Age-related changes in the sympathetic innervation of cerebral vessels and in carotid vascular responses to norepinephrine in the rat: in vitro and in vivo studies

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Address for reprint requests and other correspondence: J. Marshall, School of Clinical and Experimental Medicine, College of Medical and Dental Sciences, The Medical School, Birmingham B15 2TT, United Kingdom (e-mail: j.m.marshall@bham.ac.uk).

Omar NM, Marshall JM. Age-related changes in the sympathetic innervation of cerebral vessels and in carotid vascular responses to norepinephrine in the rat: in vitro and in vivo studies. J Appl Physiol 109: 314–322, 2010. First published May 13, 2010; doi:10.1152/japplphysiol.01251.2009.—We hypothesized that the density of sympathetic noradrenergic innervation of cerebral arteries and vasoconstrictor responses evoked in carotid circulation by norepinephrine (NE) increase with maturation and age. In rats of 4–5, 10–12, and 42–44 wk of age (juvenile, mature, middle aged), glycolytic acid applied to stretch preparations showed the density of noradrenergic nerves in basilar and middle cerebral arteries was greater in mature than juvenile or middle-aged rats. In anesthetized rats, infusion of glycolic acid applied to stretch preparations showed the density of noradrenergic nerves in basilar and middle cerebral arteries was greater in mature than juvenile or middle-aged rats. In anesthetized rats, infusion of NE (2.5 μg/kg iv) increased mean arterial pressure (ABP) to ~180 mmHg in mature and middle-aged but to only ~150 mmHg in juveniles rats. Concomitantly, carotid blood flow (CBF) decreased in mature and middle-aged rats but remained constant in juveniles because carotid vascular conductance (CVC) decreased more in middle-aged rats. We also hypothesized that nitric oxide (NO) blunts cerebral vasoconstrictor responses to NE. Inhibition of NO synthase (L-NAME (10 mg/kg iv) induced similar increases in baseline ABP in each group, but larger decreases in CVC and CBF in mature and middle-aged than juvenile rats. Thereafter, the NE-evoked increase in ABP was similar in juvenile and mature but accentuated in middle-aged rats. Concomitantly, NE decreased CVC in juvenile and mature, but not middle-aged rats; in them, CBF increased. Thus, in juvenile rats, sparse noradrenergic innervation of cerebral arteries is associated with weak NE-evoked pressor responses and weak carotid vasoconstriction that allows autoregulation of CBF. Cerebral artery innervation density increases with maturation but lessens by middle age. Meanwhile, NE-evoked pressor responses and carotid vasoconstriction are stronger in mature and middle-aged rats, such that CBF falls despite the evoked increase in ABP. We propose that in juvenile and mature rats, NO does not modulate NE-evoked pressor responses, cerebral vasoconstriction, or CBF autoregulation, but by middle age, NO limits pressor responses and prevents breakthrough of CBF in the upper part of the autoregulatory range.

norepinephrine; sympathetic nerves; vasoconstriction; cerebral vasculature; aging

IT IS RECOGNIZED that the density of the sympathetic noradrenergic innervation of blood vessels changes during maturation and ageing (3). However, for arteries supplying the brain there is no consensus. In the rabbit, the sympathetic innervation density increased progressively in carotid and basilar arteries (CA, BA, respectively) from birth to 6 wk and until 6 mo but then declined in the CA and increased in the BA until 27 mo (5). In the rat, noradrenergic innervation density decreased in the middle cerebral artery (MCA) from 10 to 30 days of age but then increased up to 25 wk (18). By contrast in another study on the rat, noradrenergic innervation density increased in the BA, MCA, anterior cerebral artery, and internal carotid artery from 1 day to 4 wk but declined between 32 and 68 wk of age (22). Further, the noradrenergic innervation density of the MCA and BA of rats was also reported to increase between 4 and 6 wk of age, continue to increase in the MCA, but decline in the BA between 8 and 12 wk (8). Clearly, it is difficult to make any deductions about the effects of maturation and ageing on individual cerebral arteries because different studies have involved different species, different arteries, and different ages over the life span of the species studied.

It has been argued that under physiological conditions, changes in sympathetic nerve activity are not an important means of evoking acute changes in cerebral blood flow (13). However, there is evidence that cervical sympathetic activity does play an important role during an abrupt increase in mean arterial pressure (ABP) within the autoregulatory range by constricting the large cerebral arteries and protecting against the breakthrough of myogenic autoregulation (13, 14, 33). Indeed, in adult rats, cerebral sympathectomy achieved by bilateral cervical ganglionectomy had no effect on resting ABP or cerebral blood flow but decreased the upper limit of the autoregulatory range to pressor responses evoked by injection of phenylephrine, from 142 to 130 mmHg and 158 to 145 mmHg in the cortex and thalamus, respectively (28). These findings raise the question of whether age-related changes in sympathetic innervation density are associated with changes in the ability of the cerebral circulation and its supplying arteries to autoregulate to systemic pressor responses or, indeed, respond actively to norepinephrine (NE). Of the few studies performed in vitro, the sensitivity to NE increased in the rabbit BA between 2 and 24 wk (31), and in rat CAs, maximal constrictor responses to phenylephrine increased from 3–8 wk to 17–29 wk of age (7). There has been no study of the effects of age on the responses of the circulation supplying the brain to NE in vivo.

Thus, in the present study, we compared the density of the sympathetic noradrenergic innervation of the BA and MCA in rats of 4–5, 10–12, and 42–44 wk of age, which correspond to the juvenile, sexually mature, and middle-age stages of life in humans (10). To test whether any age-related changes in innervation density were specific to cerebral circulation, comparisons were also made on the femoral artery (FA) and the
caudal ventral artery (CVA) of the tail. Further, in anesthetized rats of the same age groups, we compared responses evoked in carotid blood flow (CBF) and carotid vascular conductance (CVC) by systemic infusion of NE, using a dose of NE that increased ABP to at least 150 mmHg, the upper limit of the cerebral autoregulatory range in adult rats (25). Thus we were able to compare in juvenile, mature, and middle-aged rats vasoconstrictor responses evoked by NE and assess the effectiveness of autoregulation in the circulation supplied by the carotid artery, i.e., the ability to maintain blood flow despite a rise in ABP.

The tonic synthesis of NO exerts a tonic dilator influence on the resting level of ABP and cerebral circulation (e.g., 16, 17, 27). The results of our companion study suggest the tonic influence of NO on the carotid circulation is greater in mature and middle-aged than juvenile rats (24). In the rat CA in vitro, there is evidence that NO blunts the vasoconstrictor response to NE (12, 21). Thus we also tested the effect of NO synthesis inhibition on carotid vascular responses evoked by systemic infusion of NE in the three age groups. We hypothesized that the sympathetic innervation density of cerebral arterial vessels increases progressively up to middle age and that there is a parallel increase in the vasoconstrictor responses evoked by NE in the carotid circulation. Further, we hypothesized that these vasoconstrictor responses are blunted by NO, particularly in mature and middle-aged rats, such that inhibition of NO synthesis improves autoregulation of CBF in the upper part of the range and may even reveal active vasoconstriction to NE that decreases CBF despite the rise in systemic ABP.

METHODS

Experiments were performed on male Wistar rats supplied by Charles River (Hythe, Kent, UK) and maintained in the Biomedical Services Unit, University of Birmingham. All experiments were approved under the UK Animals (Scientific Procedures) Act 1986 and by the University Biomedical Ethics Review Committee. As described by Omar and Marshall (24) anesthesia was induced by passing halothane at 2.5–3.5% in O2 at a rate of 3–3.5 l/min into a box containing the rat.

In Vitro Studies

These experiments were performed on MCA, BA, FA, and CVA taken from three age groups of rats: juvenile (99.7 ± 2.9 g body wt, ∼4–5 wk of age), mature (291.7 ± 20.7 g, 10–12 wk of age), and old (775.9 ± 27.2 g, 42–44 wk of age). In each case, n = 6–8.

The rat was transferred to an operating table and killed by overdose of halothane delivered via a nose cone, followed by cervical dislocation. Thereafter, for excision of the MCA and BA, the dorsal surface of the skull was shaved and the skull opened with bone forceps. The brain was quickly removed and placed ventral surface uppermost in a dish filled with 0.9% saline. With the aid of an operating microscope, the MCA and BA were carefully freed from surrounding tissues just proximal to the circle of Willis. The FA was then exposed by a medial incision, freed, and removed. Finally, the CVA was exposed by a ventral incision, and a length of ∼3 cm at the base of the tail was freed and removed. All arteries were placed in fresh 0.9% saline. The FA and CVA were then opened longitudinally, while the CA and BA were kept intact. The arteries were incubated in glyoxylic acid (2% wt/vol) made up in 0.1 M phosphate buffer adjusted to pH 7.4 with 10 M NaOH for 45 min at room temperature (11). Each artery was then removed from this solution and stretched as a whole mount on a glass slide while the tissue dried. The procedures used to mount the arteries on the slides so as to visualize the noradrenergic innervation are essentially those described by Furness and Costa (11). Briefly, FA and CVA were placed adventitial surface uppermost. Each artery was stretched carefully and to a maximum extent by gently pulling the very edges of the tissue with fine forceps. Drying of FA and CVA was helped by use of a fan heater. The process of drying took 1–2 min for the MCA and BA and ∼3–4 min for the FA and CVA. When the tissues appeared dry, the slides were placed in an oven at 100°C for 4–6 min. On removal from the oven, the slides were allowed to cool. Then each preparation was covered with a thin film of mineral oil and a cover slip was fixed to the slide with nail varnish. The slides were kept at 4°C, as fluorescence is stable under these conditions for at least 1 mo (4).

The vessel preparations were viewed and photographed on a Zeiss ACM microscope equipped with an epi-illumination system and an Osram HB50 W mercury light source. The filters used were BP 390–440 nm, barrier filter LP 470 nm, and dichotic mirror FT 460 nm (4, 5). Photographs were taken using the microscope camera on Kodak color reversal film (at 400 ASA). All films were developed together by the Photographic and Graphics Service Unit (University of Birmingham). Photographs were then scanned into a computer (Apple Mac G3, 233, AD Instruments, Hastings, UK) and saved for image analysis by using a UMAX scanner (Power Look 2000, AD Instruments).

For analysis of the density of the perivascular nerve network, two variables were assessed: fluorescent area and surface density (see 4, 5). For each measurement, the observer was blinded to the animal group. Fluorescent area is the area of detected fluorescent nerve fibers measured as image points (pixels) expressed as a percentage of the total surface area of the image. This was obtained by using the computer software (Scion modified image 1.62 c program). The whole surface area of the image of the vessel was measured and then a threshold intensity was set to detect the nerve fibers, the threshold being lowered to ensure that only nerve fibers were detected. The density slice facility was used to highlight nerve fibers so that their area could be measured and expressed as a percentage of the whole area.

Surface density (Sv) is the intercept density calculated as the number of nerve intercepts per micrometer tissue on a grid that was superimposed on the image by using the Adobe-Photoshop 5.5 program (Pantone): the grid comprised equally spaced vertical and horizontal lines at 1 mm. The area delineated for counting was the same for each vessel in each group of rats. Because of the small size of the BA and MCA, the area chosen included most of the vessel surface that had been photographed. For the FA and CVA, the count was done within a smaller area that was judged to be representative of the whole. Those fibers that were obviously single were counted as one. When fibers ran together as a bundle but could be resolved into single fibers, they were counted individually. However, when several fibers seemed to run together but could not be individually resolved, the bundle was counted as a single fiber. The count was done three times on different occasions, and the mean of the count was taken for each vessel. Sv was calculated according to the following equation:

\[
S_v = \frac{P_r}{d \cdot P_t}
\]

where \(S_v\) is surface density (nerve intercepts/μm tissue), \(P_r\) is the number of nerve intercepts with horizontal and vertical lines of the grid within the area of measurement, \(d\) is the distance between each vertical and horizontal line of the grid at the tissue level, and \(P_t\) is the total number of intersections of the horizontal and vertical lines within the area of measurement.

In Vivo Studies

These experiments were performed on juvenile, mature, and middle-aged rats (n = 7, 7, and 8, respectively) prepared as described by Omar and Marshall (24). They were anesthetized by continuous
infusion of Saffan (Schering-Plough Animal Health) delivered at 7–12 mg·kg⁻¹·h⁻¹ via the right jugular vein. The trachea was cannulated to allow aspiration of mucus. A cannula placed in the left femoral artery allowed arterial blood pressure to be recorded and samples (150 μl) to be taken at regular intervals for blood gas analysis by using a blood gas analyzer (IL 1640; Instrumentation Laboratories). The right femoral vein was cannulated for administration of the NO synthase (NOS) inhibitor nitro-l-arginine methyl ester (l-NAME), and the right femoral artery was cannulated for infusion of NE (see below). Carotid blood flow (CBF) was recorded by means of a transonic flow probe (0.7 V) connected to a flow meter (T106, Transonic Systems Inc, Ithaca, NY, USA). Arterial pressure and CBF were sampled by a MacLab/8S at 100 Hz and connected to a Power Mac 4400/200 computer by Chart (AD Instruments, Hastings, UK). Mean arterial pressure (ABP) and heart rate (HR) were derived from the pressure signal and carotid vascular conductance was computed as CBF/ABP. At the end of the experiments, all animals were killed with an overdose of anesthetic.

Protocol. An equilibration period of at least 30 min was allowed following surgery so that all baselines stabilized. Animals in whom recorded variables were unstable or showed any respiratory distress were excluded from the study. Arterial blood gases were within normal limits throughout the experiments in juvenile, mature, and middle-aged rats (arterial partial pressure of O₂ and CO₂: 85–91 mmHg and 35–41 mmHg, respectively, pH 7.37–7.41). On stabilization, cardiovascular responses evoked by a 3-min infusion of NE (2.5 μg/kg) were recorded, NE being infused by a pump (model 100, KD Scientific). This infusion rate was chosen as the lowest dose that could consistently induce an increase in ABP to >150 mmHg without fatal effects: in pilot experiments, 10 μg/kg proved fatal to mature rats and 5 μg/kg was fatal to juvenile rats.

After the cardiovascular variables had stabilized again, a bolus dose of l-NAME (10 mg/kg iv) was given. This dose produced a maximum increase in baseline ABP and a maximum decrease in baseline femoral vascular conductance and CVC (24, 29). After a period of ~15 min, the protocol was repeated as described above. In view of the small pressor responses evoked in the juvenile rats by NE infusion at this dose (see below), at the end of the protocol, six of the seven juvenile rats received an infusion of NE at 5 μg/kg.

Chemicals

All chemicals were obtained from Sigma-Aldrich (Dorset, UK). For the in vivo experiments l-NAME and NE were dissolved in physiological saline (0.9%); they were freshly prepared on the day of the experiment.

Statistical Analyses

All results are expressed as means ± SE. For in vitro studies, comparisons were made between age groups for each vessel by using factorial ANOVA and Scheffé’s post hoc test when ANOVA indicated significance. For in vivo studies, in each age group the baseline value of each variable before NE infusion was compared with the mean value over the 3 min of the NE infusion, by Students’ paired t-test, before and after l-NAME. Students’ paired t-test was also used to compare baseline values before and 15 min after l-NAME. Comparisons between different age groups were made by using ANOVA and Scheffé’s post hoc test when ANOVA indicated significance. In all cases P < 0.05 was considered significant.

RESULTS

In Vitro Studies

The glyoxylic acid technique allowed the noradrenergic innervation to be visualized as a network of perivascular fibers on all arteries studied (Fig. 1). The specificity of the technique for noradrenergic fibers has been shown before (11). In the MCA and BA there was a substantially greater density of the nerve fibers in mature relative to juvenile rats as judged both by fluorescent area and S₀ (Fig. 2). Further, the innervation density was lower in the MCAs and BAs of the middle-aged rats relative to the mature rats such that in the middle-aged rats, the density was almost the same as in juvenile rats. By contrast in the FA, there was no difference between the innervation density in the juvenile and mature rats, whereas the density was substantially greater in the middle-aged rats relative to the other groups when judged either as fluorescent area or as S₀ (Fig. 2). On the other hand, in the CVA, the innervation density was greater in the mature than juvenile rats, and similar in the middle-aged and mature rats when considered as fluorescent area, but greater in the middle-aged than juvenile rats when considered as S₀ (Fig. 2). The disparity between the outcome of these different methods of assessment may be attributed to differences in the arrangement of the nerve fibers: individual fibers were combined into bundles in the CVAs of middle-aged rats leaving large gaps between the bundles (Fig. 1).

In Vivo Studies

Before infusion of NE there were no significant differences between the three age groups for the baseline levels of ABP, CBF, or CVC, but HR was significantly higher in the juvenile rats than in the mature and middle-aged rats (P < 0.05; see Fig. 3 and also Ref. 24). Infusion of NE at 2.5 μg/kg evoked an increase in ABP to ~180 mmHg in the mature and middle-aged rats but to only ~150 mmHg in the juvenile rats. Concomitantly, CVC decreased in all three age groups, but the reduction in CVC was greater in mature and middle-aged rats than in juvenile rats (P < 0.05). Consequently, CBF decreased in mature and middle-aged rats but did not change in juvenile rats. HR increased significantly in the middle-aged rats but not in the other groups (Fig. 3).

As expected, l-NAME induced an increase in baseline ABP, and this was accompanied in all three groups by a decrease in baseline levels of HR, CBF, and CVC (Table 1). Considered as percentage change from baseline, the increase in ABP was similar in the three groups. However, the percentage decrease in HR was greater in middle-aged than juvenile rats, and the percentage decreases in CBF and CV were greater in middle-aged and mature rats than in juvenile rats (see Table 1 and Ref. 24). From these new baselines, NE infusion at 2.5 μg/kg in juvenile rats evoked an increase in ABP to ~150 mmHg as before l-NAME; CVC fell and there was a significant decrease in CBF (Fig. 4). In mature rats, NE increased ABP to a similar level as before l-NAME, there was still a decrease in CVC, but any fall in CBF no longer reached significance (Fig. 4). By contrast, in middle-aged rats, NE evoked a larger increase in ABP after l-NAME than before, to ~210 mmHg, and there was a larger increase in HR. Concomitantly, NE no longer evoked a change in CVC, while CBF increased, rather than decreased as it had before l-NAME (Fig. 4; cf Fig. 3).

As indicated in METHODS, at the end of the experiment, i.e., after l-NAME, six of the juvenile rats received an NE infusion at 5 μg/kg. This dose did not evoke a larger response than NE at 2.5 μg/kg. Thus ABP increased from 124.1 ± 4.4 to 149.6 ± 2.3 mmHg (P < 0.001), and there was an accompanying
decrease in CVC ($P < 0.01$), with no significant change in CBF (cf Figs. 3 and 4).

DISCUSSION

The quantitative assessments we made by applying the glyoxylic acid technique to four different arteries of juvenile, mature, and middle-aged rats suggest that age-related changes occur in the density of sympathetic noradrenergic innervation in the MCA and BA of the cerebral circulation, but that very different changes occur concurrently in the FA and CVA. In our in vivo experiments systemic infusion of NE evoked a pressor response and vasoconstriction in the circulation supplied by the carotid artery in all three age groups, but the magnitude of these responses was age dependent, as were the effects of NOS inhibition. The implications of these findings must be considered in relation to the limitations of the techniques we used.

Concerning the assessment of innervation density, it is possible that the arteries showed some shrinkage after excision and that this affected measurement of fluorescent area and particularly surface density ($S_v$). However, to be able to visualize the nerve network effectively, we stretched the arterial tissue as evenly as we could against the slide during drying, so tending to counteract any shrinkage. Further, because the MCA and BA are relatively thin walled and because we did not open them, it seems likely that any shrinkage or stretch was minimal in these arteries. Indeed, in previous studies on the MCA and BA, for these very reasons, no correction was attempted for shrinkage when these arteries were similarly prepared (5, 8, 22). The FA and CVA are thicker walled and have more elastin and collagen. Moreover, the content of elastin decreases in arteries with age, whereas the relative content of collagen increases, leading to an increase in wall stiffness, at least in 78-wk-old rats relative to 12-wk-old rats (1, 10). Thus the FA and CVA may have shrunk more than cerebral arteries, and shrinkage may have been greater in mature than middle-aged rats. However, this would have resulted in an overestimation of the innervation density in mature relative to middle-aged rats. In other words, we would have underestimated the difference between them. We also acknowledge that a potential limitation in the assessment of fluorescent area is the setting of the threshold to allow the area occupied by nerve fibers to be assessed. This was done with considerable care, the threshold being adjusted for each image because of differences in fluorescent intensity, and the observer was blinded to the study design. Thus we believe we minimized any error.

Despite these limitations, the pattern of change in innervation density with age in each artery was very similar with the two methods of assessment, except in the CVA. This gives credibility to our observations, particularly as there is a reasonable explanation for the disparity in the CVA. As indicated

Fig. 1. Photomicrographs showing age-related differences in the density of sympathetic noradrenergic innervation in basilar artery (BA), femoral artery (FA), and caudal ventral artery (CVA). Note: in BA, innervation density is greater in mature than in juvenile or middle-aged rats. In FA, innervation density increases progressively from juvenile to mature to middle-age. In CVA, innervation density is greater in mature than juvenile rats, while in middle age, the fibers of the network group together to form larger bundles (see text).
in RESULTS, the fact that nerve fibers were grouped together into larger bundles in the CVA of middle-aged than in mature rats, so leaving larger spaces between fluorescent regions, may have led to underestimation of the fluorescent area in the CVA of middle-aged rats as a percentage of total area of vessel, even though the $S_V$ was increased relative to that of juvenile rats. Thus we believe we have shown for the first time in a single species that the density of sympathetic noradrenergic innervation is greater in two important cerebral arteries, the MCA and BA, of mature rats than juvenile rats, but declines again by middle age (42–44 wk). This decline is probably part of a progressive decline, for the innervation density was reported to be substantially less in MCA and BA of 68-wk-old than 32-wk-old rats (22). By contrast, in FA, which supplies hindlimb muscle, innervation density is similar in juvenile and mature rats, but greater in middle-aged rats, whereas in CVA, which supplies the thermoregulatory tail circulation, innervation density, in terms of the number of nerve fibers per unit area, probably increases progressively over the same age range.

The factors that might underlie these age-dependent and organ-dependent differences in innervation density are beyond the scope of this study. However, it may be noted, first, that Thrasivoulou and Cowen (30) attributed the lower noradrenergic innervation density of the MCA in 24-mo-old than 6-wk-old rats to a lower availability of nerve growth factor (NGF) and, second, that age-related reduction in NGF availability has been attributed to reduced synthesis by the target tissue, thickening of the basal lamina around the vascular smooth muscle hindering diffusion, and reduced ability of sympathetic neurons to accumulate NGF (19). Our results suggest that if these factors contributed to the reduction in innervation density of MCA and BA from 10–12 to 42–44 wk of age, then other factors predominate in FA and CVA. It may be they are affected by the increasing size of the rat with age and therefore in the mass of the hindlimbs and tail, respectively, for the size of the artery is a contributory factor in determining innervation density, producing proportional changes in axonal branching (34).

Considering our in vivo recordings of CBF, we discussed in our companion paper (24) the limitation of recording blood flow in the carotid artery without ligation of the external carotid artery as an index of cerebral blood flow. We took this decision to avoid damaging the carotid sinus, carotid bodies, and their innervation in the juvenile rats. However, as a consequence, we undoubtedly recorded blood flow to the facial muscles and extracranial tissues supplied by the external carotid artery, to the tongue, eye, and extracranial tissues that are supplied from the internal carotid artery (13, 29) as well as to the brain. Thus we acknowledge that our recordings of CBF gave only an indication of blood flow supplying the brain and other tissues but can argue that the regions supplied were comparable in the three age groups of rats. Moreover, it is also the case that changes in diameter of the carotid artery and its branches distal to the recording site, including the MCA, which influence gross changes in cerebral blood flow and modulate behavior of downstream intracranial resistance arteries (15, 26), must have contributed to the changes in CVC and CBF we recorded. It is clear that further studies will be required to differentiate age-dependent effects on extra- and intracranial vessels, as well as on extracerebral and intracerebral arterial vessels. Nevertheless, the present study allows us to consider

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**Fig. 2. Noradrenergic innervation density in middle cerebral artery (MCA), BA, FA, and CVA of juvenile, mature, and middle-aged rats.** *Top*: area of fluorescent nerve fiber. *Bottom*: surface density (nerve intercepts/μm tissue). For explanation, see text. Age group is indicated below each column: diagonal hatching, cross-hatching, and horizontal hatching indicate juvenile, mature, and middle aged, respectively. Columns show means ± SE. Difference between age groups as indicated by bar over top of columns: §§§ $P < 0.001$, §§ $P < 0.01$, § $P < 0.05$. 

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age-dependent changes in pressor sensitivity to NE and in responses evoked in gross carotid circulation.

The pressor sensitivity to the major sympathetic cotransmitter, NE, was weak in the juvenile rats relative to that in the mature and middle-aged rats. We could not increase ABP above 150 mmHg in the juvenile rats even when twice the dose required to raise ABP to >180 mmHg in the mature and middle-aged rats was given after L-NAME, when the tonic dilator influence of NO was inhibited (see below). This raises the possibility that in the juvenile rats, the low innervation density of the systemic circulation as indicated by the FA and CVA (see above) is associated with a low systemic density of $\alpha$-adrenoceptors relative to mature and middle-aged rats. It may also be that the greater compliance of the circulation in juvenile rats limited the magnitude of the NE-evoked pressor response (10). Nevertheless, NE infusion did raise ABP to 150 mmHg in the juvenile rats, which is the upper end of the cerebral autoregulatory range (25). Thus our finding that CBF did not change during NE infusion indicates that juvenile rats show good autoregulation of the carotid circulation as could be explained by myogenic vasoconstriction. Clearly, since CBF did not fall during NE infusion, there is no reason to suggest the juvenile rats showed additional, active vasoconstriction in carotid circulation in response to NE. It is established that cerebral vessels, including the larger extracerebral arteries, are much less sensitive to $\alpha$-adrenoreceptor stimulation than other systemic vessels (e.g., 2, 9). The present findings suggest that in juvenile rats, a weak active vasoconstrictor influence of NE on carotid circulation relative to that seen in older rats (see below) is associated with a relatively low density of noradrenergic innervation, at least of the MCA and BA of the cerebral circulation.

By contrast, the mature and middle-aged rats clearly showed decreases in CVC in response to NE that were greater than

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Table 1. Baseline values of cardiovascular variables before and after l-NAME (10 mg/kg)

<table>
<thead>
<tr>
<th></th>
<th>ABP, mmHg</th>
<th>HR, beats/min</th>
<th>CBF, ml/min</th>
<th>CVC, ml·min⁻¹·mmHg⁻¹</th>
</tr>
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<tbody>
<tr>
<td>Juvenile</td>
<td>115.0 ± 3.4</td>
<td>527.6 ± 8.17</td>
<td>1.57 ± 0.14</td>
<td>0.014 ± 0.001</td>
</tr>
<tr>
<td>Mature</td>
<td>135.9 ± 3.1</td>
<td>432.1 ± 14.7</td>
<td>2.12 ± 0.15</td>
<td>0.016 ± 0.001</td>
</tr>
<tr>
<td>Middle aged</td>
<td>136.7 ± 4.4</td>
<td>363.4 ± 9.54</td>
<td>2.19 ± 0.14</td>
<td>0.016 ± 0.001</td>
</tr>
</tbody>
</table>

**After l-NAME**

<table>
<thead>
<tr>
<th></th>
<th>ABP, mmHg</th>
<th>HR, beats/min</th>
<th>CBF, ml/min</th>
<th>CVC, ml·min⁻¹·mmHg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Juvenile</td>
<td>126.8 ± 3.4***</td>
<td>483.5 ± 10.7**</td>
<td>1.23 ± 0.07*</td>
<td>0.010 ± 0.001*</td>
</tr>
<tr>
<td>Mature</td>
<td>155.1 ± 4.6**</td>
<td>375.8 ± 10.0***</td>
<td>0.95 ± 0.09**</td>
<td>0.006 ± 0.001**</td>
</tr>
<tr>
<td>Middle aged</td>
<td>156.4 ± 4.6**</td>
<td>286.1 ± 9.3***</td>
<td>1.06 ± 0.12***</td>
<td>0.007 ± 0.001***</td>
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Values are means ± SE. ABP, arterial blood pressure; HR, heart rate; CBF, carotid blood flow; CVC, carotid vascular conductance. Significant difference between baseline values before and after nitro-l-arginine methyl ester (l-NAME): ***P < 0.001, **P < 0.01, *P < 0.05.

Fig. 3. Cardiovascular responses evoked in juvenile, mature, and middle-aged rats by systemic infusion of norepinephrine. Open columns indicate control values of mean arterial pressure (ABP) in mmHg, heart rate (HR) in beats/min (bpm), carotid blood flow (CBF) in ml/min, and carotid vascular conductance (CVC) in ml·min⁻¹·mmHg⁻¹, while hatched columns indicate mean value recorded over 3-min infusion: diagonal hatching, cross hatching, and horizontal hatching indicate juvenile, mature, and middle-aged rats, as in Fig 2, and as shown below columns. In each case, values are shown as mean ± SE. Difference between control value and value recorded during norepinephrine infusion: ***P < 0.001, **P < 0.01, *P < 0.05. Difference between groups for changes evoked by norepinephrine: §P < 0.05.
required for pressure autoregulation of CBF, such that CBF fell in both groups despite the rise in ABP. Thus, even though the innervation density of the MCA and BA was lower in the middle-aged than mature rats, and even though sympathetically mediated constriction of large cerebral arteries is considered important in preventing breakthrough of autoregulation at high systemic pressures (14, 15, 33), there was no evidence that pressure autoregulation was impaired in middle-aged rats, at least when ABP increased to 180 mmHg (see below for further discussion). Rather, the finding that CBF fell during NE infusion in mature and middle-aged rats suggests that over this age range, active vasoconstriction of the carotid circulation evoked by α-adrenoreceptor stimulation was superimposed on myogenic vasoconstriction.

Roles of NO

Administration of the NOS inhibitor L-NAME induced an increase in baseline ABP and reduction in CVC and CBF in all three groups of rats. However, as we showed in our companion paper (24), the percentage decreases in CVC and CBF were greater in mature and middle-aged rats than in juvenile rats. This indicates a greater tonic dilator influence of NO on the carotid circulation of mature and middle-aged rats than juvenile rats; it is also possible the cerebral circulation was tonically influenced by NO synthesized by neuronal, as well as endothelial, NOS (see 32). We now show that in juvenile rats, NOS inhibition had no obvious effect on the pressor response or decrease in CVC evoked by NE. Thus neither the systemic pressor response nor the vasoconstrictor response of the carotid circulation was significantly blunted by a dilator influence of tonic or NE-stimulated synthesis of NO. This is consistent with the limited adenosine-evoked release of NO from the carotid artery and lack of involvement of NO in adenosine-evoked depressor response or carotid vasodilatation in juvenile rats (24). Indeed, taken together these findings are consistent with a generally weak contribution of the tonic and agonist-stimulated dilator influence of NO in the systemic and carotid circulations of juvenile rats.

In mature rats also, NOS inhibition had no effect on the level to which ABP was increased by NE infusion, nor did it facilitate the NE-evoked decrease in CVC. The obvious interpretation of these findings is that neither tonically stimulated nor NE stimulated synthesis of NO blunts the systemic vasoconstriction that underlies the pressor response, or the carotid vasoconstrictor response to NE in mature rats. However, this interpretation must be treated with caution. First, the decrease in baseline HR caused by L-NAME was greater in mature than juvenile rats (see Table 1 and Ref. 24). Thus it may be that in mature rats, when the inhibitory influence of NO on cardiac vagal neurons was reduced by NOS inhibition (35), the ability of the baroreceptor reflex to buffer the NE-evoked pressor response was more effective. Second, it could be that because L-NAME decreased baseline CVC by removing a tonic vasodilator influence of NO and inducing myogenic vasoconstriction to the increase in systemic ABP, this limited further carotid vasoconstriction to NO. Thus our results do not actually
allow us to deduce whether tonic or NE-stimulated NO synthesis blunted the carotid vasoconstrictor response to NE. Nevertheless, the finding that CBF did not increase with the NE-evoked increase in ABP after L-NAME indicates that pressure autoregulation of the carotid circulation in the face of a substantial pressor response to ~150 mmHg is still effective in mature rats when NOS is inhibited. This is consistent with the majority of evidence which indicates that NO makes little contribution to cerebral autoregulation at the upper end of the range (32).

By contrast, in the middle-aged rats, the increase in CBF evoked by NE was substantially greater after L-NAME than before; there was a larger NE-evoked increase in HR from a lower baseline, but CVC no longer decreased and there was breakthrough of pressure autoregulation: NE evoked an increase in CBF. We can only speculate on the mechanisms responsible for the enhanced pressor response. The larger tachycardia may have contributed; the lower baseline HR probably reflected removal of strong inhibitory effect of NO on cardiac vagal activity (see 24, 35), but substantial tachycardia caused by direct stimulation of cardiac β-adrenergoreceptors may have been revealed because the cardiac component of the baroreceptor reflex is attenuated by ageing (10). In addition, contributions could have been made by age-dependent attenuation of the vascular component of the baroreceptor reflex, enhanced arterial stiffness (see 10), or loss of the systemic dilator influences of NO. Irrespective, the most obvious explanation for the lack of change in CVC in response to NE infusion after L-NAME is that baseline CVC was so low when the tonic vasodilator influence of NO had been inhibited that there was no further scope for myogenic or active vasoconstriction in the carotid circulation.

Thus we can propose that in middle-aged rats, in contrast to mature and juvenile rats and our working hypothesis (see Introduction), the cardiovascular effects of tonically synthesized NO play an essential role in keeping the ABP reached during NE infusion below the upper limit of the cerebral autoregulatory range. It may also be that when ABP is raised to the high levels evoked by NE after L-NAME (~210 mmHg), autoregulation is further compromised in middle-aged rats by a relatively weak sympathetic vasoconstrictor influence on the large extracranial cerebral arteries associated with their decreased density of sympathetic innervation (see above and 13, 15). Indeed, our results suggest that by middle age, loss of the dilator influence of NO with conditions such as hypertension and diabetes would increase the risk of cardiovascular events in the carotid circulation.

In summary, the present results indicate that the sympathetic innervation density and constrictor influence of NE on the carotid circulation that supplies the brain are weak in juvenile rats. Nevertheless, juveniles show effective autoregulation of CBF at the upper end of the autoregulatory range that is not moderated by NO. By maturity, the sympathetic innervation density of cerebral arterial vessels and the constrictor influence of NE on the carotid circulation have weakened, such that the tonic dilator influence of NO on ABP and the carotid circulation helps prevent breakthrough of CBF at the upper end of the autoregulatory range. Our companion study suggested that by middle age, the dilator influence of adenosine on the carotid circulation has weakened, and the tonic dilator influence of NO on ABP limits autoregulation of CBF at the lower end of the range (see 25). Given the rat provides a good model of the effects of maturation and aging that is not complicated by atherosclerosis (10), the results of these two studies indicate that by middle age, ageing itself has already altered several key mechanisms that regulate the carotid circulation that includes the brain.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


