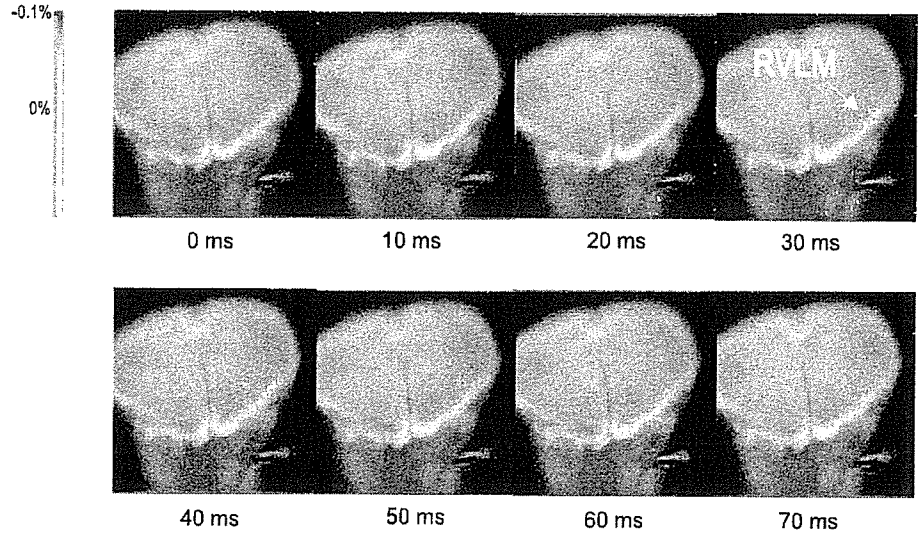


Fig. 4. Optical images of a cross section at the level of the RVLM during low-Ca high-Mg superfusion. A depolarizing response to IML stimulation was detected at the RVLM (n = 6). The latency between stimulation of the IML and the start and the peak of the depolarizing response at the RVLM was 27 ± 9 and 35 ± 10 (SD) ms, respectively.

(GABA) to the superfusion solution. IML stimulation during superfusion with low-Ca high-Mg solution containing GABA (200 μmol/l, n = 7) did not induce any depolarizing responses in the cross sections at the level of the CeVLM (Fig. 6) or of the RVLM.

Electrophysiological experiments. Other brain stem-spinal cord preparations were fashioned for the electrophysiological experiments, and during low-Ca high-Mg superfusion antidromic action potentials in response to IML stimulation were clearly detected in 9 CeVLM neurons by intracellular recordings (whole cell patch-clamp technique; Fig. 7) and in 16 CeVLM neurons by extracellular recordings. The latency between IML stimulation and detection of the antidromic action potential was 21.6 ± 7.1 ms by extracellular recording and 27.2 ± 4.6 ms by the whole cell patch-clamp technique. These latencies are almost the same as the latency between IML stimulation and the depolarizing response in the CeVLM measured by optical imaging of cross sections (see Fig. 5). The spontaneous action potentials of the CeVLM neurons (extracellular recording) collided with subsequent antidromically evoked action potentials.

Glutamate application to the CeVLM. During whole cell patch-clamp recordings of neurons in the IML with standard solution (Fig. 8*A*), glutamate was applied to the CeVLM locally after removing more rostral regions, including the CVLM and RVLM (*type 3* preparation, as in Figs. 5 and 6). Local application of glutamate to the CeVLM increased the frequency of the excitatory postsynaptic potentials (EPSPs) and induced significant depolarization (5.6 ± 2.5 mV) of the IML neurons (n = 8; Fig. 8*A*). Each EPSP was clearly revealed by increasing the speed of the sweep (Fig. 8*C*).

Histological examination. The locations of the neurons in the CeVLM in which depolarizing responses were detected by optical imaging and that exhibited an antidromic action potential in response to IML stimulation were investigated histologically (Fig. 9*A*). The neurons stained with lucifer yellow or pontamine sky blue were located in the lateral side of the lateral reticular nucleus (LRt) or in the LRt, the caudal part of

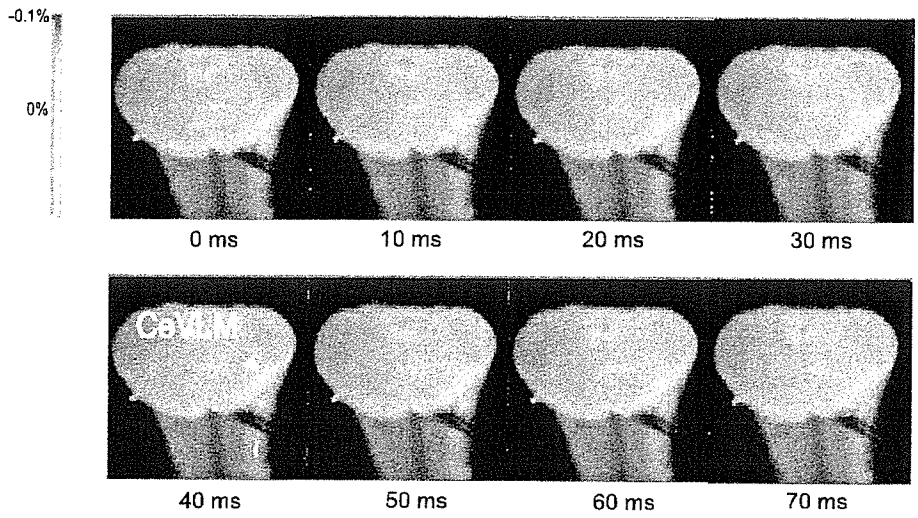


Fig. 5. Optical images of a cross section at the level of the CeVLM during low-Ca high-Mg superfusion. A depolarizing response to IML stimulation was detected at the CeVLM (n = 8). The latency (means ± SD) between stimulation of the IML and the start and the peak of depolarizing response at the CeVLM was 24 ± 5 and 36 ± 8 ms, respectively.

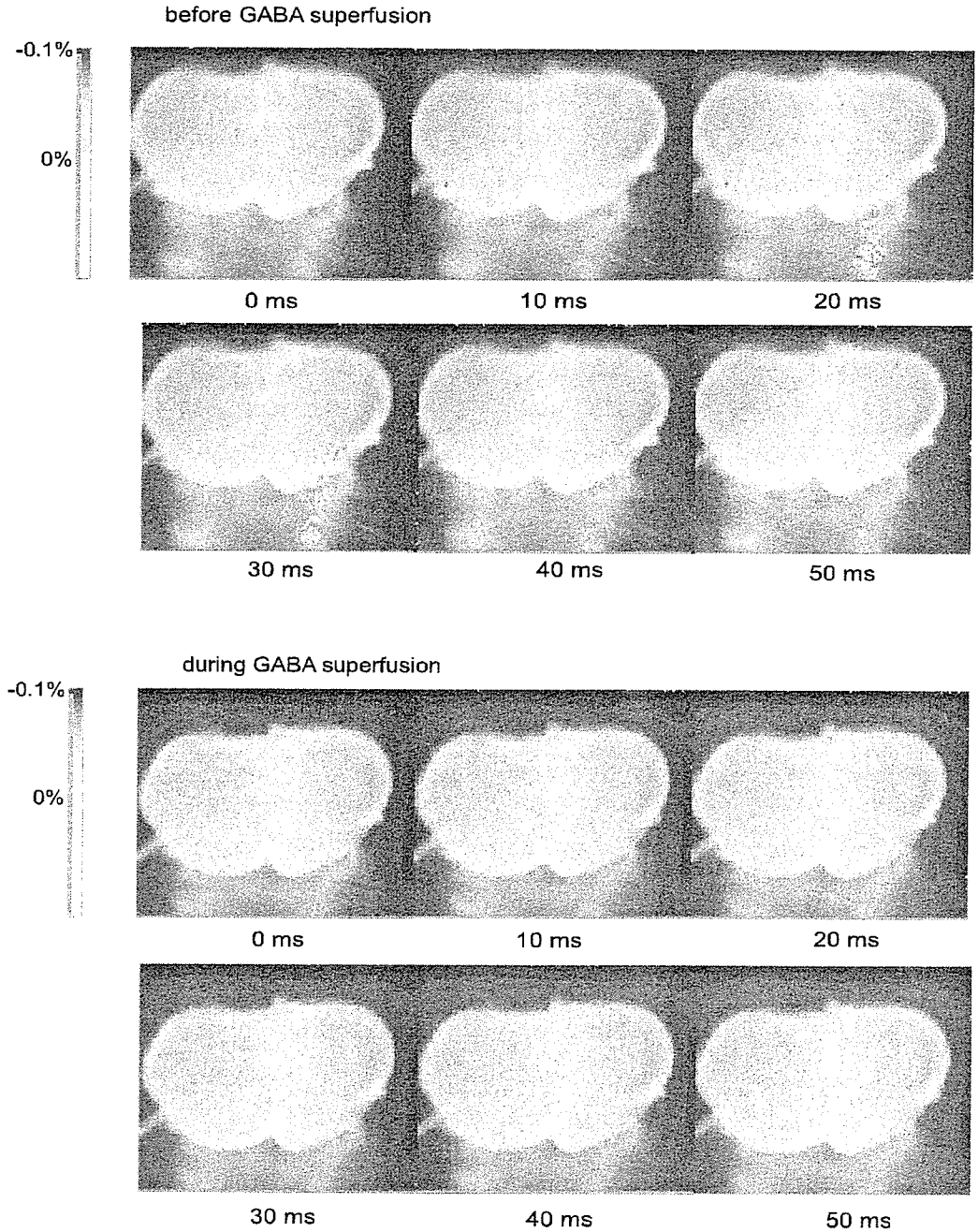


Fig. 6. Optical images of a cross section at the level of the CeVLM before (*top*) and during γ-aminobutyric acid (GABA) superfusion (*bottom*). IML stimulation during superfusion with low-Ca high-Mg solution containing GABA (200 μmol/l, $n = 7$) did not induce any depolarizing responses at the CeVLM.

the nucleus ambiguus, and the medial side of trigeminal spinal tract nucleus at the level of pyramidal decussation (Fig. 9, *B* and *C*). Thus the anatomic location of the neurons in the CeVLM satisfied the criteria for the location of the CPA described by Sun and Panneton (27, 28).

We tried to confirm that the location of the electrical stimulation in the spinal cord actually corresponded to the IML, and the results showed that location of the electrical stimulation and coagulation did correspond to the location of IML neurons (Fig. 10, *A* and *B*).

The location of neurons that exhibited depolarization and an increase in EPSP in response to local glutamate application to the CeVLM and stained with lucifer yellow in the spinal cord also corresponded to the IML (Fig. 10, *C* and *D*).

DISCUSSION

Characteristics of neurons in the CeVLM identified by optical imaging. We used optical imaging and electrophysiological methods in brain stem-spinal cord preparations of neonatal

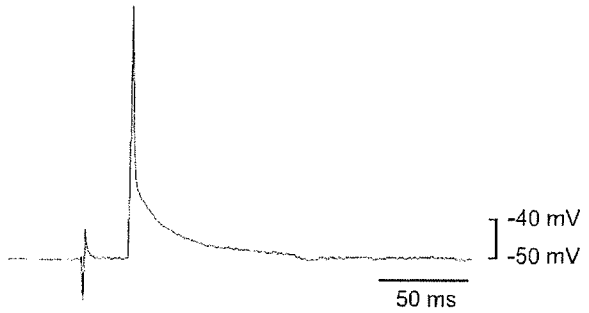


Fig. 7. Antidromic action potential of a neuron in the CeVLM recorded by the whole cell patch-clamp technique in response to electrical stimulation of the IML at the Th_2 level. The latency between stimulation and the start of the antidromic action potential was 27.2 ± 4.6 ms according to the whole cell patch-clamp recordings (n = 9).

SHRs to determine whether neurons in the CeVLM axonally project to the IML. The optical imaging revealed strong depolarizing responses in the CeVLM to IML stimulation, and CeVLM neurons fired antidromic action potentials in response to the IML stimulation. Glutamate applied in the CeVLM induced depolarization in the IML neurons. These findings demonstrated a monosynaptic axonal and excitatory projection from the CeVLM to neurons in the IML.

A number of studies, including a retrograde tracer study (10), have reported a monosynaptic projection from the RVLM, the RVMM, and the raphe nucleus to the IML (1, 4, 23, 26). Several studies have also demonstrated a direct projection from the CVLM to the IML in adult rats (7, 13), and the result of a recent tracer study using cholera toxin B subunit suggested a projection from the CPA to the IML of adult rats (11). A new region that differs from the CPA, the medullocervical pressor area, has recently been reported to project to the IML in adult rats based on an in vivo retrograde tracer study (25). Consistent with these findings, the optical imaging in our study suggested that a continuous longitudinal rostrocaudal column in the VLM, including the RVLM, the CVLM, and the CeVLM, gives rise to a monosynaptic projection to the IML. Because we detected the strongest depolarizing response in the CeVLM, we focused specifically on the CeVLM in the present study. To our knowledge, few studies have succeeded in optical visualization of the axonal projection from the CeVLM neurons that are involved in sympathetic nerve regulation to the IML. Because the existence of the projection from the CPA to the IML had been suggested only by a tracer study (11), the projection we have found must be discussed critically.

Earlier studies have shown that EPSPs or postsynaptic responses completely disappeared during superfusion with low-Ca high-Mg solution, suggesting a complete blockade of synaptic transmission (6, 17, 22). In our unpublished observations, many EPSPs were detected in the CeVLM neuron in response to IML stimulation during superfusion with standard solution, whereas, during superfusion with low-Ca, high-Mg solution, the EPSPs observed in the CeVLM neuron to IML stimulation completely disappeared. Thus use of a low-Ca high-Mg superfusion has the advantage of blocking all synapses and enabling detection of only descending monosynaptic projections alone, whereas, during superfusion with standard solution, depolarizing responses of descending polysynaptic projections and ascending projections are detected as well as descending monosynaptic projections. In the present study, the regions that exhibited depolarizing responses during low-Ca high-Mg superfusion were the same as those that displayed them during superfusion with the standard solution. In contrast, the depolarizing responses were less intense during low-Ca high-Mg superfusion, and the peak time of the depolarizing responses during low-Ca high-Mg superfusion occurred earlier than with the standard solution. These results strongly suggest that neurons in the CeVLM monosynaptically project to the IML neurons.

Previous studies found that low-Ca high-Mg solution did not affect neuronal excitability (6, 18). If neuronal excitability decreases during superfusion with low-Ca high-Mg solution, the intensity of the depolarizing responses detected in the present study should be weaker than the actual depolarizing responses. Therefore, we did not overestimate the depolarizing response by optical imaging.

Optical imaging findings on the ventral surface revealed that the depolarizing response in the CeVLM was more intense than in the RVLM. This finding suggests that, in neonatal rats, more neurons project from the CeVLM to the IML than from the RVLM and CVLM to the IML but does not imply that neuronal activity in the CeVLM is stronger than in the RVLM. It may merely reflect the characteristics of neonatal rats. However, we were unable to compare the depolarizing responses during the different developmental stages of the rats in this study.

Because the depolarizing response is the sum of the signals in the somas (cell bodies) and axon bundles, we investigated whether the depolarizing response originated in the somas or in the axons. GABA should bind to receptors on the surface of the soma, and, as shown in Fig. 6, the depolarizing response of the CeVLM was completely abolished during superfusion with GABA. This finding suggests that the depolarizing responses detected by optical imaging reflected depolarization of the

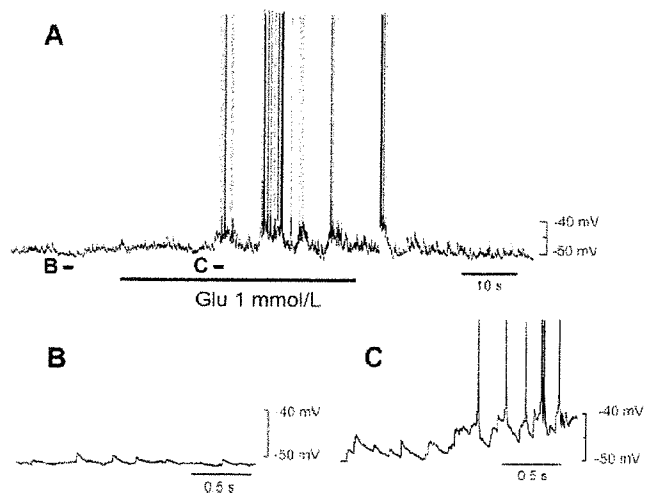
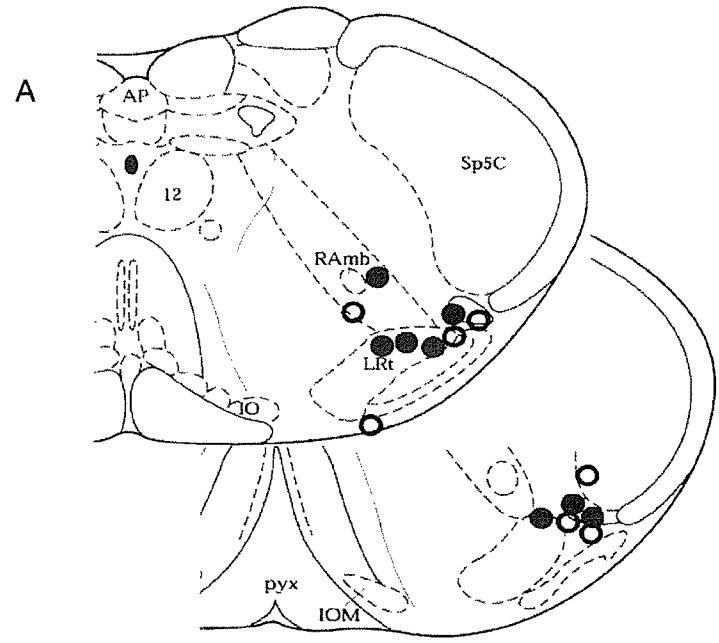
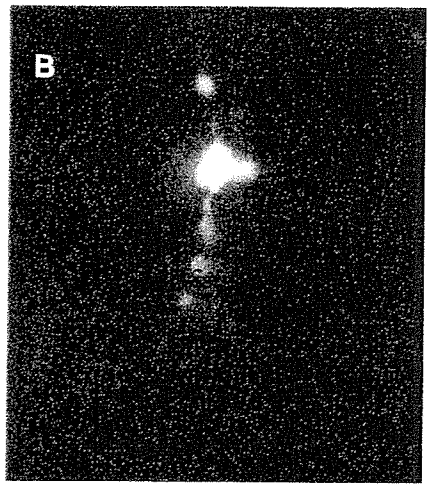


Fig. 8. Intracellular recordings of a neuron in the IML at the Th_2 level (n = 8) during superfusion with standard solution, in the *type 3* preparation, like in Figs. 5 and 6. *A*: local application of glutamate (1 mmol/l) to the CeVLM increased the number of excitatory postsynaptic potentials (EPSPs), induced depolarization (5.63 ± 2.50 mV), and increased the action potential spikes of the IML neurons. *B*: few EPSPs were seen before glutamate application. *C*: each EPSP and depolarization was clearly detected after local application of glutamate to the CeVLM.





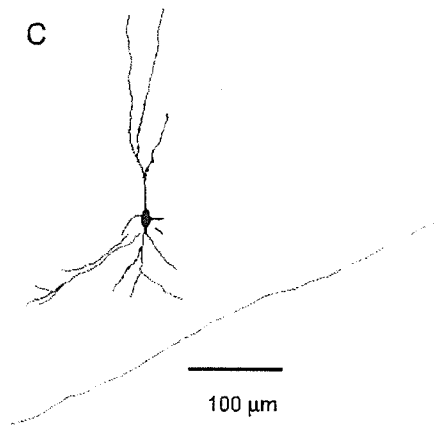


Fig. 9. *A*: location of neurons in the CeVLM that exhibited an antidromic action potential in response to electrical stimulation of the IML. This experiment was carried out during low-Ca high-Mg superfusion. ●, Extracellular recordings; ○, intracellular recordings. AP, area postrema; 12, hypoglossal nucleus; Sp5C, spinal trigeminal nucleus, caudal part; pyx, pyramidal decussation; LRt, lateral reticular nucleus; RAmb, retroambiguus nucleus; IO, inferior olivary nucleus; IOM, inferior olive, medial nucleus. *B*: fluorescence image showing a neuron in the CeVLM that exhibited an antidromic action potential and stained with lucifer-yellow. *C*: drawing of the location of a neuron that exhibited an antidromic action potential and that was stained with lucifer yellow.

somas of CeVLM neurons, not depolarization of the axons. Because we have reported the existence of the CeVLM neurons for the first time, it is unknown whether GABA receptors are present on CeVLM neurons. However, the result that the IML stimulation during superfusion with low-Ca high-Mg solution containing GABA did not induce any depolarizing responses in the cross sections of the CeVLM suggests to us that the CeVLM neurons possess GABA receptors. An earlier study reported that CPA neurons possess GABA receptors (24), and, if the CeVLM neurons correspond to CPA neurons, then CeVLM neurons probably possess GABA receptors.

We also obtained some optical imaging data in WKY rats as a control for the SHRs. The data showed similar depolarizing responses to IML stimulation in the CeVLM on the ventral surface (n = 5) and cross sections (n = 4) of WKY rats. Although careful experiments are required to make quantitative comparisons between the intensity of depolarizing responses in the CeVLM of WKY rats and SHRs, the results indicate to us that the projection from the CeVLM to the IML exists in both normotensive and hypertensive neonatal rats. It would be interesting to compare responses in the IML neurons of WKY rats and SHRs when glutamate is locally applied to the CeVLM on the preparation in the absence of the CVLM and the RVLM regions.

Methodological limitations. We cannot completely rule out the possibility that we may have stimulated regions other than

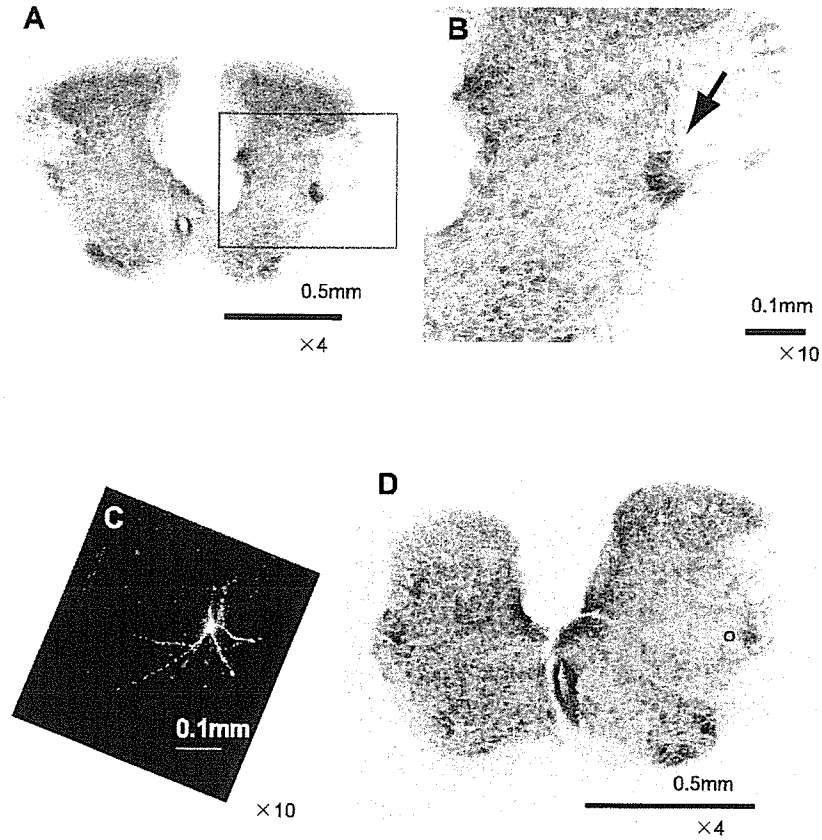


Fig. 10. *A*: histological examination of the location of IML neurons at the Th_2 level that were coagulated at the conclusion of electrical stimulation of the IML. Shown is a cross section stained with neutral red. *Top*: dorsal; *bottom*, ventral. *B*: high-magnification image of *A* showing the location of electrical stimulation of the IML. The location that was electrically stimulated and then coagulated (indicated by the arrow) actually corresponded to the IML neurons. *C*: a neuron in the IML that exhibited depolarization and an increase in EPSPs in response to glutamate application to the CeVLM. This neuron stained with lucifer yellow is the same neuron shown in Fig. 8. *D*: cross section at the Th_2 level stained with neutral red. The neuron that exhibited the increase in EPSPs, stained with lucifer yellow, and shown in *C* was actually located in the IML region (○). *Top*: dorsal; *bottom*, ventral.

the axons and neural terminals of bulbospinal neurons in the IML region (e.g., ascending neurons). However, for the following reasons, we think that the extent of the electrical stimulation in this study was relatively restricted to IML. We used very thin electrodes and carefully identified and stimulated the position of the IML neurons through the CCD camera so as to limit the stimulation point. Indeed, as shown in Fig. 10, we demonstrated that the stimulation point was accurately restricted to within the IML region. The location of the depolarizing response in Fig. 4 therefore includes RVLM neurons. The depolarizing response that was detected in the region of the RVLM in response to IML stimulation served as a good positive control.

These results also suggested that the stimulation point accurately covered axons and neural terminals of bulbospinal neurons in the IML. We were therefore able to conclude that the depolarizing responses detected in the CeVLM on both the ventral surface and cross section were depolarizations of bulbospinal neurons. As shown in Fig. 8, we also demonstrated EPSPs on the IML neurons detected during chemical stimulation of the CeVLM. The results confirmed the occurrence of orthodromic responses, demonstrating the presence of a functional projection from the CeVLM to the IML.

Developmental issues. Although a tracer study (10) found that neurons in the RVMM and in the raphe nucleus project to the IML, we did not detect any depolarizing responses in the RVMM or the raphe nucleus after IML stimulation. Because the intensity of the depolarizing response depends on the number of neurons that respond, the number of neurons projecting from these areas to the IML may be relatively small. For example, in the raphe nucleus of rabbits between *day 26* of gestation and 6 days of age, the dendrites showed expansion, increased the number and length of neurons, and developed abundant spines (5). During this period, the soma grew in size. After *postnatal day 6* to adulthood, a mature pattern of dendritic branching was achieved. Therefore, the reason that we were unable to detect depolarizing responses in the midline raphe may be because of the immaturity of the neonatal rats.

Second, a previous study showed that gap junctions between neurons may be more abundant in newborn rats than in adult rats (3), and the depolarizing responses may have been exaggerated by the gap junctions because we used newborn rats that were only 2–4 days old. The decrease in gap junctions during development may weaken the depolarizing response.

Physiological implications. In this in vitro study, we investigated whether the CeVLM corresponds to the CPA, an area that has been identified in in vivo studies (2, 8, 16, 27, 28). However, few in vitro studies have identified the anatomic location of the CPA. Our histological examination demonstrated that the location of the CeVLM neurons that exhibited an antidromic action potential and stained with lucifer yellow or pontamine sky blue corresponded to the location where depolarizing responses were observed by optical imaging. The histological examination also showed that the location of the depolarizing responses in the CeVLM neurons was almost

identical to that of the CPA reported in earlier in vivo studies (11, 27, 28).

To further confirm that the CeVLM corresponds to the CPA, we investigated whether neurons in the CeVLM are involved in peripheral sympathetic nerve regulation. The IML neurons exhibited increased EPSPs and membrane depolarization in response to local application of glutamate to the CeVLM despite the absence of the RVLM and the CVLM. These results imply that neurons in the CeVLM send excitatory input to the IML neurons and that CeVLM neurons are involved in sympathetic nerve regulation and blood pressure control. If these implications are true, we can conclude that at least some of the neurons in the CeVLM correspond to neurons in the CPA. Further study by spike-triggered averaging is needed to determine whether the spontaneously occurring action potentials of the CeVLM neurons induce EPSPs in the IML neurons.

Because earlier studies have demonstrated that the functions of the CPA are mediated via sympathoinhibitory (2) and sympathoexcitatory (16) neurons in the CVLM, the CPA neurons would be expected to project to the CVLM. However, if the CeVLM corresponds to the CPA, our optical imaging findings suggest that the CPA also projects to the IML.

Even if the CeVLM does not correspond to the CPA, the CeVLM may play a role in sympathetic nerve modulation. It is hard to imagine that the CeVLM affects sympathetic nerve activity in the same manner as the neurons in the RVLM do, and instead we postulate that the CeVLM neurons modulate sympathetic nerve activity determined by the RVLM. Horiuchi et al. (8) found that microinjection of prolactin-releasing peptide in the CPA increased blood pressure but that microinjection of ANG II did not. Thus some functions of the CeVLM, such as responses to stress, differ from those of the RVLM neurons.

In summary, the optical imaging findings in the brain stem-spinal cord preparations of neonatal SHRs suggest the existence of a continuous longitudinal column in the VLM, including the RVLM, the CVLM, and the CeVLM, that gives rise to a monosynaptic projection to the IML. The neurons in the CeVLM have a role in sympathetic nerve and blood pressure control. The projection pathway and function of the CeVLM neurons need to be investigated more precisely in a future study.

GRANTS

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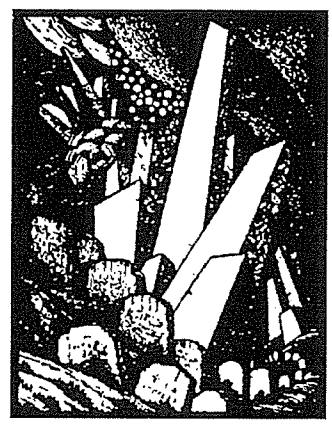

脳梗塞各論
脳梗塞の各種病型の臨床的特徴　Binswanger型脳梗塞

Binswanger型脳梗塞の概念，病理，成立機序

The notion, pathologies, and pathoetiologies of Binswanger's type infarction

冨本秀和

Key words：Binswanger型脳梗塞，白質病変，ラクナ梗塞，vascular MCI (mild cognitive impairment)，SIVD (subcortical ischemic vascular dementia)

はじめに

血管性痴呆(認知症)はその成因によって，大血管の閉塞により大小の皮質・皮質下領域の梗塞を来す多発梗塞性痴呆(multi-infarct dementia: MID)と，小血管病変に起因する小血管性痴呆に分類される．欧米では前者の比率が高く，過去にはMIDと血管性痴呆が同義に扱われた時代があったが，我が国では小血管性痴呆が血管性痴呆全体の半数以上を占めており，その重要性が認識されるようになった．小血管性痴呆は病理学的にはラクナ梗塞と虚血性白質病変が主体であり，臨床的には多発ラクナ梗塞，記憶に重要な部位の小梗塞によるstrategic infarct dementia，Binswanger型脳梗塞(いわゆるBinswanger病)の3つに分類される．多発ラクナ梗塞は多かれ少なかれ白質病変を伴っており，Binswanger型脳梗塞との境界が不明であることから，最近では両者をまとめてsubcortical ischemic vascular dementia(SIVD)と呼称されることもある．

SIVDは血管性痴呆の主要な原因であり，欧米と比べ我が国ではその割合が高率である[1]．代表的な地域疫学研究である久山町研究で，1985年に臨床的に認知症と診断された59人を2000年まで追跡した成績がある．それによれば，死亡57例(うち50例について剖検を実施)のうち，血管性痴呆，Alzheimer病の占める割合はそれぞれ40%，両方の所見を併せ持ついわゆる混合型の割合が9%であった．血管性痴呆をタイプ別にみるとBinswanger型脳梗塞が9例(39%)で最も多く，決してまれな病態でないことがわかる[2]．

1. Binswanger型脳梗塞の概念

Binswanger型脳梗塞，いわゆるBinswanger病の最初の報告は，1894年ドイツの神経科医Otto Binswangerが，ドレスデンで開催された内科医会年次総会において8症例について行った講演録に著されている．当時のドイツでは認知症の原因として神経梅毒が圧倒的多数を占めていたが，Binswangerはそれらとは別に白質の高度萎縮を認めた症例を呈示し，encephalitis subcorticalis chronica progressiva(ESCP)と命名した．その病理学的記載によれば，脳血管の動脈硬化，後頭葉・側頭葉白質の高度の萎縮，側脳室後角・下角の開大，上衣層の肥厚などの所見を認めたが，大脳皮質はほとんど異常を認めなかったとしている．臨床的には，これら患者の多くは50歳代に発症し，失語，構音障害，歩行障害などの神経症状が進行性に増悪したとし

Hidekazu Tomimoto: Department of Neurology, Kyoto University Graduate School of Medicine　京都大学大学院医学研究科　臨床神経学

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ている．

以上の報告は講演録の中に著されたもので，検索が肉眼所見にとどまるなど記載が不十分という問題があった．一方，彼はその生涯を通して記載した唯一の症例報告の中で，5年の経過で進行性に認知症を呈した男性患者についても記載しているが，26年前の梅毒罹患歴があり神経梅毒の可能性を完全に否定することが困難であった．これらの理由から，Binswangerが指摘した疾患の独立性について後世まで疑義が残ることとなった．一方，BinswangerはESCPとは別の概念としてarteriosclerotic brain degeneration(ABD)を記載している．ABDでは全身の高度の動脈硬化と心・腎障害があり，大脳白質の脱色，基底核・内包のétat criblé を認めている．顕微鏡的には動脈硬化を伴う小血管の周囲にグリオーシス，神経細胞消失があり，このような病巣が皮質，白質，基底核に散在していた．認知機能の増悪，寛解を認める点で，進行性経過を示すESCPと異なるとしているが，両者の移行型もみられることから近縁の病態である可能性が高い．

2. Binswanger型脳梗塞の病理

a. 血管病理

Binswanger型脳梗塞の患者の脳血管では，高血圧性に起因する小血管病変としてangionecrosisとfibrohyalinosisの2種類の病変がある．angionecrosisは内膜下に血漿成分の浸潤を伴い，HE染色でエオジン好性物質が沈着する．これに対し，fibrohyalinosisは中・外膜の膠原線維の増加と，中膜平滑筋細胞の変性・脱落を特徴とする．angionecrosisは基底核・視床などの穿通枝領域に多く，灰白質・白質いずれでも認められ，血管内腔の閉塞によりラクナ梗塞を来したり，微小動脈瘤を形成して脳出血の原因となる．これに対し，fibrohyalinosisはBinswanger型脳梗塞に比較的特異的で[3]，白質病変の髄質動脈に高頻度に認められ，血管内腔の閉塞はまれである．しかし，血管拡張性の刺激に対する反応性が低下する原因となり，慢性脳虚血を引き起こすことが指摘されている(**図1-A, B**)[4,5]．また，Binswanger型脳梗塞ではこれら小血管病変に平行して大脳白質領域に血液脳関門の障害が起こり，病態に関与する[6,7]．

b. 実質の病理変化

大脳白質はミエリン染色で皮質直下のU線維を除き，染色性の低下を認める．U線維が保存される点については，皮髄境界直下の白質領域は血流の二重支配を受けていることが原因として推測されている．白質病変部の髄鞘は密度が減少し，膨化した形態を呈する(**図1-C, D**)．しかし，多発性硬化症にみるような髄鞘特異性はなく，鍍銀染色を行うと軸索の染色性も低下している．髄鞘を形成するオリゴデンドログリアは細胞密度が減少し，ミクログリアは増加して突起や細胞体が肥胖化した活性化ミクログリアの形態を呈する．一方，アストログリアは軽度の白質病変部では反応性増殖を示すが，病変が高度な部位では突起が断裂し数珠状の形態変化(clasmatodendrosis)を示し，アストログリアの変性像と考えられている[8]．MRIのT2強調画像で高吸収域として観察される部位は，放射線学的にはleukoaraiosisと呼称されるが，病理学的には上記の様々な形態変化を包含している．すなわち脱髄，グリオーシス，血管周囲腔の拡大などに対応し，何らかの虚血性変化を反映していることが多い．しかし，正常白質でもleukoaraiosisが認められたとの報告があり，必ずしも虚血性変化を意味しない場合もある．

3. Binswanger型脳梗塞の成立機序

a. 慢性脳虚血に対する白質の脆弱性

白質病変の成立機転として慢性脳虚血がその原因と考えられている(**図2**)．認知症発症に伴って，Binswanger型脳梗塞では脳血流は低下する．Romanは，細動脈硬化の結果，小血管の閉塞が起きればラクナ梗塞となり，脳血管の狭窄あるいは血管反応性の低下に留まった場合は，慢性的な脳血流低下が起き白質病変が形成されるとの仮説を提唱している[9]．実際，齧歯類で脳血流を長期間慢性的に低下させると白質病変が作成可能である．マウスの両側頸動脈を狭窄させると脳血流は7割程度に低下し，急性虚血に

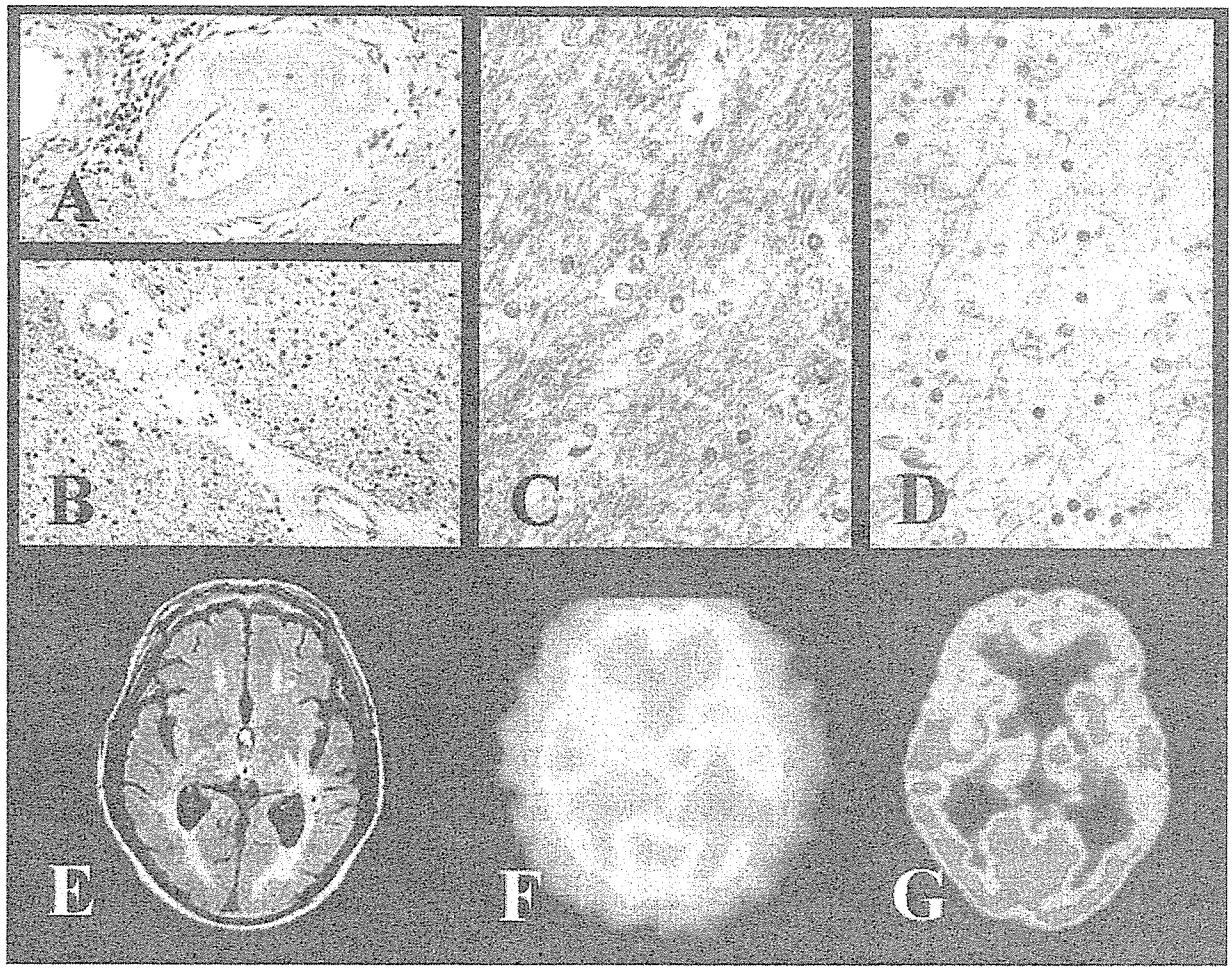

図1 Binswanger型脳梗塞の病理と神経機能画像

上段はBinswanger型脳梗塞の血管と髄鞘を示す．Binswanger型脳梗塞剖検脳の基底核のangionecrosis（A），前頭葉白質のfibrohyalinosis（B）を示す（HE染色）．髄鞘染色でBinswanger型脳梗塞（D）は対照（C）と比べ髄鞘の減少とオリゴデンドログリア細胞核の減少が明らかである．下段はBinswanger型脳梗塞患者の頭部MRI FLAIR画像（E）と脳血流SPECT（F），FDG-PET画像（G）．T2強調画像で広汎な白質病変と右視床を含むラクナ梗塞の散在を認める．SPECT，FDG-PETでは右視床の取り込み低下が明らかである．

脆弱とされる海馬・大脳皮質などの灰白質領域に梗塞を生じることなく，白質選択的に髄鞘，軸索を含む白質病変が作成される[10]．白質病変の出現に先行して病変部ではミクログリアが活性化され，様々なケミカル・メディエータが放出される．

b. オリゴデンドログリアの細胞死

白質は髄鞘をはじめとする細胞膜成分に富み，脂質の占める割合が実に55％を占め，灰白質の30％と比べ高率である．このため，虚血によって脂質過酸化反応が起こりやすくフリーラジカルが産生されやすいという特異性がある．オリゴデンドログリアの細胞死機序については不明な点も多いが，ミクログリアの活性化阻害作用があるミノサイクリンが白質保護効果を示すという事実があり[11]，虚血によって活性化されたミクログリアからフリーラジカルやTNFα，IL-1βなどの炎症性サイトカインが産生され，オリゴデンドログリアのアポトーシスを誘導する可能性がある．一方，虚血によって細胞外腔のグルタミン酸が増加し，オリゴデンドログリアのAMPA受容体刺激を介してアポトーシスを誘導する可能性も指摘されている．

c. 白質病変，ラクナ梗塞と神経ネットワーク障害

慢性脳低灌流動物の行動評価ではワーキング

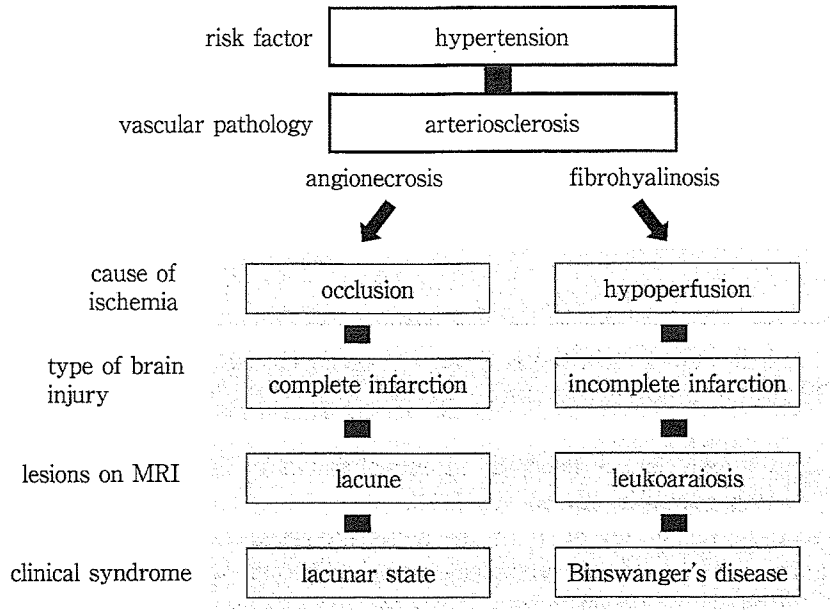

図2　白質病変とラクナ梗塞の成立機転(Romanらの仮説)(文献[9]より改変)

メモリの低下が認められており，白質病変のみでなく症候学的にもBinswanger型脳梗塞でみられる皮質下性痴呆に類似する．一方，Binswanger型脳梗塞にみられる認知症の責任病巣は白質病変かラクナ梗塞のいずれが重要か，という点は長く議論の的になってきた．欧州のLADIS研究の結果では，ラクナ梗塞と白質病変は認知機能低下のそれぞれ独立した危険因子であり，特に白質病変の関与が大きいと報告している[12]．

前脳コリン神経の起始核であるMeynert基底核は，外包を経由して大脳皮質に投射するが，大脳白質病変はその投射路にあたる外包を高頻度に障害する．したがって，Binswanger型脳梗塞ではコリン神経路のネットワーク障害があり，認知機能低下に関与する[13]．一方，記憶に重要なネットワークの一部に戦略的に小梗塞が起こった場合にも，認知機能が急速に低下することがあり，以下にその一例を示す．

[症例] 68歳，女性，左きき

1年前から人との面会を断るようになり，怒りっぽくなった．半年前から視線が定まらず無表情となった．歩行が不安定となり，転倒することがあった．3カ月前から発語が乏しくなり，パジャマの上に服を着るなどの異常行動が出現した．また，何か用事を頼もうとして何だったか忘れてしまう，など健忘が認められた．頭部MRIでは広汎白質病変とラクナ梗塞の多発を認め，特に一年前の画像検査になかった右視床の血流および糖代謝の低下が認められた(**図1-E～G**)．

白質病変が高度であっても認知症を発症しない症例が多数存在することから，白質病変は認知機能に影響しないとの考えが以前は支配的であった．しかし，最近の縦断的研究の結果では，一定の閾値を超える白質病変は認知機能障害の責任病変になると考えられるようになった[14,15]．大脳白質は一側の異なる大脳皮質領域間を連絡する連合線維，対応する半球間を結ぶ交連線維，皮質・皮質下を結ぶ投射線維より構成されている．Meynert基底核と大脳皮質を結ぶコリン神経路は投射線維に属している．一方，視床内側核群はbasolateral limbic circuitの構成要素であり，視床前核はPapetz回路の重要な中継核となっている．これら記憶に深く関連するネットワーク構成部位では，小梗塞であっても認知症の原因となり得る．したがって，認知症の発症に両者が独立に関与するとのLADIS研究の報告は妥当な結論と思われる．

おわりに

Binswanger型脳梗塞の約半数は，明らかな脳卒中発作を伴わずに認知症が進行する．また，高度の白質病変があっても認知症を呈さない時期が存在し，血管性軽度認知機能障害(vascular MCI)と呼ばれている[16]．今後は無症候性白質病変の中で，特に認知機能障害を来しやすい群を早期に検出すること，これらの群に対して危険因子の管理を徹底することと平行して，有効な治療法を開発することが重要と考えられる．

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Are Cerebrovascular White Matter Lesions an Early Sign of Vascular Cognitive Impairment and Vascular Dementia?

Hidekazu Tomimoto*

Department of Neurology, Kyoto University Graduate School of Medicine, Kawahara-cho 54, Shogoin, Sakyo-ku, Kyoto 606-8504, Japan

Abstract: Vascular dementia is a heterogeneous syndrome resulting from large vessel disease or small vessel disease. Multi-infarct dementia (MID) is due to large vessel disease, and is characterized by multiple cortical infarctions and motor disabilities. Prompt treatment during the acute stage for stroke and appropriate strategies for stroke recurrence are pivotal for the prevention of MID.

In contrast, subcortical ischemic vascular dementia (SIVD) is caused by small artery disease. Patients with SIVD may show abulia, apathy, and a loss of verbal fluency and executive function, but the amnesia is less severe as compared to those with Alzheimer's disease. These patients do not necessarily exhibit a stroke episode, and may eventually evolve from a latent condition with vascular mild cognitive impairment (MCI-V) and radiological abnormalities such as extensive cerebrovascular white matter lesions (WMLs). These lesions consist pathologically of a dilatation of the perivascular space, gliosis, demyelination and incomplete infarction of the cerebral white matter. Their clinical significance has remained unclear for a long time, since WMLs are frequently observed in elderly asymptomatic subjects. However, recent studies indicate that these lesions are predictors of a future risk of stroke and dementia.

This review discusses the diagnosis, treatment and prevention of vascular dementia. The recent advances in neuroimaging techniques which may enable the identification of patients susceptible to developing vascular dementia and motor disabilities among the population with extensive WMLs are also described.

Keywords: White matter lesion, subcortical ischemic vascular dementia, multi-infarct dementia, magnetic resonance imaging.

INTRODUCTION

Vascular dementia is caused by heterogeneous pathophysiology, including small vessel disease and large vessel disease. Occlusion or stenosis of the major vessels, which are attributable to atherosclerosis or cerebral embolism, results in cortical cerebral infarction. This type of vascular dementia has been designated as multi-infarct dementia (MID), and was previously thought to be a synonym of vascular dementia. Tomlinson *et al.* reported that cognitive deterioration was correlated with the volume of the cerebral infarction, and dementia occurs if the volume exceeds 50 ml [1]. Patients with MID show a step-wise deterioration with each stroke episode, characterized by severe motor disabilities. The strategies to prevent MID are aimed at stroke prevention.

In contrast, small vessel disease is the other mechanism of vascular dementia, and is responsible for lacunar infarctions and white matter lesions (WMLs), the latter of which consist of demyelination, axonal loss, dilatation of perivascular space and incomplete infarctions [2]. Patients may become amnesic suddenly if the lacunar infarction damages the brain sites specifically related to memory acquisition and retention. This condition is termed acute onset amnesia, and represents a type of vascular dementia, i.e. strategic single infarct dementia.

*Address correspondence to this author at the Department of Neurology, Kyoto University Hospital, Kawahara-cho 54, Shogoin, Sakyo-ku, Kyoto 606-8504, Japan; Tel/Fax: 81-75-751-3766;
E-mail: tomimoto@kuhp.kyoto-u.ac.jp

However, more than half of the patients with vascular dementia exhibit a slowly progressive deterioration of their neurological symptoms, including cognitive impairment and gait disturbances [3]. Most of these patients suffer from WMLs and lacunar infarctions, and this can be designated as subcortical ischemic vascular dementia (SIVD), which ranges from multiple lacunar infarctions to Binswanger's disease, a form of SIVD characterized by extensive WMLs, depending on the severity of the WMLs.

1. VASCULAR DEMENTIA AND VASCULAR COGNITIVE IMPAIRMENT (VCI)

Several diagnostic criteria have been proposed for vascular dementia: Hachinski's ischemic score (HIS), the Diagnostic and Statistical Manual for Mental Disorders, Fourth Edition (DSM-IV), the National Institute of Neurological Disorders and Stroke-Association Internationale pour Recherche et l'Enseignement en Neurosciences (NINDS-AIREN), the State of California Alzheimer Disease Diagnostic and Treatment Centers (ADDTC), and International Classification of Diseases, and the Tenth Edition (ICD-10) criteria. Unfortunately, none of these criteria have enough sensitivity and specificity for the diagnosis of vascular dementia, and the degree of inconsistency is not trivial among these criteria; the difference in the rate of diagnosis for vascular dementia ranges several folds within the same subjects [4, 5]. The common features of the DSM-IV, NINDS-AIREN, and ADDT-C criteria are the keynote definition,

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which depends on a temporal profile of cognitive impairment emerging within 3 months after the stroke.

Esiri *et al.* reported that one third of patients develop dementia within 1 year after a stroke [6]. Henon *et al.* showed a cumulative incidence of post-stroke dementia for 3 years was 28.5%, among which two thirds of patients met the criteria for vascular dementia [7]. Indeed, all of these patients with post-stroke dementia are not necessarily affected by pure vascular dementia. Instead, the evidence suggests a spectrum of underlying pathologies, which include neurodegenerative diseases. In the autopsied brains from the Nun study, vascular lesions and senile changes including senile plaques and neurofibrillary tangles contributed in an additive fashion to cognitive impairment [8]. In addition, recent studies have shown that common risk factors may coexist between vascular dementia and Alzheimer's disease. These factors include hypertension, diabetes mellitus, hypercholesterolemia, atherosclerosis, hyperhomocysteinemia and the apolipoprotein E4ε allele, and suggest a complex relationship between vascular factors and the pathogenesis of Alzheimer's disease [9]. Thus, vascular dementia consists of several interrelated conditions; post-stroke dementia, mixed Alzheimer's disease and vascular dementia, and vascular mild cognitive impairment (MCI). As an umbrella term, vascular cognitive impairment has been proposed, and comprises vascular MCI, vascular dementia, mixed dementia (mixed Alzheimer's disease and vascular dementia), and post-stroke dementia [10].

2. DOES CHRONIC CEREBRAL HYPOPERFUSION INDUCE VASCULAR COGNITIVE IMPAIRMENT?

Roman hypothesized that chronic cerebral hypoperfusion is a trigger of vascular cognitive impairment [11]. Long standing hypertension leads to arteriolosclerosis of the small arteries, which induces lacunar infarction after complete occlusion and WMLs after stenosis of the small vessels. Angionecrosis and fibrohyalinosis are responsible predominantly for lacunar infarctions and WMLs, respectively. Angionecrosis is caused by the subendothelial accumulation of hyaline material, often occluding the lumen, and is distributed in both the gray and white matter. In contrast, fibrohyalinosis is characterized by the proliferation of fibroblasts and collagen fibrils in the tunica media and adventitia [12]. It rarely occludes the luminal spaces, and is found mostly in the white matter. In brains with SIVD, fibrohyalinosis is the cardinal pathology of the medullary arteries [13], with smooth muscle cells degrading in the proximal arteries but proliferating in the terminal arterioles [14]. Fibrohyalinosis after longstanding hypertension may impair the vasomotor reactivity of the medullary arteries [15, 16], and causes cerebral hypoperfusion in the absence of vessel occlusion. Several lines of experimental data support this hypothesis. First, the stroke-prone spontaneously hypertensive rat (SHR-SP), a genetic model for hypertension and stroke, exhibits WMLs after sustained hypertension [17]. Second, chronic cerebral hypoperfusion models can be induced experimentally in gerbils, rats and mice [18-20]. These animals exhibit WMLs and cognitive impairment after a latent period.

3. THE IMPACT OF LACUNES AND WMLS ON COGNITIVE IMPAIRMENT

The neuropathological substrates of cognitive impairment in small vessel disease are still under discussion. Grey matter lacunes are common in elderly brains, however, most of them are asymptomatic from a cognitive point of view. At baseline, there are no correlations between the presence of lacunes and cognitive functions, but yet their presence is associated with a cognitive decline when a new lacunar infarction occurs during the follow-up [21]. In the Nun study, lacunar infarction has been shown to accelerate the cognitive dysfunction in Alzheimer's disease [8]. More recently, Gold *et al.* showed that lacunes are an independent predictor of cognitive impairment in the absence of senile changes [22].

The contribution of WMLs to cognitive impairment is documented in more detail as compared to lacunes. This causal relationship has been under investigation previously, since WMLs are frequently observed even when the subjects are free of any neurological symptoms [23]. In cross-sectional MRI studies, no or a subtle association has been shown between the severity of the WMLs and cognitive impairment [24, 25]. In a follow-up for 4 years of 19 patients with WMLs, none showed any neurological deterioration [26]. These points are in good agreement with the daily clinical experience that patients with marked WMLs often do not show any neurological abnormalities. However, in more recent longitudinal studies, a weak but significant correlation between WMLs and cognitive impairment has been described [27, 28]. Thus, further investigations are warranted on whether WMLs are responsible for cognitive impairment, and apparently a more stratified analysis is needed in terms of the baseline severity of WMLs and specific cognitive domains.

In the Cardiovascular Health Study, patients with worse WML grades show more cognitive decline during the follow-up period [29]. This worsening of the WMLs depends on the baseline severity of the WMLs, being initially latent but progressive at later confluent stages [30]. Therefore, it is reasonable that earlier WMLs are not associated with cognitive impairment, with a threshold before developing symptoms [31]. The most affected cognitive domains are executive functions and the speed of mental processing, which may secondarily result in impaired memory and visuospatial functions [32], thereby making it difficult to assess the cognitive dysfunction in terms of amnesia.

4. PREDICTION OF COGNITIVE IMPAIRMENT IN PATIENTS WITH EXTENSIVE WMLS

Among the subjects with extensive WMLs, those with and without cognitive impairment can be differentiated based on hemorheology [33-36], cerebral blood flow and metabolism [37-42] and functional neuroimaging [43-45]. Patients with SIVD have an activation of their platelet aggregation and coagulation-fibrinolysis system [35, 36]. In contrast, these systems remain within normal ranges in subjects with extensive WMLs but without dementia [33]. The SIVD patients also have increased levels of fibrinogen and plasma viscosity [34].

The relationship between WMLs and cerebral blood flow and metabolism has been studied extensively. Patients with extensive WMLs show a decreased cerebral blood flow (CBF), even when cognitive function is not impaired [37, 38]. Similarly, the vasomotor reactivity to CO_2 and postural changes are also decreased with extensive WMLs [14, 15]. However, global [39, 40] and frontal [41] cortical metabolism is decreased in patients with SIVD, but not in asymptomatic patients. In a previous positron emission tomography (PET) study, asymptomatic patients with extensive WMLs showed a decreased CBF but normal cerebral metabolic rate for oxygen (CMRO2), with an increased oxygen extraction fraction (OEF), whereas demented patients showed decreased CBF and $CMRO_2$ values [42]. We also demonstrated impaired neuronal viability in the frontal and temporal cerebral cortices in SIVD using a flumazenil PET scans as markers [43].

Using MR spectroscopy, N-acetylaspartate (NAA), a marker of neuroaxonal components, and choline-containing compounds (Cho), a marker of cellular membrane turnover, were found to be well-preserved in those subjects with normal cognitive function. In contrast, these markers were decreased in patients with SIVD, implying that both demyelination and axonal damage occur in SIVD [44]. Diffusion tensor MR imaging is a method in which the fractional anisotropy (FA) of water molecules can be an index of the degradation of white matter pathways. An FA map is inversely correlated with executive functions better than conventional MRI, and may provide a better index of WMLs [45]. Thus, cognitive impairment is associated with hypometabolism and decreased neuronal viability in the cerebral cortices and both demyelination and axonal damage in extensive WMLs.

5. THE MECHANISM OF COGNITIVE IMPAIRMENT IN PATIENTS WITH WMLS

The dysfunction of the cerebral cortex in SIVD has also been implicated in a neuropathological study which demonstrated that the synaptic density was decreased in proportion to synaptophysin immunohistochemistry in autopsied brains [46]. Hippocampal and cortical gray matter volumes are also reduced in patients with SIVD without AD pathology [47].

The mechanism of cortical dysfunction may be due to a direct and/or indirect effect of ischemic insults. Microinfarctions, noncystic lesions with a diameter less than 4 mm, directly involve the cerebral cortex, and these lesions may impair cognitive function directly in vascular dementia [48]. These lesions have been shown to be the best predictors of cognitive impairment (19.9%), followed by periventricular and diffuse WMLs (9.7% and 5.4%, respectively) in 45 elderly persons with various degrees of cognitive impairment, but without significant neurofibrillary tangle pathology or macrovascular lesions [49]. Microinfarctions may be related to small vessel diseases, but this idea needs to be studied further, since concurrent MR techniques have failed to detect these lesions.

An alternative and more likely mechanism responsible for cortical dysfunction is subcortico-cortical disconnection, which is mediated by a variety of pathophysiologies. Involvement of neural networks closely related to memory and other cognitive functions may cause a cognitive decline [50, 51]. Indeed, loss of NAA in the cerebral cortex in SIVD is correlated regionally with the subcortical lesion load, and suggests that subcortical lacunes and WMLs cause functional deafferentation of the cerebral cortex, which is sometimes termed subcortical-cortical diaschisis [52]. Disconnection of the cholinergic pathway may also be caused by cortical and subcortical lesions. The diagonal band of Broca and the septal nucleus provide a cholinergic input to the hippocampus, and the nucleus basalis of Meynert to the cerebral cortices passing through the subinsular regon [53]. The latter pathway may be involved by WMLs strategically in the subinsular region [54, 55, 56], or ischemic foci in the nucleus basalis of Meynert [57]. Large vessel cortical infarction may also impair cholinergic neurotransmission by retrograde degeneration of the neurons in the nucleus basalis of Meynert [58]. Moreover, cholinergic neural disconnection may impair a positive regulation of cortical CBF by the nucleus basalis of Meynert [59, 60], and result in an impairment of CBF regulation in the cerebral cortex.

6. DOES VASCULAR MCI EVOLVE INTO SIVD?

Patients with SIVD may show abulia, apathy, loss of verbal fluency, impaired attention and executive functions, with a slowing of motor performance and information processing [61]. Episodic memory is also relatively spared in SIVD as compared with Alzheimer's disease [62].

The symptoms in subjects with extensive WMLs are nonspecific, with dizziness, a tendency to fall down, depression, and emotional incontinence. Neurological examinations in these patients may reveal abnormalities including dysarthria, a laterality of the deep tendon reflexes, a small steppage gait, apathy, and incontinence in the absence of dementia. Thus, vascular MCI has distinctive features separate from amnesic MCI, which is prodromal for Alzheimer's disease; 15-20% of these patients also exhibit dementia annually [63]. Radiologically, vascular MCI is characterized by extensive WMLs and the absence of cortical infarctions on MRI [64]. SIVD patients do not necessarily exhibit a stroke episode, but 54% of SIVD patients eventually evolve from a latent condition without stroke [3]. In contrast, most patients with MID or strategic single infarct dementia, which is related to lacunar infarcts strategically important for memory, develop dementia without any preceding symptoms (Fig. **1**). Meyer *et al.* reported that during the course of vascular dementia, 55% of these patients evolved from vascular MCI and most of their subtype was SIVD [64]. Patients with vascular MCI exhibit lower scores in word fluency and the Wisconsin card sorting test, but milder amnesia as compared to patients with amnesic MCI. The clinical profiles of vascular MCI show more severe motor disabilities and a shorter life expectancy than amnesic MCI [65]. Therefore, therapeutic intervention for vascular MCI at an earlier stage is pivotal for controlling vascular dementia.

7. PREVENTION OF SIVD

With respect to primary preventive strategies for SIVD, the control of hypertension is crucial because hypertension and aging are the major risk factors for WMLs. An increased diastolic blood pressure is often associated with a worsening of WMLs [29, 66]. In a clinical trial, the Systolic Hyperten-

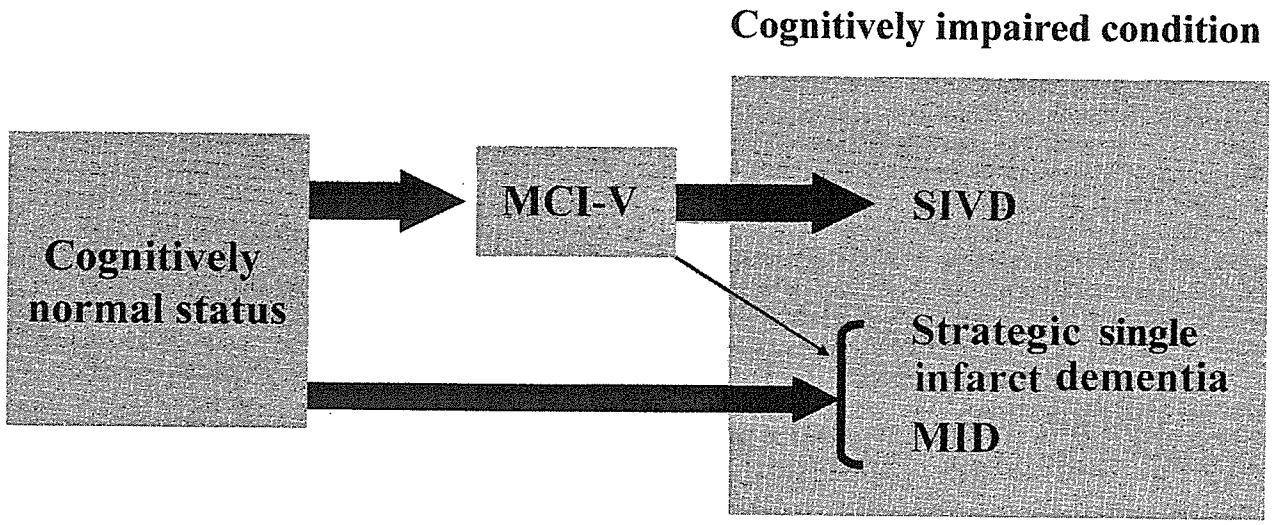

Fig. (1). Temporal profiles of each subtype of vascular dementia. The patients with MCI-V are gradually transformed into those with SIVD, whereas strategic single infarct dementia and MID show a sudden onset. MCI-V: vascular mild cognitive impairment, SIVD: subcortical ischemic vascular dementia, MID: multi infarct dementia.

sion in Europe (Syst-Eur), an alleviation of the dementia by anti hypertensive treatment has been reported, but remains inconclusive [67, 68]. Hyperlipidemia is a marginal risk factor for SIVD, but lipid-lowering agents, statins such as atorvastatin, cerivastatin, fluvastatin, pravastain, or simvastatin, substantially lowered the risk of dementia due to undetermined etiologies [69]. Other putative vascular risk factors includes diabetes mellitus, coronary heart disease, atherosclerosis, smoking, atrial fibrillation and hyperhomocystinemia [70].

Secondary prevention is targeted to management of cardiovascular risks and prevention of stroke recurrence. A limited number of tools are available for the secondary prevention of SIVD. Several drugs to date have shown positive results, however, various drugs for vascular dementia, including antithrombotics, vasodilators, Ginkgo biloba extracts, nootropics, ergot alkaloids and antioxidants, were mostly disappointing [71]. Perindopril, an angiotensin-converting enzyme inhibitor showed a prominent benefit in the management of hypertension and reduction of the risk for dementia among patients with recurrent episodes of stroke [72]. Ischemic neuronal damage is mediated by excitotoxicity, and therefore the non-competitive *N*-methyl-D-aspartate (NMDA) antagonist memantine has been tested for the treatment of vascular dementia with some beneficial effects in cognitive stabilization [73]. The calcium antagonist nimodipine was effective in improving some of the neuropsychological scores in SIVD as compared to MID [74]. Propentofylline, a phosphodiesterase inhibitor, showed a long term efficacy in patients with mild to moderate vascular dementia [75].

Cholinergic pathways represent another target for pharmacological modulation. Choline esterase inhibition has a potency to restore CBF in the cerebral cortex after lesioning of the nucleus basalis of Meynert [77], and cerebral ischemia [78], and also upregulate cholinergic neurotransmission in the cerebral cortex. Concurrent evidence supports the efficacy of cholinesterase inhibitors in vascular dementia, which include donepezil [79, 80] and ribastigmine [81, 82]. Galantamine is an acetylcholine esterase inhibitor that also modulates central nicotinic receptors and enhance cholinergic neurotransmission. This agent showed benefits on cognition, activities of daily living, behavioral symptoms and global function in patients with Alzheimer's disease plus cerebrovascular disease, or with probable vascular dementia [83]. Results from further trials are pending, but the evidence thus far suggests that cholinesterase inhibitors may be a useful tool in the treatment of vascular dementia.

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Matrix Metalloproteinase-2 Plays a Critical Role in the Pathogenesis of White Matter Lesions After Chronic Cerebral Hypoperfusion in Rodents

Kayoko Nakaji, MD; Masafumi Ihara, MD; Chiaki Takahashi, MD; Shigeyoshi Itohara, PhD; Makoto Noda, PhD; Ryosuke Takahashi, MD; Hidekazu Tomimoto, MD

Background and Purpose—Cerebrovascular white matter (WM) lesions contribute to cognitive impairment and motor dysfunction in the elderly. A disruption of the blood–brain barrier (BBB) is believed to be a critical early event leading to these WM lesions. Previous studies have suggested the involvement of matrix metalloproteinase-2 (MMP-2) in BBB disruptions and the upregulation of MMP-2 after chronic cerebral hypoperfusion in a rat model. In the present study, we asked whether MMP-2 is involved in the BBB disruption and the subsequent WM lesions after chronic cerebral hypoperfusion.

Methods—We compared the severity of white matter lesions in rats after chronic cerebral hypoperfusion with or without an MMP inhibitor. Then, we also induced the chronic cerebral hypoperfusion in wild-type and MMP-2-null mice.

Results—In the rats treated with a relatively selective MMP-2 inhibitor, AG3340, the WM lesions after chronic cerebral hypoperfusion were significantly less severe, and the number of activated astroglia and microglia were also significantly lower as compared with the vehicle-treated rats. Gene knockout of MMP-2 also reduced the severity of the WM lesions and the number of activated astroglia and microglia in a mice system. In both rodents, the disruption of BBB function, as assessed by IgM staining and the Evans blue extravasation test, was less severe when MMP-2 activity was attenuated.

Conclusions—These findings indicate that MMP-2 plays a critical role in the BBB disruption, glial cell activation, and WM lesions after chronic cerebral hypoperfusion and suggest the potential value of MMP-2 inhibitors as a therapeutic tool in cerebrovascular WM lesions. (***Stroke.* 2006;37:2816-2823.)**

Key Words: blood–brain barrier ■ chronic cerebral hypoperfusion ■ MMP inhibitor ■ MMP-2 ■ white matter lesion

Cerebrovascular white matter (WM) lesions, a neurodegenerative condition characterized by hyperintense signals on magnetic resonance images, are frequently associated with aging and cerebrovascular disease and are responsible for the cognitive decline of the elderly. Chronic cerebral ischemia is likely to cause these WM lesions, because cerebral blood flow is decreased in these patients.[1] Indeed, similar WM lesions can be induced in rats and mice after chronic cerebral hypoperfusion, the experimental conditions mimicking chronic cerebral ischemia in humans.[2,3]

Matrix metalloproteinases (MMPs) are a family of endopeptidases that can degrade most of the major constituents of the extracellular matrix.[4] MMP-2 and MMP-9 represent a subgroup of the MMP family and degrade several extracellular matrix components, including type IV collagen, fibronectin, and gelatin. Deregulated MMPs have been implicated in the tissue destruction associated with cancer, arthritis, and multiple sclerosis.[4] MMPs may also play a role in neurologic disorders. For instance, MMP-9 is increased in human brains after stroke,[5] and MMP-2 and MMP-3 are increased in cerebrovascular WM lesions from patients with vascular dementia.[6] A reduction in the basement membrane components, including type IV collagen, is associated with the blood–brain barrier (BBB) disruption during cerebral ischemia.[7] In our previous study on chronic cerebral hypoperfusion, the BBB disruption was accompanied by an upregulation of MMP-2 but not MMP-9,[8] suggesting the specific involvement of MMP-2 in the WM lesions. We hypothesize that the MMP-2 upregulation after chronic cerebral hypoperfusion correlates with BBB damage, which leads to glial activation and subsequent WM lesions. To clarify the cause–effect relationship among MMP-2 upregulation, BBB disruption, and WM lesions, we used 2 strategies to attenuate MMP-2 activity: an MMP inhibitor, AG3340, and MMP-2

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From the Department of Neurology (K.N., M.I., R.T., H.T.), Horizontal Medical Research Organization (M.I.), the Department of Molecular Oncology (M.N.), and The 21st Century Center of Excellence Program, Department of Oncology (C.T.), Kyoto University Graduate School of Medicine, Kyoto, Japan; and the Laboratory for Behavioral Genetics (S.I.), RIKEN Brain Science Institute, Wako, Japan.

Correspondence to Kayoko Nakaji, MD, Department of Neurology, Graduate School of Medicine, Kyoto University, 54 Kawaharamachi, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. E-mail kann@kuhp.kyoto-u.ac.jp

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knockout. The results from both experiments strongly supported the idea that MMP-2 plays a critical role in BBB disruption and WM lesions.

Materials and Methods

Chronic Cerebral Hypoperfusion in Rats and Treatment With an Matrix Metalloproteinase Inhibitor

Chronic cerebral hypoperfusion with bilateral common carotid artery occlusion (BCAO) was induced in male Wistar rats (weight 150 to 200 g; Shimizu Experimental Supply; Kyoto, Japan) by double ligation of the common carotid arteries as previously described.[2] After the operation, the rats were kept in animal quarters with food and water ad libitum.

AG3340 (Agouron Pharmaceuticals) was dissolved at 75 mg/mL in 50% DMSO in propylenglycol. The rats were treated twice a day with an intraperitoneal injection of AG3340 (100 mg/kg) or vehicle (DMSO/propylenglycol) from just before the operation until 14 days after the operation. Similar doses and treatment paradigms have been shown to be effective in inhibiting MMP activity in gliomas in model animals.[9] Because our previous study demonstrated that the number of microglia peaked on 3 days and WM lesion started to become evident on 14 days after BCAO,[2] the animals were subjected to the analyses described subsequently.

Mice

The generation of C57BL/6J mice carrying the MMP-2-null allele has been described elsewhere.[10] In this mutant allele, a region containing the promoter and the first exon of the MMP-2 gene is replaced by the pgk-neo cassette. MMP-2$^{\pm}$ parents were mated to obtain both wild-type and MMP-2$^{-/-}$ (MMP-2-null) littermates. Genotyping was performed by polymerase chain reaction using the following primers: wild-type forward, CAACGATGGAGGCACGAGTG; wild-type reverse, GCCGGGGAACTTGATGATGG; mutant forward, CTTGGGTGGAGAGGCTATTC; and mutant reverse, AGGTGAGATGACAGGAGATC.

Chronic Cerebral Hypoperfusion in Mice and Cerebral Blood Flow Measurement

Adult male mice (weight 20 to 25 g) were subjected to bilateral common carotid arteries stenosis (BCAS) by applying the microcoils with an inner diameter of 0.18 mm to both common carotid arteries as previously described.[3] The cerebral blood flow (CBF) was recorded by laser Doppler flowmetry by placing a straight probe (OmegaFLO-N1; Neuroscience Inc) on 1 mm posterior and 2 mm lateral from bregma perpendicular to the skull bone through the guide cannula. The baseline CBF recordings were obtained just before and at 2 hours and 3, 7, 14, and 30 days after the surgery. The CBF values were expressed as a percentage of the baseline value.

Histochemical Evaluation of White Matter Lesions and Glial Activation

Under deep anesthesia, the animals were perfused with 10 mmol/L phosphate-buffered saline (300 mL for rats, 100 mL for mice) and then with a fixative consisting of 4% paraformaldehyde, 0.2% picric acid, and 0.1 mol/L phosphate buffer at pH 7.4 (300 mL for rats, 100 mL for mice). The brains were removed and postfixed for 24 hours in 4% paraformaldehyde in 0.1 mol/L phosphate buffer and then stored in 15% sucrose in 0.1 mol/L phosphate buffer. The fixed brains were embedded in paraffin and sliced into 2-μm-thick coronal sections. Klüver-Barrera staining and Bielschowsky staining were used to visualize the myelin sheaths and axons, respectively. As previously described,[2] the severity of the WM lesions was semiquantitatively graded as normal (grade 0), disarrangement of the nerve fibers (grade 1), formation of marked vacuoles (grade 2), and disappearance of myelinated fibers (grade 3) by an investigator blind to the experimental condition. For immunohistochemistry, serial sections (20-μm-thick) were cut in a cryostat and incubated overnight with a primary antibody at 4°C followed by incubation with the appropriate biotinylated secondary antibody (1 hour, room temperature), treatment with an avidin–biotin complex (diluted 1:200; Vector Laboratories), and visualization with 0.01% diaminobenzidine tetrahydrochloride and 0.005% H_2O_2 in 50 mmol/L Tris-HCl (pH 7.6). The primary antibodies used were as follows: monoclonal anti-rat glial fibrillary acidic protein (GFAP) (diluted 1:5000; Sigma-Aldrich; Mo, USA), polyclonal rabbit anti-mouse GFAP (diluted to 1:5000; Dako Cytomation, Denmark), polyclonal rabbit anti-MMP-2 (diluted to 1: 1,000, Chemicon International, Inc), monoclonal rat anti-mouse MHC class II antigen antibodies (diluted to 1:5000; Dako Cytomation), and rabbit anti Iba-1 antibody (1 μg/mL; Wako Pure Chemical Industries, Ltd; Osaka, Japan). Some sections were incubated with a biotinylated goat anti-rat IgM (μ), biotinylated goat anti-mouse IgM (μ) (diluted 1:1000; Kirkegaad & Perry Laboratories; Md, USA), or biotinylated Ricinus communis agglutinin-1 (diluted 1:1000; Vector Laboratories; Calif, USA) and were incubated directly with the avidin–biotin complex. To confirm the cellular source of IgM, sections were labeled by biotinylated anti-mouse IgM and rabbit anti-mouse GFAP followed by fluorescein isothiocyanate-labeled avidin (diluted 1:100; Dako Cytomation) and rhodamine-labeled goat anti- rabbit IgG (2.5 μL/mL; Dako). In the sections immunostained for Ricinus communis agglutinin-1, MHC class II antigen, Iba-1, GFAP, and IgM, we counted the number of immunopositive cells in at least 6 representative fields (per 0.25 mm^2) in the corpus callosum, the caudoputamen, and the optic tract for the quantitative analysis.

Zymography and Matrix Metalloproteinases-2 Activity Assay

Minced forebrain tissues were incubated with gentle rotation at 4°C for 20 hour in an extraction buffer consisting of 0.5% Triton-X 100, 0.5 U/mL aprotinin, and 0.01% sodium azide in 0.01 mol/L phosphate-buffered saline. The samples were then centrifuged at 14 000 rpm for 15 minutes at 4°C and the supernatants were collected. The protein content was adjusted to 10 mg/mL. The gelatinolytic activity of these samples was detected by SDS-PAGE zymography as described elsewhere,[8] although MMP-2 activity in the gray matter may interfere a sensitive detection of the activity in the WM. Equal amounts of tissue extract (50 μg) were then subjected to electrophoresis. To restore the activity of the protein, sample gels were agitated in 0.01 mol/L Tris-HCl (pH 8.0) containing 2.5% Triton X-100 (30 minutes×2). After washed in 0.05 mol/L Tris-HCl (pH 8.0) for 30 minutes, the gels were incubated overnight twice at 37°C in 0.05 mol/L Tris-HCl (pH 8.0) containing 0.5 mmol/L $CaCl_2$ and 1.0 mol/L $ZnCl_2$. After incubation, the gels were stained with Coomassie blue R-250. The amount of activated and latent forms of MMP-2 in the whole forebrain extracts were also assessed using the Matrix Metalloproteinase-2 Biotrak Activity Assay System (Amersham Biosciences), which is based on a 2-site enzyme-linked immunosorbent assay "sandwich" format and recognizes both the proform and active form of MMP-2.

Evans Blue Extravasation

The mice were killed at 3 hours and 1, 3, 5, 7, and 14 days after BCAS. One hour before each time point, 1 mL of 4% Evans blue (EB; Nakalai Chemicals Ltd) in normal saline was injected intraperitoneally. The animals were anesthetized and then perfused transcardially with 200 mL of 10 mmol/L phosphate-buffered saline. The brains were snap-frozen, sectioned into 20-μm-thick slices, and examined by fluorescence microscopy. For quantitative measures, the images were analyzed within 4 structurally similar areas (2 paramedian portions of the corpus callosum on each hemisphere) in each mouse and digitally level-adjusted by Adobe Photoshop (Adobe Systems) so that intravascular EB would be reported as white (pixel value 255) on a black background (pixel value 0). Using the public domain NIH Image 1.61 program (National Institutes of Health), the images were then binarized with intensity threshold set at pixel value 50 so that the white pixels represent intravascular and extravasated EB. The number of white pixels was divided by the total pixel