textsuperscript{R169C, Notch3−/−}, Notch3−/−; TgPAC-Notch3\textsuperscript{R169C, Notch3−/−}, and Notch3−/−; TgPAC-Notch3\textsuperscript{R169C} were littermates obtained from the F3 cross. The genetic background of all mice used in the study was in the same hybrid background of 88% C57BL/6:12% FVB/N overall.

All mice were bred at the University Paris Diderot, site Villemin, animal facility (Paris, France). All experiments described in this study were conducted in full accordance with the guidelines of our local Institutional Animal Care and Use Committee (Lariboisière-Villemin), with every effort made to minimize the number of animals used.

Gene Expression Analysis

Mice were overdosed with isoﬂurane and decapitated, and the brains were harvested. Cerebral pial arteries (15–150 μm diameter) were dissected under the microscope (Figure II in the online-only Data Supplement), immediately snap-frozen in liquid nitrogen, and stored at −80°C until use. Brain arteries from 2 mice were pooled to prepare 1 sample. Quantitative polymerase chain reaction (PCR) was performed in triplicate on a CFX connect Single-Color Real-Time PCR detection system (BioRad) using gene-specific primers showing efficient ampliﬁcation and the SYBR Green PCR master mix (BioRad) as previously described.\textsuperscript{18} Expression levels were normalized for β-actin or transgelin mRNA.

Brain Parenchyma Histchemical and Immunohistochemical Analyses

Anesthetized mice were perfused transcardially with sodium phosphate buffer, followed by 4% parafomaldehyde in phosphate buffer. Brains were removed, half cut sagitally, and postfixed. One half was processed for paraffin embedding. Representative 7-μm-thick paraffin sections were stained with hematoxylin and eosin or Kläwer-Barrera Luxol fast blue. Vacuoles are deﬁned as well-delineated cavities in the brain tissue, distinct from vascular lumens, and infarcts are deﬁned as areas of focal brain necrosis.

The other half brain was cryoprotected in 15% to 30% sucrose/phosphate buffer at 4°C and embedded in optimal cutting temperature (Tissue-tek). Free-floating sections (16 μm) were incubated with mouse monoclonal anti–myelin basic protein (t-myelin basic protein; t:2000; SM94R, Covance) followed by secondary antibody (Alexa Fluor 594 anti-mouse IgG; 1:500; Invitrogen). This t-myelin basic protein antibody recognizes both the normal and degraded myelin basic protein with a lower and higher affinity, respectively (E. Cognat, MD, and A. Joutel, MD, PhD, manuscript in preparation, 2013). Myelin debris are deﬁned as foci of hyperintense t-myelin basic protein immunoreactivity, as previously reported in patients with CADASIL.\textsuperscript{19} Sections from mutant and wild-type animals were processed in parallel.

Notch3ECD Staining

Mice were overdosed with isoﬂurane and decapitated, and the brains were harvested. Acetone-fixed cryosections (12 μm) were incubated with mouse monoclonal anti-Notch3ECD (clone 5E1, 1:2 dilution) and following by appropriate Alexa-conjugated secondary antibody (Alexa Fluor 594 anti-mouse IgG; 1:500; Invitrogen). This α-Notch3ECD antibody recognizes Notch3ECD in mouse and rat NOTCH3, respectively and conducted molecular analyses of the brain arteries as well as neuropathological studies. Together, the data argue against a critical contribution of a loss of NOTCH3 function in the pathogenesis of CADASIL.

Imaging Analysis

Brain sections were analyzed by an investigator blinded to the genotypes. Detailed information on imaging analysis is provided in the online-only Data Supplement.

Statistical Analysis

Data are expressed as mean±SEM. Multiple comparisons were evaluated by 1-factor ANOVA followed by the Tukey post-hoc test. Values of p<0.05 were considered significant.
Results

A Molecular Toolbox to Directly Assess the In Vivo Consequence of CADASIL Mutations on NOTCH3 Transcriptional Activity

Recently, we identified a core set of novel NOTCH3-RBPJ-regulated genes in the context of brain artery development. These genes, which are predominantly expressed in arterial SMC, are downregulated in the brain arteries of mice completely lacking NOTCH3 from the point of conception (A. Joutel, MD, PhD, unpublished data, 2012). Because of the dominant nature of CADASIL mutations, we first investigated whether profiling expression of NOTCH3-RBPJ target genes could enable detecting a 50% reduction in NOTCH3 activity. We dissected the brain arteries of mice with half-normal dosage of NOTCH3 (Notch3+/−) and wild-type littermates (1 month old) and quantified the expression level of previously reported and novel NOTCH3-RBPJ-regulated genes. Of interest, mutant arteries do not exhibit any structural abnormalities in both young and old animals (data not shown). Nevertheless, we found that 11 among the 18 genes analyzed were significantly downregulated in the brain arteries of Notch3+/− mice (Figure 1).

Because loss of NOTCH3 function may occur with aging, we examined whether profiling expression of NOTCH3-RBPJ target genes could enable detecting reduced activity after normal completion of arterial development. To this end, we generated mice with tamoxifen-induced deletion of RBPJ in SMC from 10 weeks of age once arterial maturation is achieved (Figure 2A; Figure 1A–IC in the online-only Data Supplement). SMC deletion of RBPJ in the adult did not produce any structural defects of brain arteries (Figure ID in the online-only Data Supplement). Importantly, a subset of 5 genes among the 18 genes tested were significantly downregulated in arteries of tamoxifen-treated SMMHC-CreERT2,Rbpjflox/flox mice compared with SMMHC-CreERT2,Rbpjflox/flox mice, including Notch3, as well as Grip2 and Nrip2, which were downregulated in Notch3−/− mice (Figure 2B). Particularly, downregulation of these genes results from the deletion of RBPJ but not from the tamoxifen treatment as previously established (E. Cognat, MD, and A. Joutel, MD, PhD, unpublished data, 2013). Together, these findings provide us with an appropriate molecular toolbox to detect a haploinsufficient loss of NOTCH3-RBPJ that may occur with aging.

Brain Arteries of Arg170Cys Knock-in Mice Display Prominent Notch3CD Accumulation But Unaltered NOTCH3 Activity

We next used the molecular toolbox defined above to investigate whether the archetypical Arg169Cys mutation mitigates NOTCH3 activity in the brain arteries. Recently, we engineered a strain of mice harboring a heterozygous missense Arg170Cys mutation (corresponding to Arg169Cys mutation in human NOTCH3) at the endogenous Notch3 locus (Notch3Arg170Cys), analogous to the situation in affected humans. Considering that Notch3CD aggregates formation may interfere with NOTCH3 activity, we first verified that Notch3Arg170Cys heterozygous mice developed the characteristic deposition of Notch3CD in the brain arteries. Mutant mice, aged 6 to 7 months, showed marked granular anti-Notch3CD staining outlining the SMCs compared with wild-type mice, which exhibited diffuse and faint anti-Notch3CD staining (Figure 3A and 3B). We dissected the brain arteries of these mice and performed quantitative reverse-transcription PCR analysis. The results showed that expression level of all the genes tested did not differ between Notch3Arg170Cys heterozygous and wild-type littermates (Figure 3C; Figure III in the online-only Data Supplement). We then assessed Notch3Arg170Cys homozygous mice, which exhibit far more pronounced Notch3CD deposits in the brain arteries compared with the heterozygous mice (Figure 3A and 3B). Brain arterial structure was unaltered in mutant mice (Figure IV in the online-only Data Supplement). Notably, the quantitative reverse-transcription PCR results showed that the expression level of all the genes tested was not further reduced in Notch3Arg170Cys homozygous compared with Notch3Arg170Cys heterozygous and wild-type littermate mice (Figure 3C; Figure III in the online-only Data Supplement). Thus, the data suggest that brain arteries of mice with the Arg170Cys mutation retain normal NOTCH3 activity despite extensive Notch3CD deposition.

Eliminating Endogenous Wild-Type NOTCH3 Does Not Exacerbate White Matter Degeneration in a Mouse Model With Arg169Cys Mutation, and Notch3 Null Mice Do Not Develop White Matter Degeneration

The earliest and most consistent brain abnormalities in CADASIL are white matter lesions, which precede the onset of clinical symptoms by 10 to 15 years. The TgPAC-Notch3R169C
mice, but not the Notch3<sup>Arg169Cys</sup> mice, develop a spontaneous age-dependent white matter phenotype. To assess further the possibility that the Arg169Cys mutant loses its activity or even exhibits a dominant-negative behavior with aging, and that loss of essential NOTCH3 function contributes to the CADASIL phenotype, we assessed the influence of eliminating wild-type NOTCH3 in TgPAC-Notch3<sup>R169C</sup> mice on the onset and burden of white matter lesions.

TgPAC-Notch3<sup>R169C</sup> mice were crossed to Notch3KO mice to generate mice expressing Notch3<sup>169C</sup> in the absence of endogenous Notch3, along with littermates with both endogenous Notch3 alleles. TgPAC-Notch3<sup>R169C</sup> Notch3<sup>−/−</sup> mice develop normally and display, like the TgPAC-Notch3<sup>R169C</sup>, Notch3<sup>−/−</sup> mice, no overt clinical phenotype. Interestingly, the load of Notch3<sup>EDC</sup> deposits in the brain arteries was comparable in TgPAC-Notch3<sup>R169C</sup>, Notch3<sup>R169C</sup>, and TgPAC-Notch3<sup>R169C</sup>, Notch3<sup>−/−</sup> mice (Figure V in the online-only Data Supplement).

A prominent white matter pathology observed in TgPAC-Notch3<sup>R169C</sup> mice, on a normal background, is the vacuolation of white matter tracts that manifests at ≈20 months of age. Vacuolation was assessed in the internal capsule and the fimbria at 12 (data not shown) and 20 months of age (Figure 4A). In TgPAC-Notch3<sup>R169C</sup>, Notch3<sup>−/−</sup> mice, vacuolation was absent at 12 months (data not shown) and was comparable with the one seen in TgPAC-Notch3<sup>R169C</sup>, Notch3<sup>R169C</sup> mice at 20 months (Figure 4B). Remarkably, histological analysis demonstrated no significant vacuolation in mice completely lacking NOTCH3 at ≤20 months (Figure 4). Furthermore, none of the mice, be they Notch3<sup>−/−</sup> or TgPAC-Notch3<sup>R169C</sup>, showed lacunar infarct.

Another prominent white matter pathology in the TgPAC-Notch3<sup>R169C</sup> mice is the accumulation of myelin debris, indicating myelin degradation (E. Cognat, MD, and A. Joutel, MD, PhD, manuscript in preparation, 2013). Density of myelin debris was assessed in the corpus callosum at 20 months of age (Figure 5A). Twenty-month-old TgPAC-Notch3<sup>R169C</sup>, Notch3<sup>−/−</sup> mice showed abundant myelin debris, although their density was comparable between TgPAC-Notch3<sup>R169C</sup>, Notch3<sup>−/−</sup> and TgPAC-Notch3<sup>R169C</sup>, Notch3<sup>−/−</sup> mice at the same age (Figure 5B). Notably, myelin debris were essentially absent in 20-month Notch3-null mice as in age-matched wild-type mice (Figure 5A and 5B). Hence, the results indicate that complete absence of NOTCH3 does not result in white matter degeneration (ie, vacuolation and myelin degradation) at ≤20 months and that white matter degeneration in transgenic mice with the Arg169Cys Notch3 mutation is not exacerbated in the complete absence of NOTCH3.

**Discussion**

Since the discovery that NOTCH3 mutations cause CADASIL, the question of whether malfunction of NOTCH3 receptor causes the disease in all patients has been the subject of intense debate. This issue is directly relevant to future therapeutic strategies based on the modulation of NOTCH3 activity or the reduction of mutant NOTCH3 level. The controversy came from the finding that, on the one hand, several mutations, including those located in the mutational hotspot region, seem not to impair NOTCH3 activity, and that, on the other hand, all the mutations cause misfolding and aggregation of NOTCH3, which may inactivate the receptor, and a few mutations unambiguously result in a loss of NOTCH3 activity. The recent observation that a mutant allele can exhibit loss-of-function properties in an age-dependent manner has raised the hypothesis that, actually, all CADASIL mutations reflect hypomorphic NOTCH3 activity. Nevertheless, it is important to stress that such a scenario does not necessarily imply that phenotypic manifestations of the disease are driven by the loss of an essential NOTCH3 function. Interestingly,
Rutten et al recently reported 2 human subjects carrying an hypomorphic NOTCH3 allele that did not exhibit any of the CADASIL phenotypic features.

Herein, we reinvestigated this critical issue, taking advantage of new molecular reagents allowing direct assessment of NOTCH3 activity in vivo and of the availability of mouse models that accurately mimic naturally occurring CADASIL mutation and spontaneously develop brain lesions relevant to CADASIL. Importantly, we present 3 lines of evidence arguing against the general conclusion that all CADASIL mutations reflect hypomorphic NOTCH3 activity and that reduction in NOTCH3 activity is a major driving force of the brain manifestations of this disease. First, using mice harboring the Arg170Cys mutation (corresponding to Arg169Cys mutation in human NOTCH3) at the endogenous Notch3 locus, we show that the archetypal Arg169Cys NOTCH3 mutation does not mitigate NOTCH3 activity in the brain arteries at an age marked by extensive deposition of Notch3ECD aggregates. Notably, NOTCH3 activity was unaltered in homozygous mutant mice, although these mice are devoid of wild-type NOTCH3 and exhibit far more pronounced Notch3ECD aggregates than the heterozygous mutant mice. Previous studies have examined the consequence of CADASIL mutations on NOTCH3 activity either in vitro, in cultured cells, or in vivo using indirect assays based on the ability of mutant NOTCH3 to rescue the arterial defects of Notch3KO mice or their consequences.7–9,11,14 The approach we used here, quantifying in the brain arteries the expression level of NOTCH3 target genes relevant to NOTCH3 function in SMCs, is quite unique in this regard. Significantly, the subset of genes we have tested has proven to be appropriate to detect NOTCH3-RBPJ haploinsufficiency, which could occur with aging (ie, after normal completion of arterial maturation). Furthermore, the Notch3Arg170Cys mice we analyzed are especially relevant because they accurately mimic naturally occurring CADASIL mutation, expressing endogenous level of wild-type and mutant

Figure 3. Brain arteries of Notch3Arg170Cys knock-in mice display prominent Notch3ECD accumulation but unaltered NOTCH3 signaling. A, Brain sections of 6-month-old to 7-month-old wild-type (WT), heterozygous (R170C+/+), and homozygous Notch3Arg170Cys (R170C/R170C) mice were labeled with the 5E1 Notch3ECD antibody. Shown are representative pial arteries with granular deposition of Notch3ECD in mutant mice. For purposes of clarity, a dashed line delineates the outline of the vessels. Scale bar, 20 μm. B, Quantification of Notch3ECD deposits in the brain arteries, including both the pial and the intraparenchymal arteries, of 6-month-old to 7-month-old WT, R170C/+ , and R170C/R170C mice (n=4 mice per genotype). C, Expression level of Notch3, Grip2, and Nrip2 mRNA in arteries of R170C/+ (n=7 samples, 14 mice) and R170C/R170C (n=4 samples, 8 mice) mice normalized to β-actin mRNA, expressed as fold change to arteries of WT (n=5 samples, 10 mice) littermate mice. All mice are 6 to 7 months old. L indicates lumen; and ns, nonsignificant.
NOTCH3 protein. Second, we found that the elimination of wild-type Notch3 in mice overexpressing the Arg169Cys mutant Notch3 did not exacerbate the onset and burden of white matter lesions and did not produce lacunar infarcts. Remarkably, this finding cannot be attributed to differences in the Notch3ECD vascular load, which is comparable between TgPAC-Notch3R169C, Notch3+/− and TgPAC-Notch3R169C, Notch3−/−. Third, an approach to directly elucidate the contribution of Notch3 loss of function to the brain manifestations is to assess the phenotype of Notch3 null mice. Herein, we showed that Notch3 null mice develop neither spontaneous white matter degeneration nor lacunar infarct at ≤20 months. Notably, we previously established that Notch3 protein and activity are completely absent in this line.17 Lack of spontaneous brain lesions in these mice is intriguing and awaits further studies, given the presence of major structural and functional arterial defects. Finally, the data in both mouse models with the Arg169Cys mutation demonstrated that the wild-type Notch3 protein was not required for aggregation, either for stabilizing the mutant or for contributing to aggregates nucleated by mutant Notch3.

The phenotypic expressivity shows marked variability among patients with CADASIL. Particularly, the data suggest that the volume of white matter hyperintensities is influenced by genetic factors, although the specific genetic variants underlying this variability are still unknown.23 In this study, we had the opportunity to analyze the phenotype of TgPAC-Notch3R169C mice on 2 different genetic backgrounds. Interestingly, we noticed that transferring the Notch3R169C mutation from an FVB/N into a C57BL/6 background was associated with an attenuation of the myelin degradation phenotype (data not shown). Hence, this result supports the notion of genetic modifiers in CADASIL. Furthermore, it points out that the genetic strain must be carefully considered when modeling CADASIL, and possibly other SVD, in the mouse.
We acknowledge several limitations in this study. First, we did not assess whether eliminating wild-type NOTCH3 in the TgPAC-Notch3R169C mice exacerbates cerebrovascular dysfunction, which has been recognized as one of the earliest manifestations in mouse models of CADASIL.\textsuperscript{21} The TgPAC-Notch3R169C mice and the Notch3\textsuperscript{−/−} mice exhibit profound vascular dysfunction, including decreased myogenic responses and impaired autoregulation of cerebral blood flow.\textsuperscript{17,18} Using a technical point of view, we estimated that the chances to detect further reduction of myogenic responses and alteration of cerebral blood flow responses in the double transgenic mice (TgPAC-Notch3R169C, Notch3\textsuperscript{−/−}) were low. Using a scientific point of view, we estimated the issue less relevant in the absence of exacerbation of brain lesions in the TgPAC-Notch3R169C, Notch3\textsuperscript{−/−} mice. A second potential caveat is that the TgPAC-Notch3R169C mice still have wild-type Notch3 activity on a Notch3 null background (the present study and data not shown). Consequently, we could not elucidate whether reduced Notch3 activity may play a modifying role on the disease course or whether brain manifestations of CADASIL can develop without any Notch3 signaling activity. The Cys428Ser and Cys455Arg mutations map within the ligand-binding domain of Notch3 and both mutants lack Notch3 activity in vitro and in vivo. However, because the SM22-mediated expression of both Notch3 mutants in the mouse does not cause overt spontaneous brain lesions,\textsuperscript{10,11} these transgenic lines likely do not comprise an alternative strategy. Therefore, novel mouse models or approaches are needed to address these issues.

In conclusion, our present findings in the mouse provide strong evidence that CADASIL can develop without impairment of NOTCH3 signaling and argue against a loss of NOTCH3 function as the driving mechanism for white matter lesions in CADASIL. Instead, the data are consistent with a model that invokes a toxic gain of function.

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Disclosures

None.

References

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SUPPLEMENTAL MATERIAL

The archetypal Arg169Cys mutation in NOTCH3 does not drive the pathogenesis in CADASIL via a loss of function mechanism

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Supplementary Methods

Histological analysis

β-galactosidase staining

Whole-mount staining for β-galactosidase activity was performed as previously described \(^1\).

High resolution microscopy

The middle cerebral artery and surrounding brain tissue were dissected under the microscope from CARSON fixed brains and embedded in Epon E812 resin as previously described \(^1\). Tissue was sectioned at 1-μm thickness using an ultracut, placed on microscope slides, stained with Toluidine blue and examined under a DMR microscope (Leica).

Imaging analysis

Samples were imaged using a Nikon 80i eclipse microscope and captured using a DXM1200C digital camera (Nikon) and NIS Elements BR v 3.0 software (Nikon), with identical settings across compared groups. We used NIS-Element BR 3.0 (Nikon) software for vacuolation analysis and ImageJ (NIH, v1.46j) for all other analyses.

White matter vacuolation was assessed in \(n=5-6\) mice per genotype (5-8 sections per mouse) at the level of the internal capsule on paraffin sections stained by Klüver-Luxol-Barrera, and at the level of the fimbria on H&E stained sections. White matter tracts of the internal capsule where automatically delineated and the area was measured (total white matter area), then, vacuoles within the white matter tracts were manually pointed, automatically delineated and the area measured (total vacuolated area). Fimbria was delineated by automatic thresholding (Otsu method) then vacuoles within the fimbria borders were automatically selected after background subtraction, followed by automatic and manual (if needed) correction for non-vacuoles objects improperly selected. Fimbria (total white matter area) and total vacuolated area were measured. Vacuolation was expressed as the ratio of total vacuolated area to total white matter area.

Myelin debris density was semi-automatically quantified in \(n=5-6\) mice per genotype (4-6 sections per mouse) at the level of the internal capsule on sections immunostained with \(\alpha\)MBP. The corpus callosum was manually delineated and the area was measured. Hyperintense foci were automatically detected (local maxima approach) and counted. Debris density was expressed as the ratio of hyperintense foci number to the corpus callosum area.

The load of Notch3\(^{ECD}\) deposits was semi-automatically quantified in \(n=4\) mice per genotype on two channel images (\(\alpha\)Notch3\(^{ECD}\), \(\alpha\)CollIV) of pial and intraparenchymal arteries (≥ 10 arterial rings over 6 brain sections per mouse). Vessel borders were manually delineated on the collagen IV channel and vessel area was measured (varea). Mean vessel background (vcbk) and mean vessel (accu) intensities were assessed on the Notch3\(^{ECD}\) channel. Mean parenchyma background intensity (pcbk) was assessed within a manually defined ROI representative of image background on the Notch3\(^{ECD}\) channel. Image was binarized (threshold value= pbck+vbck). Notch3\(^{ECD}\) accumulation area (accu.area) was obtained by measurement of the area above the threshold value, within the vessel bounds. Notch3\(^{ECD}\) accumulation was expressed either as the corrected mean fluorescence intensity (accu – pbck) or as the Notch3\(^{ECD}\) accumulation area ratio (accu.area / varea).
Supplementary Figures

A-B: *SMMHC-CreERT2* mice were crossed with *Rosa26-Stopfl-LacZ* reporter mice, which express β-galactosidase after removal of a Floxed cassette by Cre recombinase. Mice were injected with tamoxifen at 10 weeks of age during 5 consecutive days and analyzed 6 weeks later; the brain was dissected and stained with X-gal. Shown is a ventral view of the brain of representative *Rosa26-Stopfl-LacZ* (A) and *SMMHC-CreERT2, Rosa26-Stopfl-LacZ* mice (B) with strong staining of the arteries in the latter.

C: *SMMHC-CreERT2, Rbpj$^{flx/flx}$* mice were treated at 10 weeks of age with tamoxifen (SM-RBP-J-KO) or oil (control) and analyzed at 16 weeks of age. Genomic DNA of blood vessels was assessed for Cre/loxP recombination by PCR. Shown is a representative gel electrophoresis of PCR products demonstrating efficient recombination (deleted band) in the SM-RBP-J-KO mice.

D: Shown are representative semi-thin sections of middle cerebral arteries with adjacent brain tissue from a control (left panel) and a SM-RBP-J-KO mouse (right panel) stained with toluidine blue. Scale bar: 30µm
Supplementary Figure II: Imaging of brain arteries dissected for Q-PCR analysis

A: Phase contrast imaging of pial brain arteries, 15 to 150 μm in diameter, dissected under the microscope.

B-C: Fluorescent imaging of DAPI-stained nuclei (B) and α-smooth-muscle actin immunostaining (C) of the brain vessels preparation. Insets show high magnification of the boxed areas.

D: Semi-thin section of a representative pial artery stained with toluidine blue.

Scale bar: A-C: 1 mm; D: 20μm
Supplementary Figure III: Expression level of NOTCH3 target genes is unaffected in heterozygous and homozygous Notch3<sup>Arg170Cys</sup> mice

Quantitative reverse transcription polymerase chain reaction (QRT-PCR) analysis of NOTCH3-target genes in arteries of heterozygous Notch3<sup>Arg170Cys</sup> mice (R170C/+)(n=7 samples, 14 mice) and homozygous Notch3<sup>Arg170Cys</sup> mice (R170C/R170C) (n=4 samples, 8 mice) normalized to SM22 mRNA, expressed as fold change to arteries of wildtype (WT) littermates mice (n= 5 samples, 10 mice). ns, non significant
Supplementary Figure IV: Brain arterial structure of R170C mutant mice is unaltered

Shown are representative semi-thin sections (toluidine blue staining) of middle cerebral arteries with adjacent brain tissue from a heterozygous R170C/WT (A) and a homozygous R170C/R170C mouse (B) aged 6-7 months old. Brain arterial structure of mutant mice is unaltered (see supplementary Fig. I D for appearance of a WT artery). Scale bar is 20µm
Supplementary Figure V: Elimination of wildtype NOTCH3 does not affect the amount of Notch3ECD deposits in TgPAC-Notch3R169C mice

A-B Brain sections of 1 month-old TgNotch3R169C, Notch3+/+ and TgNotch3R169C, Notch3-/- mice were labeled with the 5E1 anti-Notch3ECD antibody. Shown are brain arteries with granular deposition of Notch3ECD aggregates in mutant mice. Scale bar is 50 µm

C- Quantification of Notch3ECD deposits in the brain arteries including pial and intraparenchymal arteries (n=3 mice per genotype, ≥ 15 arterial rings per mouse) expressed as corrected relative mean fluorescence intensities.

Supplementary references