Notch3 Arg170Cys Knock-In Mice Display Pathologic and Clinical Features of the Neurovascular Disorder Cerebral Autosomal Dominant Arteriopathy With Subcortical Infarcts and Leukoencephalopathy


Objective—Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is an adult-onset neurovascular disorder caused by stereotyped mutations in the NOTCH3 receptor. Elucidation of its pathobiology is still incomplete and remains a challenge, in part because the available preclinical mouse models to date do not reproduce the full spectrum of CADASIL pathology and clinical disease.

Methods and Results—Here, we report a novel knock-in mouse with Arg170Cys substitution in murine Notch3, corresponding to the prevalent Arg169Cys substitution in CADASIL. The Notch3Arg170Cys mice displayed late-onset, dominant CADASIL arteriopathy with typical granular osmiophilic material deposition and developed brain histopathology including thrombosis, microbleeds, gliosis, and microinfarction. Furthermore, Notch3Arg170Cys mice experienced neurological symptoms with motor defects such as staggering gait and limb paresis.

Conclusion—This model, for the first time, phenocopies the arteriopathy and the histopathologic as well as clinical features of CADASIL and may offer novel opportunities to investigate disease pathogenesis. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: cerebrovascular disorders ■ gene mutations ■ CADASIL ■ Notch3 ■ arteriopathy
only 1 of them (Arg169Cys rat Notch3) developed brain histopathology, albeit limited to leukoaraiosis and astrogliosis, and none of the models developed neurological symptoms. Here, we report a novel mutant mouse carrying an Arg170Cys knock-in mutation in the endogenous murine Notch3 that, to the best of our knowledge, constitutes the first model comprising both CADASIL histopathology and clinical disease.

Methods

Generation of the Mice

For details on the generation, genotyping, and expression analysis of the Arg170Cys knock-in mice, see the supplemental material, available online at http://atvb.ahajournals.org. Experimental mice were littermates from heterozygous breeding (genetic background overall 50% 129EvSv:50% Swiss). Given the dominant phenotype (see below), experiments were done with heterozygous or homozygous mice, except for expression analyses, which were consistently performed with material from homozygous mutant mice. This study was approved by the ethical committee for animal experimentation of the K.U. Leuven, Belgium.

Phenotypic Analysis

Expression analysis by quantitative reverse transcription–polymerase chain reaction and by Western blotting was as described in the supplemental material. Notch3 signaling functionality was evaluated by target expression analysis in brain tissue and in vitro in an activation assay using cocultures of embryonic fibroblasts derived from wild-type and mutant embryos, with human umbilical vein endothelial cells (see supplemental material). Tissue preparation and (immuno)histochemical analysis, vascular morphometric analysis, and ultrastructural analysis of brain and peripheral tissues are detailed in the supplemental material.

Mice were monitored during aging for spontaneous motor abnormalities (reduced spontaneous mobility, tremor, ataxia as observed by staggering gait, and limb paresis as observed by dragging of a limb when stimulated to walk on a tabletop). Arterial remodeling or regrowth was assessed in the lung during pulmonary hypertension and in the skin during healing of a full-thickness skin wound, as detailed in the supplemental material.

Behavioral testing was performed using cognitive and anxiety/exploratory tests (elevated plus maze, open field exploration, social exploration, passive avoidance test, contextual fear conditioning, radial maze test) as described.

Statistical Analysis

Data represent mean±SEM. Statistical significance was calculated by the Student t test or by the Fisher exact test, considering P<0.05 as statistically significant.

Results

Generation, Viability, and Fertility of Arg170Cys Mutant Knock-In Mice

Introduction of an Arg170Cys substitution in murine Notch3, corresponding to the prevalent CADASIL Arg169Cys substitution, was achieved by knock-in of the corresponding cgto-tgt mutation in exon 4 of the murine gene (Supplemental Figure IA and IB). The resulting mutant mice had normal expression levels of the mutant Notch3 and did not compensatorily upregulate other Notch receptors (Supplemental Note I and Supplemental Table I). The mutant mice displayed normal growth, viability, and fertility (data not shown). Given the dominant effect of the mutation in our model (see below and Supplemental Note II), heterozygous and homozygous mice are collectively named Notch3Arg170Cys mice.

Arg170Cys Knock-In Mice Develop Cerebral and Systemic CADASIL Arteriopathy With GOM Deposition

To verify whether the Notch3Arg170Cys mice developed the typical ultrastructural abnormalities of CADASIL arteriopathy, brains of aged mice were analyzed by electron microscopy. Mice ~20 months old (9 Notch3Arg170Cys mice and 5 wild-type littermate controls) were analyzed to maximize the likelihood of disease development. Typical abnormalities were observed in Notch3Arg170Cys mice but not wild-type littermates (Figure 1). GOM deposits in the basal lamina of the SMCs and surrounding matrix, a hallmark of CADASIL, were found (Figure 1A and 1C–1E). GOM was frequently located in indentations of the SMC membrane (Figure 1D and 1E), which was often associated with abundance of caveolae and endosomal vesicles (Figure 1E), as reported in CADASIL patients. We occasionally also observed GOM-like material inside caveolae (Figure 1F). Enlargement of the subendothelial space (Figure 1G), enlargement of the SMC intercellular space, and the presence of a multilayered endothelial basement membrane (Figure 1G and 1H) was seen, as also reported in CADASIL. SMC and endothelial cell vacuolization, indicating cellular degeneration, and occasional SMC necrosis were also observed (Figure 1D and data not shown). These CADASIL-like ultrastructural abnormalities were found in 67% (6 of 9) of the mutant mice analyzed at this age but never in wild-type littermates (n=5) (Figure 1B). They were most easily detected in the brain slices corresponding to ~2 mm on either side of bregma. No abnormalities were seen in Notch3Arg170Cys mice analyzed at the age of 4 to 5 months (n=4; data not shown).

Unlike the case in CADASIL patients and most of the other mouse models, we did not obtain evidence for accumulation of the Notch3 ectodomain (by Western blotting for Notch3 ECD and Notch3 intracellular domain; Supplemental Figure IC).

Arteriopathy in CADASIL patients is systemic, allowing diagnosis by skin biopsy analysis. In accordance with this, we also observed ultrastructural abnormalities in tail arteries in Notch3Arg170Cys mice (Supplemental Figure II). This peripheral arteriopathy was observed from 8 to 9 months onward in 67% (4 of 6) of the mutants, equaling the incidence of arteriopathy observed in the brain.

Thus, Notch3Arg170Cys mice displayed typical CADASIL ultrastructural abnormalities in cerebral and peripheral vessels. The arteriopathy was detected in both females and males and regardless of homo- or heterozygosity (see Supplemental Note II), indicating a dominant effect of the mutation, as in CADASIL.

Arg170Cys Knock-In Mice Display CADASIL-Like Brain Histopathology

To define whether the mutant mice developed the cerebral histopathology observed in CADASIL, histopathologic analysis was carried out on a large group of mice (n=73), again at more advanced age (20–22 months) to maximize detection
of cerebral events. Abnormalities were detected in 23% (17) of the Notch3<sup>Arg170Cys</sup> mice but not in wild-type brains (n=19) (for overview of incidence and features, see Supplemental Table II). Brain abnormalities included perivascular microbleeds (12%) (Figure 2A and 2B); hemosiderin deposits or hemosiderin-containing macrophages (visible by Perl’s iron staining), indicating previous microbleeds<sup>42</sup> (5.5%) (Figure 2C); perivascular inflammatory infiltrates (CD4<sup>+</sup>; data not shown) (8%) (Figure 2D and 2E), which have been reported in some CADASIL cases<sup>41</sup>; gliosis (3%), as evidenced by glial fibrillary acidic protein staining, sometimes correlating with an area of microbleeding (Figure 2F and 2G); and thrombosis (7%) and perivascular fibrin(ogen) deposits (3%) (Figure 2H and data not shown). Notably, in 9 of 73 (12%) Notch3<sup>Arg170Cys</sup> mice, distinct parenchymal lesions were seen, including microinfarctions characterized by foci with pallor and cell loss around small cystic cavities (5 mice) (Figure 2I–2L). Enlargement of the Virchow-Robin spaces creating perivascular lacunae, which is also frequently detected in CADASIL,<sup>44</sup> was also found (Figure 2M and 2N and data not shown). All microinfarctions detected were located in the motor cortex area (primary [M1] motor area [4 mice] or supplementary [M2] motor area [1 mouse]) (Figure 2I–2L). All abnormalities were located within the area spanning ≈2.5 mm anterior to ≈2 mm posterior to the bregma (Supplemental Table II).

Given the systemic nature of the arteriopathy in the Notch3<sup>Arg170Cys</sup> mice, extracerebral organs were analyzed for pathological signs (kidney, liver, heart, lung); however, this analysis revealed no overt abnormalities (data not shown).

**Arg170Cys Knock-In Mice Display Motor Defects**

Clinical manifestations of CADASIL include recurrent ischemic episodes frequently causing a classic lacunar syndrome, leading to motor disability.<sup>34,41</sup> We therefore monitored the same group of 73 Notch3<sup>Arg170Cys</sup> mice up to 22 months of age for signs of motor impairment. Because variations in body weight among the mice were relative large at advanced age (eg, weight range of 35–55 g at 18 months) and influenced specific motor performance tests (eg, no differences were observed between genotypes in a grid wire hanging test or turnover test<sup>45</sup> after correction for body weight; data not shown), monitoring was restricted to directly observable spontaneously occurring disabilities. Nine mice (12%) developed overt permanent motor disability, ataxia, or both (Supplemental Table II). Eight of them had paresis of 1 or more limbs, as observed by dragging of the affected limb(s) during movement. Three mice showed staggering ataxic gait. The earliest onset of motor defects observed was 13 months. Among the affected mutant mice, only 2 displayed obvious cerebral pathology on analysis after euthanization (Supplemental Table II). Notably, this involved mice with microinfarction in the motor area of the right (M2 area) or left (M1 area) hemisphere (Figure 2I and 2J and data not shown), which displayed paresis of the left or right hindlimb, respectively. No loss of motor function was observed in 19 wild-type littermates monitored in parallel.

Evaluation of changes in cognitive and complex behavioral performance, a typical symptom in CADASIL patients,<sup>41,46</sup>
Figure 2. Mice carrying the Arg170Cys mutation display brain histopathologic features of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. Panels show representative coronal sections from 7 individual Notch3Arg170Cys (R170C) mutant mice at 20 months of age. A and B, Hematoxylin/eosin (H&E)–stained section of a Notch3Arg170Cys mutant mouse with a microbleed in the somatosensory cortex (arrow). B is an enlargement of the boxed zone in A. C, Perl’s iron reactivity in the external capsule, indicative of possible previous microbleeds with hemosiderin deposit or presence of hemosiderin-containing macrophages. D and E, Leukocyte accumulation at the junction between the lateral dorsal nucleus (LD) and fimbria (Fi) (D) as compared with a normal wild-type control (WT) (E). F and G, Glial fibrillary acidic protein (GFAP) staining of a section adjacent to the section shown in A. Positive signal (arrowheads) suggested gliosis around the location of the microbleed. An age-matched wild-type control brain is shown in G. H, Massive thrombus in the caudate putamen. The inset shows the same thrombus stained for fibrin(ogen) (Fn). I to N, Parenchymal lesions in different mutant mice including microinfarctions in the motor area. J, L, and N are enlargements of the boxed zones indicated in I, K, and M, respectively. Note the enlargement of the Virchow-Robin space around the vessel in N. CA3 indicates cornu ammonis 3 field of the dorsal hippocampus; CPU, caudate putamen; Scale bars=100 μm (A, C–I, K, M); 25 μm (B, J, L, N, and insets of D and E).

Arg170Cys Knock-In Mice Retain Normal Receptor Signaling

To evaluate functionality of the Arg170Cys mutant Notch3, expression of target genes (Hey1, Hey2, Nrarp, HeyL) was analyzed in murine embryonic fibroblasts, derived from wild-type and homozygous mutant embryos and cultured in the absence and presence of Jagged1-expressing human umbilical vein endothelial cells to induce the Notch pathway.23 Coculture of the fibroblasts with human umbilical vein endothelial cells caused a moderate increase of Notch3 expression (1.37-fold in wild-type and somewhat higher, 2.6-fold, in the mutant fibroblasts), in line with previous reports.23 However, coculture with human umbilical vein endothelial cells induced Notch3 targets to a more pronounced level (as much as >10-fold). Induction in mutant fibroblasts was somewhat reduced for Hey2 and HeyL but equal to or surpassing that in wild-type fibroblasts for the other tested targets, Hey1 and Nrarp (Figure 3A). Furthermore, no differences in target mRNA expression (Hey1, Hey2, Nrarp, HeyL, HeyL, HeyS) were observed in brain extracts of aged mice (20 months) (Figure 3B). Thus, the Arg170Cys substitution in mouse Notch3 did not affect signaling, in accordance with previous observations with the counterpart mutation in rat Notch3, which was shown to display normal surface expression, ligand binding, and signaling.34,47

Arg170Cys Knock-In Mice Display Normal Vascular SMC Remodeling or Growth and Normal Cerebral Capillary Densities

SMC α-actin immunostaining did not reveal overt loss of SMCs in small cortical arteries in the Arg170Cys knock-in mice, which is in line with recent observations in mice with transgenic expression of the counterpart rat mutation33 (SMC α-actin” wall thickness of arterioles of 50–150 μm inner diameter: 7.3±1.8 μm for Notch3Arg170Cys mice versus 5.2±1.1 for wild-type mice; mean±SEM, n=3–5, P=not significant between genotypes). We also did not detect genotypic differences in expression levels of platelet-derived growth factor receptor-β, a key molecule in vascular SMC biology, which was recently identified as a direct target gene of Notch320 (copies of platelet-derived growth factor receptor-β mRNA/10^3 copies of β-actin mRNA in brain extracts of 20-month-old mice were 8.9±0.90 for homozy-
gous Notch3Arg170Cys mice versus 8.2±0.59 for wild-type mice; mean±SEM, n=3–4, P=not significant). Subtle SMC defects might be present, however, as suggested by the SMC vacuolization and SMC necrosis seen by electron microscopy (see above).

Notch3 has been implicated in the development of pulmonary hypertension and associated remodeling of the small pulmonary arteries.22 To further assess a potential impact of the Arg170Cys mutation on SMC responses, we exposed Notch3Arg170Cys mice to chronic normobaric hypoxia (10% O2) for 28 days and assessed pulmonary artery remodeling by quantification of partially and fully muscularized small peripheral lung vessels.35 Wild-type and Notch3Arg170Cys mice (aged 3–4 months) exposed to hypoxia developed right ventricular hypertrophy as indicated by the 1.7-fold increase in the ratio of the mass of the right ventricle to that of the left ventricle plus septum as compared with normoxia exposure (Figure 3C). This was associated with pulmonary arterial remodeling as revealed by the up to 3.3-fold higher fraction of completely muscularized vessels, from ~10% in normoxia to ~30% in hypoxia (Table). No differences were observed, however, between wild-type and mutant mice (Table), indicating that the presence of the Arg170Cys substitution per se...
did not impede the pulmonary arterial SMC response to hypoxia exposure.

We also subjected aged mice (12–13 months) to a skin wound healing model and evaluated the wound healing response and associated vessel regeneration. Healing of a full-thickness incision wound inflicted on the back of the mice proceeded similarly in wild-type and mutant mice, as indicated by comparable wound closure rates (Figure 3D). Immunostaining of skin wound sections 14 days after wound-indication by comparable wound closure rates (Figure 3D). Total blood vessel density (CD31 staining) in the wound and wound border zone also did not differ between genotypes (Figure 3F).

Joutel et al recently reported capillary rarefaction in the cortex and corpus callosum in their mouse model with transgenic expression of mutant Arg169Cys rat Notch3, at stages before white matter lesion. We therefore compared capillary densities in the brain of Notch3Arg170Cys and wild-type control mice aged 12 to 13 months (after onset of arteriopathy) as well as in mice aged 20 to 22 months and with confirmed brain pathology, including mice with parenchymal pallor and loss. In both age groups, cortical capillary densities did not differ significantly between mutant and wild-type mice (CD31+ capillary density in number per mm²: 242±9.5 for Notch3Arg170Cys mice versus 264±17 for wild-type mice at 12–13 months and 283±22 for Notch3Arg170Cys mice versus 330±17 for wild-type mice at 20–22 months; mean±SEM, P<not significant between genotypes).

Thus, cerebral arterioles and capillaries appeared grossly normal, and neither young nor aged Notch3Arg170Cys mice displayed overt impairment of SMC responses in selected models of vascular repair or remodeling.

Discussion
Here, we generated a mouse model of CADASIL arteriopathy and clinical disease by introducing an Arg170Cys substitution in murine Notch3, corresponding to the prevalent Arg169Cys substitution in CADASIL patients. This knock-in approach resulted in unperperturbed expression levels in the Notch3Arg170Cys mice and allowed direct comparative phenotyping of mutant and wild-type control littermates. Arteriopathy was observed in both the brain and peripheral sites (tail) in Notch3Arg170Cys mice and was characterized by typical GOM deposition and enlargement of endothelial subcellular and SMC intercellular space. Histological analysis of the brain revealed microbleeds, thrombosis, fibrillar gliosis, and microinfarction, all typical features in CADASIL. As in CADASIL, neurologic consequences affecting motor ability (ataxia, paresis) were observed. Additional similarities with CADASIL and the Notch3Arg170Cys mouse mutant phenotype were the late onset, with hallmarks of arteriopathy observed at around 8 months (after not being present at 4 to 5 months) and the earliest signs of motor decline at 13 months, and the dominant nature of the disease.

As in CADASIL, electron microscopy of the Notch3Arg170Cys mice revealed the presence of typical GOM-like material in the basal lamina and membrane enfoldings of the SMCs but also inside caveolae. The latter, to the best of our knowledge, has not been reported before, although recently, intracellular GOM-like inclusions were observed in the Cys455Arg and Arg1031Cys human Notch3 transgenic models. It is tempting to speculate that these findings may reflect stages of GOM development or may relate to a postulated defective ubiquitin-dependent (trans)endocytosis of mutant Notch3. The latter is a plausible scenario, implying the presence of Notch3 ECD in GOM. This remains controversial, however, as the detection of Notch3 ECD in GOM by immuneelectron microscopy on patient material was not confirmed by mass spectrometry analysis. Interestingly, in vitro studies showed that CADASIL-mutant but not wild-type Notch3 ECD fragments can spontaneously form multimer aggregates, in which other proteins can coaggregate. This aggregation could contribute to the observed Notch3 ECD accumulation in CADASIL and might sequester potentially critical factors by coaggregation. Unlike in CADASIL and in other CADASIL mouse models, we did not obtain evidence for overt Notch3 ECD accumulation in the Notch3Arg170Cys knock-in mice using a commercially available antibody. Whether and how such accumulation is critical for disease development remains to be investigated.

Common to several CADASIL mutations and to findings in other CADASIL models, the Notch3Arg170Cys knock-in mice developed CADASIL-like pathology in the absence of Notch signaling defects. These data are in line with the prevailing hypothesis that the effect of Notch3 mutation in CADASIL may be largely based on a neomorphic function rather than on altered signaling, without excluding the fact that reduced signaling reported for specific mutations can modify and further aggravate disease. Unique so far to the Notch3Arg170Cys knock-in model is the development of multiple features of CADASIL brain pathology, including the occurrence of microinfarctions, creating small cavities, and the development of neurological symptoms (Supplemental Table II). Among the previously reported models, the Arg169Cys rat Notch3 transgenic model develops some of the same parenchymal pathology, primarily comprising leukoaraiosis and astrogliosis but not lacunar infarcts and possibly reflecting presymptomatic disease stage. Interestingly, these Arg169Cys rat Notch3 transgenic mice display white matter capillary rarefaction, which together with cerebrovascular dysfunction has been proposed to lead to the cerebral hypoperfusion observed in these mice and which possibly contributes to the white matter alterations. The normal capillary densities observed in this study in aged Notch3Arg170Cys brains, including those from mice with confirmed brain pathology, suggests that capillary rarefaction may be an important contributing factor but possibly not a general event in mouse models of CADASIL-like disease.

Notch3Arg170Cys mice experienced CADASIL-like neurological consequences affecting motor ability, including mice with microinfarction in the motor area displaying paresis of the contralateral hindlimb. In contrast, none of the other models reported so far developed neurological symptoms, despite marked overexpression of the mutant receptor in
several of these transgenic lines. Data obtained with the Cys455Arg human Notch3 transgenic model linked this particular hypomorphic mutation to susceptibility to stroke, but the mice did not develop spontaneous infarcts. The absence of brain pathology and neurological consequences in these models was accounted for by potential differences between human and mouse cerebral vasculature, brain architecture, and vulnerability or by the short life span of mice, limiting disease development to its earlier stages. Although such factors might well be influencing disease outcome, the Notch3Arg170Cys knock-in mouse model demonstrates that CADASIL-like pathology and clinical disease can develop within the life span of this species and without requiring enhanced expression of the mutant protein.

One observation on the Notch3Arg170Cys knock-in model is the incomplete penetrance of the neurological and the brain histopathologic phenotype despite occurrence of the typical CADASIL arteriopathy in the majority of the mice. This finding suggests that arteriopathy does not cause pathological and neurological consequences per se but that modifying factors, yet to be identified, may critically influence disease development or progression. Also, the reason for the absence of cerebral pathology in some of the mice with motor disability remains unclear. We cannot, however, exclude the possibility that subtle pathological abnormalities might have escaped our analysis. Vice versa, some Notch3Arg170Cys mice with microinfarction in the motor area did not display overt motor deficits or ataxia. However, the phenotypic outcome of similar events might differ between individual mice, in line with the highly variable clinical presentation of the disorder in CADASIL patients, even among individuals with identical mutation. The incomplete penetrance of the Notch3Arg170Cys phenotype may in part be accounted for by the partially outbred, mixed genetic background of the colony (129SvEv:Swiss), with differences in potential modifying genetic factors among individual mice. Genetic background differences may also contribute to the difference in phenotype and phenotype severity between the Notch3Arg170Cys knock-in model and the other Notch3 mutant models (C57BL/6, FVB-C57BL/6.Balb/C26).22

In summary, this functional genetic study showed that Notch3Arg170Cys knock-in mice developed most of the characteristic features of CADASIL neurovascular disease. Pathology and disease was late-onset, was dominant, and included not only the cerebral and peripheral arteriopathy but also several of the typical CADASIL brain pathologies, including microinfarction, as well as neurological symptoms. This novel mouse model thus may offer new opportunities to study CADASIL pathogenesis.

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References


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SUPPLEMENTAL MATERIAL

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Wallays, Nuyens, et al.

SUPPLEMENTAL METHODS

GENERATION OF THE MICE

A targeting vector (Supplement Figure IA) was constructed to replace exon 4 with a mutated exon 4 sequence containing a CGT-to-TGT point mutation of nucleotide 567 of the murine Notch3 cDNA (Genbank accession number X74760). This mutation causes an Arg to Cys substitution of amino acid 170 in epidermal growth factor (EGF) repeat 4 and corresponds to the prevalent Arg169Cys CADASIL mutation in humans. The 5’ homology in the targeting vector consisted of a 6.2-kb XhoI-PstI fragment comprising the major part of intron 2, exon 3, the mutated exon 4, exon 5 and part of intron 5. The 3’ homology consisted of the adjacent downstream 2.8-kb Bsu36I-SacI fragment spanning the remaining part of intron 5, and exon 6 through approximately half of intron 8 (Supplemental Figure IA). A loxP-flanked neomycin resistance (neo’) cassette was inserted in intron 5 between the PstI and Bsu36I site. A thymidine kinase cassette was cloned downstream of the 3’ homology to allow for negative selection against random integration events. Along with the CADASIL mutation, a silent mutation creating a HincII site was introduced in exon 4 for
genotyping purposes. Sequencing of all exons confirmed the presence of the mutations and the otherwise correct Notch3 DNA sequence.

The vector was linearized with NotI prior to electroporation in 129SvEv ES cells as described. Correctly targeted clones were identified by appropriate Southern blot analysis (Supplemental Figure IA,B and not shown). The presence of the introduced HindIII site was verified by PCR using primers spanning the mutated region followed by HindIII restriction digestion of the amplicons, and the presence of the CADASIL-like mutation was finally confirmed by sequencing (not shown). The targeted clones were then used for embryo aggregation with Swiss morula stage embryos as described previously to generate chimeric and transgenic animals, still carrying the neo' cassette in intron 6 (Notch3^+/neo and Notch3^neo/neo mice). In parallel, a correctly targeted clone was subjected to transient transfection with the Cre-expression plasmid pOG231 to excise the loxP-flanked neo' cassette in vitro. This yielded Notch3^+/m clones with correct excision of the intronic neo' cassette as identified by PCR analysis. These Cre-excised ES cells likewise were used for aggregation with Swiss morula embryos to generate mice (Notch3^+/m and Notch3^m/m mice). Both colonies were maintained by heterozygous breeding to generate the respective wild type, heterozygous and homozygous mutant littermate offspring (mixed genetic background of overall 50% 129EvSv:50% Swiss in both colonies) for phenotypic analysis.

All mutant genotypes (Notch3^+/m, Notch3^m/m, Notch3^+/neo, Notch3^neo/neo) were obtained at the expected Mendelian distribution (not shown) and displayed a normal viability (up to 24 month survival) and fertility (not shown).
Mutant mice of either colony displayed identical phenotypes, irrespective of homo- or heterozygocity of the mutation (Supplemental Note I, Supplemental Note II), thus indicating a dominant effect, similar to the situation in CADASIL patients. Therefore, heterozygous and homozygous mutant mice of either colony were considered equally as mutant mice and are further named Arg170Cys mutant (Notch3^{Arg170Cys}) mice. Expression analyses were consistently performed with material from homozygous mutant mice.

Housing and all experimental animal procedures were approved by the Institutional Animal Care and Research Advisory Committee of the K.U. Leuven.

**Genotyping of the mice**

Tail biopsies were lysed overnight at 55°C using a proteinase K containing lysis buffer as described. PCR genotyping was performed on purified genomic DNA using pure Taq ready-to-go PCR beads (Amersham biosciences, UK). Oligos located in exon 5 (sense 5′-GTC AAC GTG GAT GAC TGT CCT GGA C-3′) and exon 6 (antisense 5′-ATA GGG CAG GCA CAG TAG AAA GAG-3′) were used to detect the remnant loxP site in the Notch3^{+/m} and Notch3^{m/m} mutant mice (giving a 550-bp band on the mutant allele as compared to a 450-bp band on the wild type allele) (Supplemental Figure IB). To reveal the presence of the neo-gene in the Notch3^{+/neo} or Notch3^{neo/neo} mice, oligos located in the neo-gene (neo sense 5′-CGC ATC GCC TTC TAT CGC CTG CTT GAC-3′) and the exon 6 antisense primer were used, giving a 700-bp band specific for the neo-mutant allele (Supplemental Figure IA).
RNA ANALYSIS

RNA analysis was done on samples from wild type and homozygous mutant mice. Total RNA was isolated from cells or from fresh frozen brain biopsies using Trizol reagent (Invitrogen, Merelbeke, Belgium) and was reverse transcribed using random hexamer primers (Amersham Pharmacia Biotech) and Superscript II (Invitrogen). Quantitative real-time RT-PCR was performed using the Taqman Universal PCR master kit (Taqman Technology, PE Applied Biosystems, Foster City, CA) or SYBR Green (Applied Biosystems, Lennik, Belgium) on the ABIPRISM 7700 sequence detector (Applied Biosystems, Carlsbad, CA) as described.\(^7,8\) Copy numbers of the analyzed mRNAs were normalized using HPRT or β-actin mRNA levels. Home-designed primers and probes or premade Taqman primer sets (Applied Biosystems, Carlsbad, CA) were used as listed below.

For murine Notch3: forward primer 5’-ATC TTG TGG CCC GCT ATC TC-3’, reverse primer 5’-TGA ACG CCT GAG TCC AAG GA-3’, and probe 5’-FAM-CGT CCT CAT TGA TCT CAC AGA GAA-TAMRA-3’, specifically annealing in exon 21/22 of Notch3 cDNA. For murine HPRT: forward primer 5’T-TTA TCA GAC TGA AGA GCT ACT GTA ATG ATC-3’, reverse primer 5’T-TTA CCA GTG TCA ATT ATA TCT TCA ACA ATC-3’, and probe 5’-FAM-TGA GAG ATC ATC TCC ACC AAT AAC TTT TAT GTC CC-TAMRA-3’. For murine PDGFRβ: forward primer 5’-GCA GTT GCC TTA CGA CTC CAC-3’, reverse primer 5’-AAG AGT GCG TCC CAG AAC AAG-3’, and probe 5’-FAM-TGG TCC CGT GGC AGC TCC CA-TAMRA-3’. For murine Hes5 (SYBR Green): forward primer
5'-GAG ATG CTC AGT CCC AAG G-3' and reverse primer 5'-CGA AGG CTT TGC TGT GTT TC-3'. For murine HeyL/Hrt3 (SYBR Green): forward primer 5'-CGC AGA GGG ATC ATA GAG AAA C-3' and reverse primer 5'-ATT CCC GAA ACC CAA TAC TCC-3'. Premade Taqman primer set ID numbers (Applied Biosystems, Carlsbad, CA) were for murine β-actin: Mm00607939_s1, murine Notch1: Mm00435245_m1, murine Notch2: Mm00803077_m1, murine Notch4: Mm00440510_m1, murine Hey1: Mm00468865_m1, murine Hey2: Mm00469280_m1, murine Hes1: Mm00468601_m1 and murine Nrarp: Mm00482529_m1.

WESTERN BLOT ANALYSIS

Protein was extracted from brain using detergent-containing buffer (1% Triton) and equal-loading SDS-PAGE gels were used for immunoblotting as described. Antibodies used were the mouse Notch3 ECD specific antibody AF1308 (R&D Systems Europe, Abingdon, UK) and mouse Notch3 ICD antibody (Upstate Biotechnology, Lake Placid, NY, USA). ECD/ICD ratios were determined by densitometry of the bands using the NIH Image J software.

COCULTURE ASSAY

Primary murine embryonic fibroblasts (MEF) were prepared from E.13.5 wild type and homozygous mutant embryos. For co-culture assay, MEFs were seeded at 150,000 cells per well in 6-well plates without or with 200,000 human umbilical vein endothelial cells (HUVEC) (Lonza, Braine-l’Alleud, Belgium). After
incubation for 24 hours, the cells were processed for RNA extraction and qRT-PCR expression analysis, using primers with confirmed specificity for the murine genes.

**PULMONARY HYPERTENSION MODEL**

Mice were exposed to hypoxia by placing them in a tightly sealed chamber under normobaric hypoxia (10% O₂), which was maintained by a continuous inflow of 2 l/min N₂ and 2 l/min normal air (21% O₂). Normoxia mice were kept in normal air (21% O₂). After exposure for 28 days, mice were weighed and immediately used for determination of right ventricular (RV) hypertrophy expressed as the ratio of right ventricle weight to left ventricle (LV) plus septum (S) weight [RV/(LV+S)].

Pulmonary arterial remodeling was assessed on lung sections immunostained for murine smooth muscle cell alpha-actin (SMA), by counting the number of partially muscularized (incomplete coverage of SMA⁺ cells) and completely muscularized (complete coverage of SMA⁺ cells) peripheral vessels that were located distally to the bronchi. At least 15 areas equivalent to 100 alveoli were counted on 2 to 3 non-adjacent sections per mouse.

**SKIN WOUND MODEL**

Vessel regeneration was studied using a model of skin wound healing in which a standardized 15-mm full-thickness skin wound was inflicted on the back of the mice taking care not to damage the underlying muscle. Wound healing was monitoring by daily measurement of the length and width of the wound. At 14 days after wounding, skin tissue was dissected for histology. CD31⁺ total and
SMA⁺ arterial vessel densities were determined in the wound and border area by counting the number of vessels (expressed as number/mm²) using the KS300 morphometric software (Zeiss). The border area is constituted of the two optical fields flanking the wound area; per mouse, the averages of the two flanking areas were taken as data for the border area.

**HISTOLOGY, IMMUNOHISTOCHEMISTRY AND TRANSMISSION ELECTRON MICROSCOPY**

*Tissue preparation.* For light microscopical analyses, tissue was dissected from PBS and zinc fixative (Anatech Ltd., Battle Creek, MI) perfused mice, paraffin-embedded and sectioned at 10 µm thickness.

For transmission electron microscopy, tissues (whole brain, tail biopsies) were fixed with 3% PFA and 2% glutaraldehyde in 0.1 M cacodylate, pH7.4. Brain tissue was sliced into 1 to 2 mm thick slices using a brain slicer before post-fixation in 2% osmium tetroxide in 100 mM cacodylate. Postfixed tissue was dehydrated, treated with propylene oxide and embedded in epoxy resin (EMS Inc., Hatfield, PA). Ultra-thin sections (80 nm), counterstained with 1 % lead citrate and 0.5 % uranyl acetate, were examined on a Hitachi H7650 electron microscope (Hitachi High Technologies America, Inc.).

*Histochemical stainings.* All tissue sections were stained with haemotoxylin and eosin (H&E) for histopathological analysis. For brain analysis, 4-5 slides containing up to 70 coronal sections at 100-µm intervals and spanning the brain from ca Bregma +3 through -4 were H&E stained and inspected in order to obtain a representative overview of each brain. For Perl's
iron staining, paraffin sections were deparaffinized, incubated for 40 minutes in a solution of equal volumes 20% HCl-solution (Merck, Darmstadt, Germany) and 10% potassium ferrocyanate solution (Sigma-Aldrich, St-Louis, MO), rinsed in demineralised water, and counterstained with Nuclear Fast Red.

*Immunostaining.* Sections were immunostained for fibrinogen, CD45, glial fibrillary acidic protein (GFAP), smooth muscle alpha actin (SMA) or CD31 using immunofluorescent or colorimetric staining as described.\(^3,8,10,11\)

*Morphometric analysis* of lung arteries (pulmonary hypertension model) and skin vessels (wound healing model) is described above. Capillary density in cerebral cortex was quantified by counting CD31\(^+\) capillaries (expressed as number/mm\(^2\)) using the KS300 morphometric software (Zeiss). At least 10 optical fields were counted on 2-3 non-adjacent sections per mouse.

Images were acquired with the Zeiss AxioVision 4.6 software on a Zeiss Axioplan 2 imaging microscope equipped with a Zeiss AxioCam MrC5 digital camera (Carl Zeiss, Zaventem, Belgium).

**SUPPLEMENTAL RESULTS**

**SUPPLEMENTAL NOTE I: NORMAL EXPRESSION OF NOTCH RECEPTOR**

*Expression of Notch3*

Expression of mutant Notch3 in Notch3\(^{m/m}\) or Notch3\(^{neo/neom}\) mice was comparable to the level of wild type Notch3 expression in the respective wild type littermates as confirmed by quantitative real-time RT-PCR on brain extracts of young mice (copies mRNA Notch3/10\(^3\) copies mRNA HPRT: 0.93 ± 0.13 for
Notch3\(^{m/m}\) versus 1.2 ± 0.17 for the corresponding wild type littermates; mean ± SEM, N=4, \(P=NS\); and 2.1 ± 0.58 for Notch3\(^{\text{neo/neo}}\) mice versus 1.9 ± 0.39 for their wild type littermates; mean ± SEM, N=4-6, \(P=NS\). Similar Notch3 mRNA expression was also observed in cultures of embryonic fibroblasts derived from wild type or Notch3\(^{m/m}\) embryos (copies mRNA Notch3/10\(^5\) copies mRNA β-actin: 14.3 ± 0.93 for Notch3\(^{m/m}\) fibroblasts versus 11.3 ± 0.94 for wild type cells; mean ± SEM, N=3, \(P=NS\)). Thus, the Arg170Cys mutation per se nor the presence of the intronic loxP site in Notch3\(^{m/m}\) mice or of the neo\(^r\) cassette in Notch3\(^{\text{neo/neo}}\) mice interferes with gene transcription and RNA processing. The latter was further verified by sequencing of cDNA from the mutant mice which confirmed that exon 5/exon6 splicing occurred correctly.

Furthermore, equal expression was retained during aging as shown by comparable Notch3 mRNA levels in extracts from 20-month old wild type and Notch3\(^{m/m}\) mice (Supplemental Table I). This was confirmed at the protein level using Western blotting of protein extracts from aged brain with an antibody against the Notch3 intracellular domain, revealing comparable levels by densitometry (arbitrary units: 3,320 ± 350 for Notch3\(^{m/m}\) mice versus 4,200 ± 420 for their wild type littermates; mean ± SEM, N=5-4, \(P=NS\)).

Expression of other Notch receptors

No compensatory upregulation of other receptors was observed in homozygous Notch3\(^{R170C}\) mice. qRT-PCR analysis on embryonic skin fibroblasts and on brain from 20-month old mice showed no genotypic differences, except for a 4-fold higher expression of Notch4 in Notch3\(^{R170C}\) embryonic fibroblasts as compared to wild type embryos; this elevated
expression was however not seen in the brain of the mutant mice (Supplemental Table I).

SUPPLEMENTAL NOTE II: COMPARABLE PHENOTYPE IN HETEROZYGOUS AND HOMOZYGOUS MUTANT MICE

Characterization of heterozygous and homozygous mutant mice revealed comparable phenotypes, with respect to the arteriopathy, brain pathology as well as neurological symptoms. This indicated that the Arg170Cys mutation in mice is dominant, similar to its counterpart Arg169Cys mutation and the other known CADASIL mutations in humans.5,6

Indeed, the ultrastructural analysis of the tail samples showed the occurrence of the arteriopathy in 2 out of 3 heterozygous mutant mice analyzed as well as in 2 out of 3 homozygous mutant mice analyzed. For ultrastructural analysis of brain samples, only homozygous samples were used.

The histopathological analysis of the brain was performed on 33 heterozygous and 40 homozygous mutant mice. In both groups, a similar incidence of CADASIL-like brain histopathology was observed, i.e. in 6 out of 33 heterozygous (18%) and 11 out of 40 homozygous mice (27%) (P=NS by Fisher’s exact test).

Observation of this same group of mice for motor ability revealed abnormalities in 15% of the heterozygous mutants (5/33) and 10% of the homozygous mutant mice (4/40) (P=NS by the Fisher exact test).
Behavioral testing (see also Supplemental Note III) was done on 10 heterozygous and 12 homozygous mutant mice, revealing an approximately 4-fold reduced center visit frequency in the social exploration test for the pooled mutant population as compared to wild type controls (Supplemental Figure III). Among the mutant mice, center visit frequency did not differ between heterozygous and homozygous mutant mice (5 ± 3.2 for heterozygous mutant mice versus 6 ± 2.6 for homozygous mutant mice, mean ± SEM, P=NS).

**SUPPLEMENTAL NOTE III: REDUCED EXPLORATIVE BEHAVIOR**

Progressive cognitive decline and changes in complex behaviors is a typical symptom in CADASIL patients.\textsuperscript{12,13} To evaluate complex behavioral performance, we compared wild type (N=10) and Notch3\textsuperscript{Arg170Cys} female mice (N=22) of 15-18 months (i.e. beyond the age of onset of arteriopathy) in cognitive and anxiety/exploratory tests (elevated plus maze, radial maze test, passive avoidance test, contextual fear conditioning, open field exploration, social exploration\textsuperscript{14,15}). No obvious difference between genotypes was observed in memory/learning tests (elevated plus maze, radial maze test, passive avoidance test, contextual fear conditioning; not shown). In the social exploration test, Notch3\textsuperscript{Arg170Cys} mice showed somewhat decreased total path length (possibly related to their ataxia). Furthermore, their relative distance moved in the center was decreased, and a pronounced reduction in center visit frequency was measured (P<0.05) while corner visits were comparable to those of wild type control mice (Supplemental Figure III). A tendency of decreased exploratory behavior was also seen in the open field test (reduced center visit
frequency and center visit duration) (not shown). Reduced exploratory behavior may relate to changes in motivational and/or affective functions, which may be in accordance with the observed narrowing of the field of interest in CADASIL patients.\textsuperscript{13}
REFERENCES TO THE SUPPLEMENTAL MATERIAL


SUPPLEMENTAL FIGURES

SUPPLEMENTAL FIGURE I: Generation of Notch3<sup>Arg170Cys</sup> knock-in mutant mice.

**A**, Strategy for introduction of the Arg170Cys (R170C) mutation into the murine Notch3 gene by homologous recombination in embryonic stem cells. The scheme shows the targeting vector, containing the cgt-to-tgt mutation in exon 4 (denoted by the asterisk), and the wild type (WT) allele (top); the targeted allele after homologous recombination containing both the mutation and an intronic neomycin resistance cassette (neo<sup>r</sup>) (middle); and the mutant allele after Cre-recombinase mediated excision of neo<sup>r</sup> (bottom). Introns and exons are indicated by the shaded bar and dark boxes in the genes, respectively. Relevant diagnostic restriction sites are indicated on the genes. Hybridization probes (black bars), and the diagnostic restriction fragments with their respective length are indicated under the genes. Black arrows under the genes denote the primers used for PCR genotyping, with indication of the expected amplicon lengths. **B**, PCR on genomic DNA from wild type, heterozygous and homozygous mutant mice generated with a targeted ES cell clone after in vitro Cre-recombinase mediated excision of neo<sup>r</sup>, revealing amplicons of the expected size (see A). **C**, Immunoblotting of brain extracts for Notch3 ECD and ICD and densitometric quantification of the ECD/ICD ratio, revealing comparable ratios for wild type and Notch3<sup>Arg170Cys</sup> knock-in mice. Aged mice of 20-22 months were analyzed; homozygous Notch3<sup>Arg170Cys</sup> mice were used. Graph shows mean ± SEM, N=8-10. Representative blots are shown under the graph.
SUPPLEMENTAL FIGURE II: Notch$^{\text{Arg170Cys}}$ mutant mice develop systemic CADASIL-like arteriopathy.

A to D, Overview of the vessel wall of tail arteries of Notch$^{\text{Arg170Cys}}$ mutant (R170C) (A,C) or wild type (WT) mice (B,D) at 8-9 months of age, indicating enlarged smooth muscle cell intercellular space in the mutant mice as compared to wild type mice (white lines in A and B). The Notch$^{\text{Arg170Cys}}$ mutant mice also display more irregular and looser connections of the endothelial cells and basement membrane (arrows in C and D). Note the vacuolization of the endothelial cells in panel C (arrowheads) indicating degeneration. Panel C is an enlargement of the boxed zone 1 indicated in panel A; panel D is an enlargement of the boxed zone in panel B. E, Granular osmiophilic material (GOM) deposition in the matrix surrounding vascular smooth muscle cells (arrows) and higher vacuolization of the smooth muscle cells in mutant mice (arrowheads; compare with wild type mice in panel D). Panel E is an enlargement of the boxed zone 2 indicated in panel A. F, Multilayered basement membrane (asterisks) in a tail vessel of a Notch$^{\text{Arg170Cys}}$ mutant mouse. EC indicates endothelial cell; IEL, internal elastic lamina; N, nucleus; SMC, smooth muscle cell. Scale bars: 20 $\mu$m (A,B), 10 $\mu$m (C-F).

SUPPLEMENTAL FIGURE III: Notch$^{\text{Arg170Cys}}$ behavioral analysis

Social exploratory behavior of wild type (WT) and mutant Notch$^{\text{Arg170Cys}}$ (R170C) mice, revealing significantly reduced center visit frequency for the mutant mice as compared to wild type littermates, suggesting decreased
explorative behavior. Female mice of 15-18 months of age, randomly chosen from the colony, were tested. Mean ± SEM, N=10-22, * $P<0.05$ vs WT.
SUPPLEMENTAL TABLES

SUPPLEMENTAL TABLE I. mRNA expression of Notch receptors in embryonic fibroblasts and in brain of aged wild type (WT) and Arg170Cys (R170C) knock-in mice (20 months) as determined by qRT-PCR.

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<td>Notch4</td>
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Values are mean ± SEM, N=3-4. * P<0.001.
SUPPLEMENTAL TABLE II. Incidence and features of brain histopathology and motor deficits in Notch3<sup>Arg170Cys</sup> mice.
Seventy three mutant mice were monitored for motor defects and were sacrificed at 20-22 months of age for histopathological analysis; location and approximate bregma coordinate of the sections showing abnormalities is indicated. The motor or brain abnormalities were never observed in a group of 19 age-matched wild type littermates.

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| # (%) | 17 (23%) | 9 (12%) | 4 (5.5%) | 6 (8%) | 2 (3%) | 5 (7%) | 2 (3%) | 4 (5.5%) | 5 (7%) | 9 (12%) | 3 (4%) | 8 (11%) |

Locations represent 1 detected site unless otherwise indicated. B, bregma; Ext, external; Int, internal; L, left; R, right.
**Supplemental Figure I**

**Targeting vector**

*WT allele*

*targeted allele*

*mutant allele*

**Panel A:** Depiction of the targeting vector with restriction sites (BglII) and exons (3-8) marked. The vector includes a neo' gene and a TK gene. 

**Panel B:** Gel electrophoresis showing differences in band profiles between WT (+/+) and targeted (R170C/R170C) alleles. The targeted allele has bands at -550 bp and -450 bp. 

**Panel C:** Western blot analysis of ECD/ICD ratios comparing WT and R170C alleles. The ECD/ICD ratio is near 1, indicating no significant difference between the two conditions. The blot shows bands at 220 kDa N3ECD and 90 kDa N3ICD.
Supplemental Figure II
Supplemental Figure III