Anat Embryol (2000) 201:383–397

ORIGINAL ARTICLE

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Arteether-induced brain injury in *Macaca mulatta*. I. The precerebellar nuclei: the lateral reticular nuclei, paramedian reticular nuclei, and perihypoglossal nuclei

Accepted: 20 October 1999

Abstract Malaria poses a threat across several continents: Eurasia (Asia and parts of Eastern Europe), Africa, Central and South America. Bradley (1991) estimates human exposure at 2,073,000,000 with infection rates at 270,000,000, illnesses at 110,000,000, and deaths at 1,000,000. Significant mortality rates are attributed to infection by the parasite *Plasmodium falciparum*, with an estimated 90% among African children. A worldwide effort is ongoing to chemically and pharmacologically characterize a class of artemisinin compounds that might be promising antimalarial drugs. The U.S. Army is studying the efficacy and toxicity of several artemisinin semi-synthetic compounds: arteether, artemether, artelinic acid, and artesunate. The World Health Organization and the U.S. Army selected arteether for drug development and possible use in the emergency therapy of acute, severe malaria. Male Rhesus monkeys (*Macaca mulatta*) were administered different daily doses of arteether, or the vehicle alone (sesame oil), for a period of either 14 days, or 7 days. Neuropathological lesions were found in 14-day arteether treated monkeys in the precerebellar nuclei of the medulla oblongata, namely: (1) the lateral reticular nuclei (*subnuclei magnocellularis*, *parvicellularis*, and *subtrigeminalis*), (2) the paramedian reticular nuclei (*subnuclei accessorius*, *dorsalis*, and *ventralis*), and the perihypoglossal nuclei (*n. intercalatus of Staderini*, *n. of Roller*, and *n. prepositus hypoglossi*). The data demonstrate that the simian medullary precerebellar nuclei have a high degree of vulnerability when arteether is given for 14 days at dose levels between 8 mg/kg per day and 24 mg/kg per day. The neurological consequences of this treatment regimen could profoundly impair posture, gait, and autonomic regulation, while eye movement disorders might also be anticipated.

Key words Lateral reticular nuclei · Paramedian reticular nuclei · Perihypoglossal nuclei · Malaria · Arteether · Artemisinin · Neurotoxicity · Rhesus monkey · Antimalarial drugs · Cerebral malaria · *Macaca mulatta* · *Plasmodium falciparum*

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Introduction

Cerebral malaria (unrousable coma) is characterized by fever, delirium, convulsions, coma, and vasculature congestion caused by a marked parasitemia with *Plasmodium falciparum*, while *P. vivax* infections are less common. Mortality overall varies between 20 and 40%, but may reach 80% where convulsions and coma coexist. The clinical experience with childhood cerebral malaria in the tropics can be devastating. Brewster et al. (1990) reported 604 children diagnosed with falciparum malaria. Of these, 308 (51%) presented with cerebral malaria and 203 (34%) were severely anemic. Deaths occurred in 14% of the children with cerebral malaria and 7.8% with severe anemia. Residual neurological deficits in 35 patients included hemiplegia (66%), ataxia (17%), aphasia (26%), blindness (31%), spasticity (9%), tremors and psychosis (6%). Follow-ups were made on 23 children and full recovery was achieved in 52%; severe cerebral palsy was found in 2 cases (9%), blindness in 2 (9%), alphasia in 2 (9%), epilepsy in 3 (13%), and hemiplegia in 6 (26%) cases. Neurological sequelae appeared closely correlated in children with prolonged and recurrent convulsions and this was seen in 17 children and in

7 cases with treatment-refractory status epilepticus. Brewster et al. (1990) also reviewed the results obtained in 15 clinical studies performed in Malawi, Senegal, Kenya, Tanzania, Uganda, Zaire, Madagascar, India, and Papua New Guinea. In children with cerebral malaria, the incidence of neurological sequelae varied between 0 and 21%, and deaths occurred between 5 and 38%. Taken together with the Brewster et al. (1990) study, the overall death rate was 20% while neurological deficits occurred in 7% of the cases. Mortality, due to infection by *P. falciparum*, has been estimated among African children at half a million (van Hensbroek et al. 1996) and generally at 1–2 million deaths annually across Africa (Campbell 1997). Once concentrated in rural areas, the infection has spread to African urban populations (Campbell 1997). Over the past several decades the *Plasmodium falciparum* malaria parasite has developed resistance to a host of drugs: 4-aminoquinolines, chloroquine and amodiaquine, pyrimethamine-sulfadoxine (Fansidar), quinine, and biguanide, and proguanil (Campbell 1991; White 1992a, b, 1994, 1996).

A worldwide effort is in progress to identify and evaluate a number of artemisinin compounds that may aid in the control and treatment of human falciparum malaria infections. A class of compounds derived from artemisinin (qinghaosu) has been found to be effective against the erythrocytic stages of chloroquine-resistant *Plasmodium falciparum* malaria. The compounds include arteether, artemether, artelinic acid, and artesunate. In 1985 the World Health Organization and the U.S. Army selected arteether as a potential agent for the emergency treatment of acute, severe malaria. The laboratories of the Walter Reed Army Institute of Research initiated animal experiments to investigate the efficacy and toxicity of several artemisinin compounds and initially included studies of arteether, artemether, and dihydroquinghaosu (DQHS). Together with the World Health Organization, the U.S. Army specifically investigated the usefulness of arteether for the treatment of severe multi-resistant falciparum malaria. This report further documents one portion of this effort and describes the neurotoxic effects of arteether on the medullary precerebellar nuclei of the simian brainstem.

Materials and methods

Eleven male *Macaca mulatta* monkeys weighing between 3.4 and 5.3 kg were prepared for study. Six monkeys were intramuscularly injected with arteether for 14 days and arranged in three dose pairs: 24 mg/kg per day (monkey case numbers DA-472; DA-514); 16 mg/kg per day (DA-484; DA-490), and 8 mg/kg perday (DA-475; DA-508). Two monkeys served as vehicle controls and were given intramuscular (IM) doses of sesame oil (vehicle) daily for 14 days (DA-504, DA-505). Three additional monkeys were treated for 7 days. Two were injected intramuscularly with AE at either 24 mg/kd per day (DA-387, 168 mg total dose) or 8 mg/kg per day (DA-356, 56 mg total dose), and the third monkey was given IM sesame oil (DA-386). Injection volume was less than 2 ml for drug treated and vehicle (control) monkeys.

At the end of the treatment period, all monkeys were administered ketamine (12 mg/kg, im) prior to the infusion of intravenous (IV) pentobarbital sodium (23 mg/kg) titrated to achieve deep surgical anesthesia. The descending aorta was exposed at the level of the renal artery following laparotomy. An aortic catheter was threaded cranially to the heart. Transaortic perfusion immediately followed. The vena cava was opened to effect euthanasia by exsanguination. Two sequential perfusions were conducted; a buffered clearing solution (Zahm and Munger 1985), and then with the fixative solution. Bouin's fluid was used to fix the brains of all 14-day arteether-treated monkeys (and the controls) for the study of neuronal injury and gliosis. Formalin (10%) was used to fix the brains of all 7-day arteether-treated monkeys (and control) for the study of neuronal injury, gliosis, and the presence of axonopathy. After completing the perfusions, the central nervous system remained *in situ* for 2 h before dissection was begun. This technique prevents the development of neuronal hyperchromatosis (the "dark neurons" of Cammermeyer (1960, 1961, 1962, 1978). The brains and spinal cords were dissected *in toto* except for transection of the spinal cord at the cranial end of the C_1 segment. The brains were immersed overnight in fresh Bouin's fluid (14-day cases) or formalin (7-day cases).

Brains perfused with Bouin's fluid were blocked transversely or mid-sagittally, then immersed in daily changes of ethanol (70%) to remove excess picric acid. Dehydration in ascending grades of ethanol followed, and clearing was accomplished using cedarwood oil followed by brief treatment with xylene prior to paraffin embedding. A rotary microtome was used to cut and collect 10 µm serial sections. Three sets of serial sections (10 µm) were prepared and stained according to the methods of Nissl [cresylechtviolett, CV; Drury and Wallington 1980), Klüver-Barrera (Klüver and Barrera 1953; luxol fast blue, and cresylechtviolett (manufactured by Chroma-Gesellschaft) counterstained; KB], and hematoxylin and eosin (H&E). All stained brainstem serial section sets (CV, KB & H&E), through the medulla oblongata and pons, were examined microscopically for evidence of cellular pathology.

The numerical data were obtained from: (1) three 14-day arteether-treated (DA-472, 24 mg/kg per day; DA-484, 16 mg/kg per day, and DA-475, 8 mg/kg per day); (2) two 14-day vehicle treated (DA-504, DA-505) monkeys; (3) two 7-day arteether-treated (DA-387; DA-356); and (4) one 7-day vehicle treated monkey (DA-386). Cresylechtviolett, KB, or H&E stained brain sections, separated by no less than 110 µm to 220 µm, were scanned systematically. Neurons with visible nucleoli were counted until the target of 100 or more cells were identified (sum of affected and unaffected neurons). The ratio of affected (numerator) to total neurons (affected and unaffected, the denominator) ×100 provided the percent of affected neurons. In the absence of neuronal injury the structure under study was scored as unaffected, that is, given a value of zero percent affected.

The percent of neurons that were injured was calculated for 9 subnuclear groups selected from three precerebellar functional systems: lateral reticular nuclei; paramedian reticular nuclei, and perihypoglossal nuclei, and for selected cranial nerve nuclei. The nuclei studied included the following cell groups: (1) the lateral reticular nuclei (*n. reticularis lateralis subnuclei magnocellularis, parvicellularis et subtrigeminalis*); (2) the paramedian reticular nuclei (*n. reticularis paramedianus subnuclei dorsalis, ventralis, et accessorius*); (3) perihypoglossal nuclei (*n. intercalatus of Staderini, n. of Roller*, and *n. prepositus hypoglossi*); and (4) selected motor cranial nerve nuclei, the hypoglossal nucelus (*n. nervi hypoglossi*, nNHyp); the abducens nucleus (*n. nervi abducentis*, nNAb); the facial nucleus (*n. nervi facialis*, nNFac); and the motor trigeminal nucleus (*n. nervi trigemini motorius*, nNTrigm).

Statistical Analysis

The SAS general linear models analysis of variance (ANOVA) procedure SAS Institute 1989 with a repeated measures option was used to compare mean percent injury for arteether-treated monkeys and mean percent injury for vehicle treated monkeys within the perihypoglossal and lateral and paramedian reticular

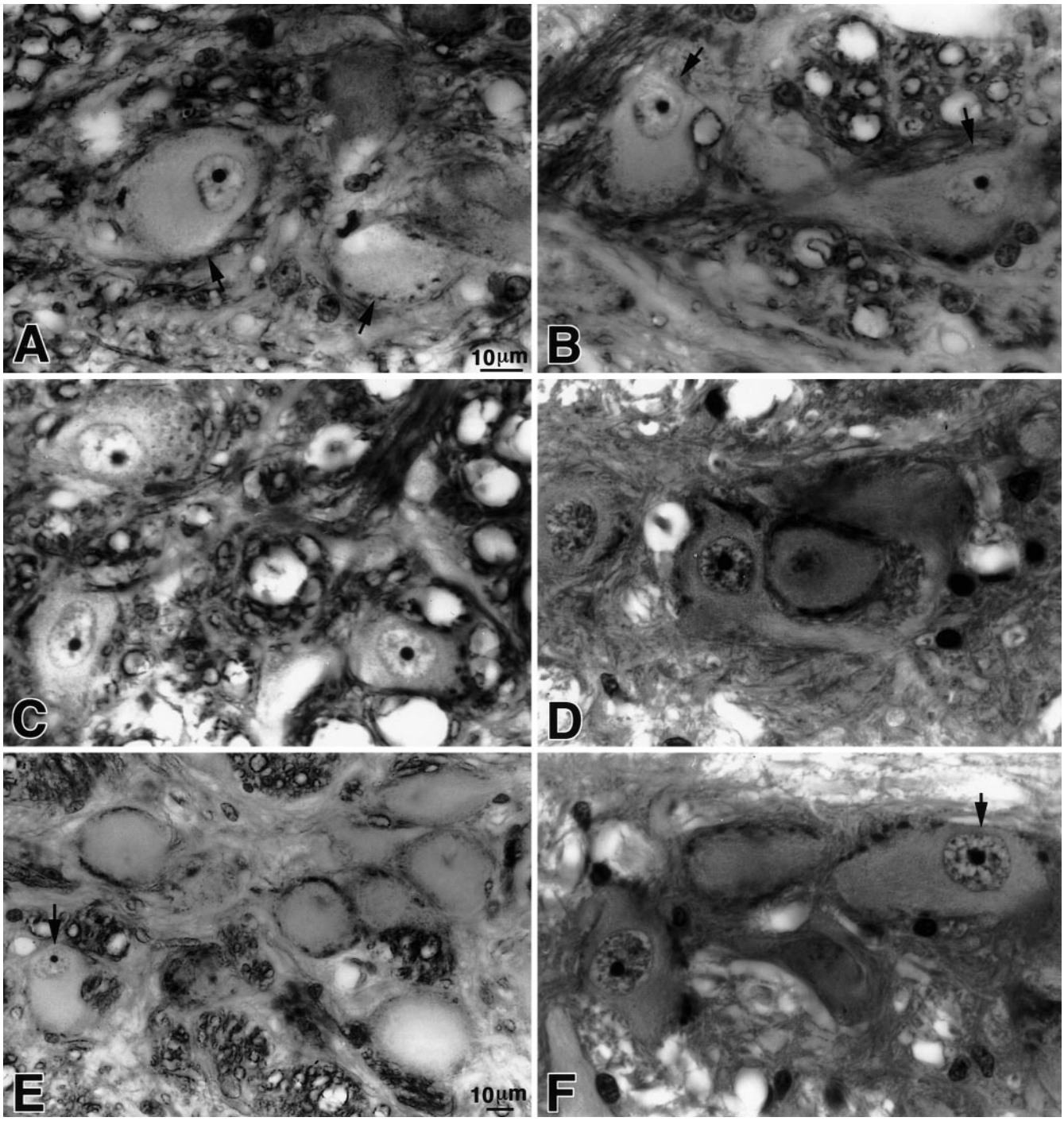

Fig. 1A–F These photomicrograph illustrate the presence of central chromatolysis among neurons of the lateral reticular nuclei (LRN) following 14 days of IM administration of the antimalarial compound arteether. Affected neurons (some indicated with *arrows*) from the subnucleus subtrigeminalis (LRNsub) in **A**; from the subnucleus magnocellularis (LRNm) in **B**, **C**, while **D**, **F** are from the subnucleus parvicellularis (LRNp). Stains: Klüver-Barrera method, **A**–**C**, **E**; H&E; H&E, **D**, **F**. *Bars* 10 µm. The neurons shown in panel E were photographed with a lower-power objective

nuclear regions. An F score was considered to be significant if its probability of occurrence under the null hypothesis was 0.05 or less. If an F-score was significant for a region, Dunnett's test was used to determine whether the mean injury scores for the arteether and vehicle treated groups were significantly different within the individual nuclei of that region.

Use of animals

The research was conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the NIH publication 86-23 Guide for the care and use of laboratory animals.

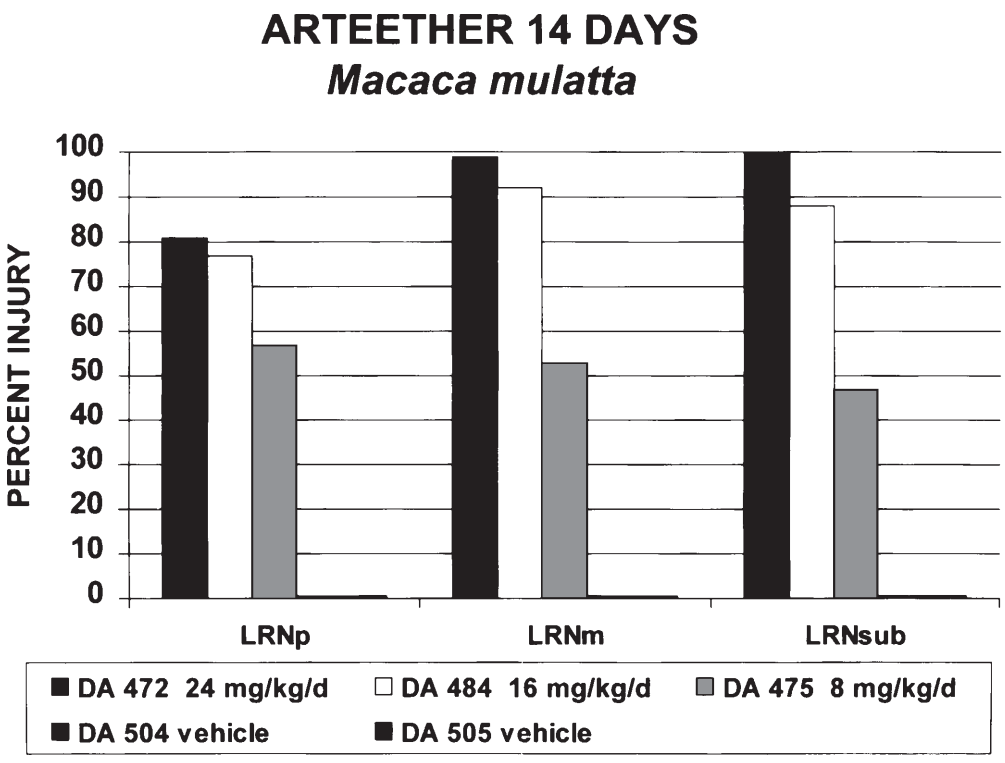

Fig. 2 Bar graph illustrating the percent of injured neurons present in the *nucleus reticularis lateralis, subnuclei magnocellularis* (LRNm), *parvicellularis* (LRNp), and *subtrigeminalis* (LRNsub) following 14-d treatment with arteether. No evidence of injury was observed in the vehicle control cases

Results

We earlier reported the results of arteether-induced injury affecting the neurons in 14 nuclear groups distributed topographically in the medial medullary and pontine reticular formation, the vestibular nuclei, and the auditory system of male Rhesus monkeys administered arteether for 14-days (Petras, et al. 1997). Using the same monkeys, the present report extends the previous descriptive and numerical data by studying nine additional subnuclear populations constituting the precerebellar nuclei of the medulla oblongata. The subnuclear divisions are: (1) the *n. reticularis lateralis subnuclei magnocellularis, parvicellularis* and *subtrigeminalis*; (2) the *n. reticularis paramedianus subnuclei dorsalis, ventralis, and accessorius*, and (3) the *n of Roller, n. intercalatus of Staderini*, and *n. prepositus hypoglossi* constitute the perihypoglossal nuclei.

Descriptive neuropathology

Cellular pathology

All subnuclear groups of the lateral reticular (Fig. 1), the paramedian reticular (Fig. 3), and the perihypoglossal nuclei (Fig. 5) contained neurons whose cell somas displayed pallor of the central cytoplasm (14-d AE-treated cases). The severity of neuronal **central chromatolysis** (or axonal reaction) varied according to dose level, and the nuclear groups under investigation. At the high (24 mg/kg/day) and middle (16 mg/kg/day) dose levels injury was most widspread in all precerebellar nuclei. Affected neurons were characterized by a swollen **cell soma** and a reduction in the number of Nissl bodies (Figs. 1, 3, 5). More severe neuronal injury was characterized by a further reduction of the number of Nissl bodies in the central and peripheral cytoplasm with perhaps a few remaining large clump-like Nissl bodies located inside the cell membrane (for examples see Fig. 1A–F; Fig. 3A, F). An achromatic cytosol was observed in many severly affected neurons. The **cell nucleus** was located in the central cytoplasm (e.g., Fig. 3F), and was mildly (Fig. 3A) or markedly eccentric (Fig. 1B). Chromatolytic neurons contained nuclei which were either spherical, monoconcave, or biconcave in profile. In cases of severe central chromatolysis, the cytosol was achromatic and the nucleus either monoconcave or biconcave and located adjacent to the cell membrane (Fig. 1E; Fig. 3B). A bright pink-colored cytosol (H&E stain; Fig. 3E) was observed in some chromatolytic neurons and in necrotic neurons. For example, at the 24 mg/kg per day for 14-days, sections stained with the H&E method revealed pink neurons present bilaterally in small numbers in the paramedian reticular nuclei (ventral and dorsal subnuclei), and were bilaterally abundant in the *n. prepositus hypoglossi.* The **nucleolus** appeared darkly stained and elongated (Fig. 3C, E) with karyopyknosis observed in some neurons. Satellitosis and neuronophagia was absent in the precerebellar nuclei. This stands in marked contrast to the widespread satellitosis and neuronophagia observed in the brainstem reticular formation of the high dose 14-day cases as reported previously (Petras et al. 1997).

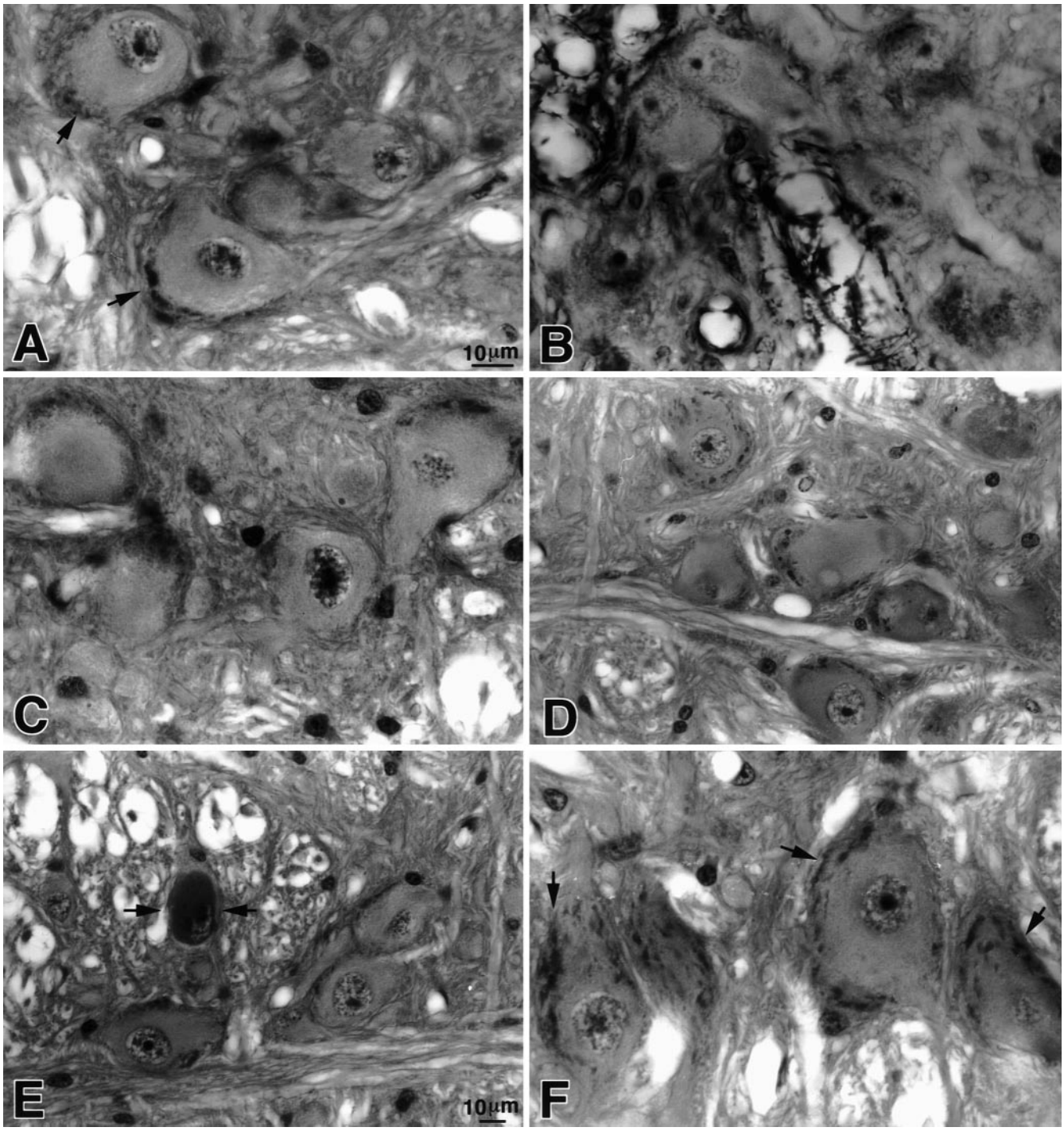

Fig. 3A–F Photomicrographs illustrating the presence of central chromatolysis among neurons in the paramedian reticular nuclei (PRN) in a 14-day arteether-treated Rhesus monkey. Affected neurons (*arrows* indicating loss of central chromatin or Nissl bodies) from the accessory subnucleus (**A**, **B**, **C**) and from dorsal subnucleus (**D**–**F**). A neuron with strongly eosinophilic cytoplasm ("pink cell") is seen in panel E (*double arrow*). Klüver-Barrera method in panels **A**, **B**, **D**, **F**, and H&E stained sections in panels **C**, **E**. *Bars* 10 μm. **A**–**C**, and **F** were photographed at the same magnification, while panels **D** and **E** were photographed at lower magnification

Systems neuropathology: distribution of the brainstem injury

The lateral reticular nuclei

In all 14-day arteether (AE) treated Rhesus monkeys, neuronal pathology was found in: (1) the *magnocellular*, *parvicellular* and *subtrigeminalis* subnuclei of the lateral reticular nuclei; (2) the *accessory*, *dorsal*, and *ventral* subnuclei of the paramedian reticular nuclei; (3) the *n. intercalatus* of *Staderini*, the *n. of Roller*, and the *n. prepositus hypoglossi* of the perihypoglossal group.

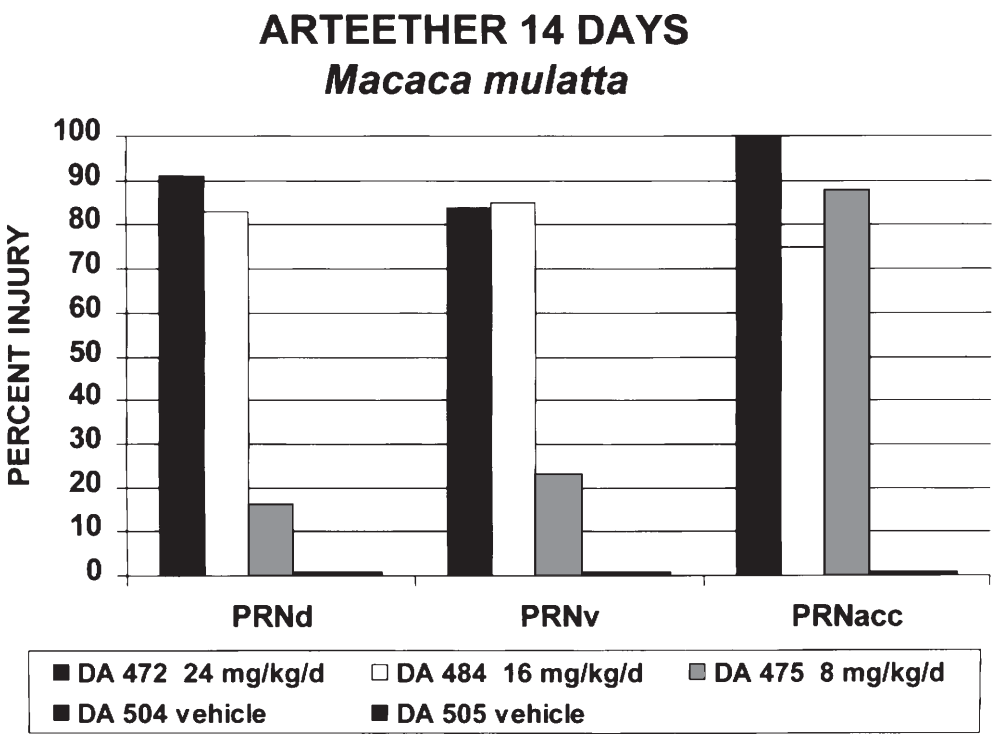

Fig. 4 Bar graph illustrating the percent of injured neurons present in the *nucleus reticularis paramedianus, subnuclei dorsalis* (PRNd), *ventralis* (PRNv), and *accessorius* (PRNacc) following 14-day treatment with arteether. No evidence of injury was observed in the vehicle control cases

The lateral reticular nucleus (LRN; *n. reticularis lateralis*, or nucleus of the lateral funiculus) is a prominent nuclear group in the ventrolateral tegmentum of the caudal medulla oblongata located between the inferior olivary nuclear complex and the tract and nucleus of the trigeminal nerve. Three subnuclear groups are distinguished: *subnucleus magnocellularis* (LRNm), *parvicellularis* (LRNp), and *subtrigeminalis* (LRNsub). According to Olszewski and Baxter (1982) the human brainstem contains two subdivisions of the lateral reticular nucleus: (1) the *n. medullae oblongatae lateralis subnucleus dorsalis* (L.d), and (2) *the subnucleus ventralis* (*L.v*). The subtrigeminalis subnucleus is regarded as a separate nuclear group and is named *n. medullae oblongatae subtrigeminalis*, by Olszewski and Baxter (1982). We will refer to the three subnuclear groups magnocellularis, perivicellularis, and subtrigeminalis.

Microscopic study of our 14-day arteether treated monkeys at the high (24 mg/kg per day cases DA-472 and DA-514), middle (16 mg/kg per day (DA-484 and DA-490), and low (DA-475, and DA-508) dose levels disclosed severe bilateral injury within each subnuclear group of the lateral reticular neurons: *n. reticularis lateralis subnucleus magnocellularis* (LRNm), *n. reticularis lateralis subnucleus parvicellularis* (LRNp), and *n. reticularis subnucleus subtigeminalis* (LRNsub). No evidence of neuronal injury was observed in either the 7-day arteether-treated high (24 mg/kg per day DA-387) or low (8 mg/kg per day DA-356) dose monkeys. Similarly, no evidence of injury within the lateral reticular subnuclear groups was observed in either the 14-day (DA-504, DA-505) or the 7-day vehicle treated (DA-386) monkeys.

The numerical data revealed that the degree of injury differed between the subnuclei. The LRNm and LRNsub were the most severely affected, with 99–100% of the population injured at the high dose level and 88–92% injured at the middle dose level. At the low dose level the LRNp was more severely affected with 57% of the neurons injured while 53% and 47% of the neurons were affected in the LRNm and LRNsub cell groups, respectively.

At the high and middle dose levels the number of affected neurons in all nuclear groups remained high, thus varying between 68 and 100%. Approximately half (47–57%) of the cell population at the low dose level were affected in all three subnuclear groups, perhaps indicative of the sensitivity of all these nuclei. No evidence of injury was found in the magnocellular, parvicellular or subtrigeminal subnuclei in the 14-day sesame oil vehicle control monkeys.

The paramedian reticular nuclei

The paramedian reticular nuclei (PRN) are craniocaudally coextensive with, and somewhat cranial to the hypoglossal nuclei and mediolaterally located in the medulla oblongata between the midline raphe nuclei and the medial reticular formation. Cytoarchitectonically three subnuclear groups have been defined (Brodal 1953; Brodal and Torvik 1954; Brodal and Gogstad 1957): (1) *n. reticularis paramedianus pars ventralis* (PRNv), (2) *pars dorsalis* (PRNd), (3) *pars accessorius* (PRNacc).

Neuronal injury was present bilaterally in the *accessory*, *dorsal*, and *ventral* subnuclei at all three dose lev-

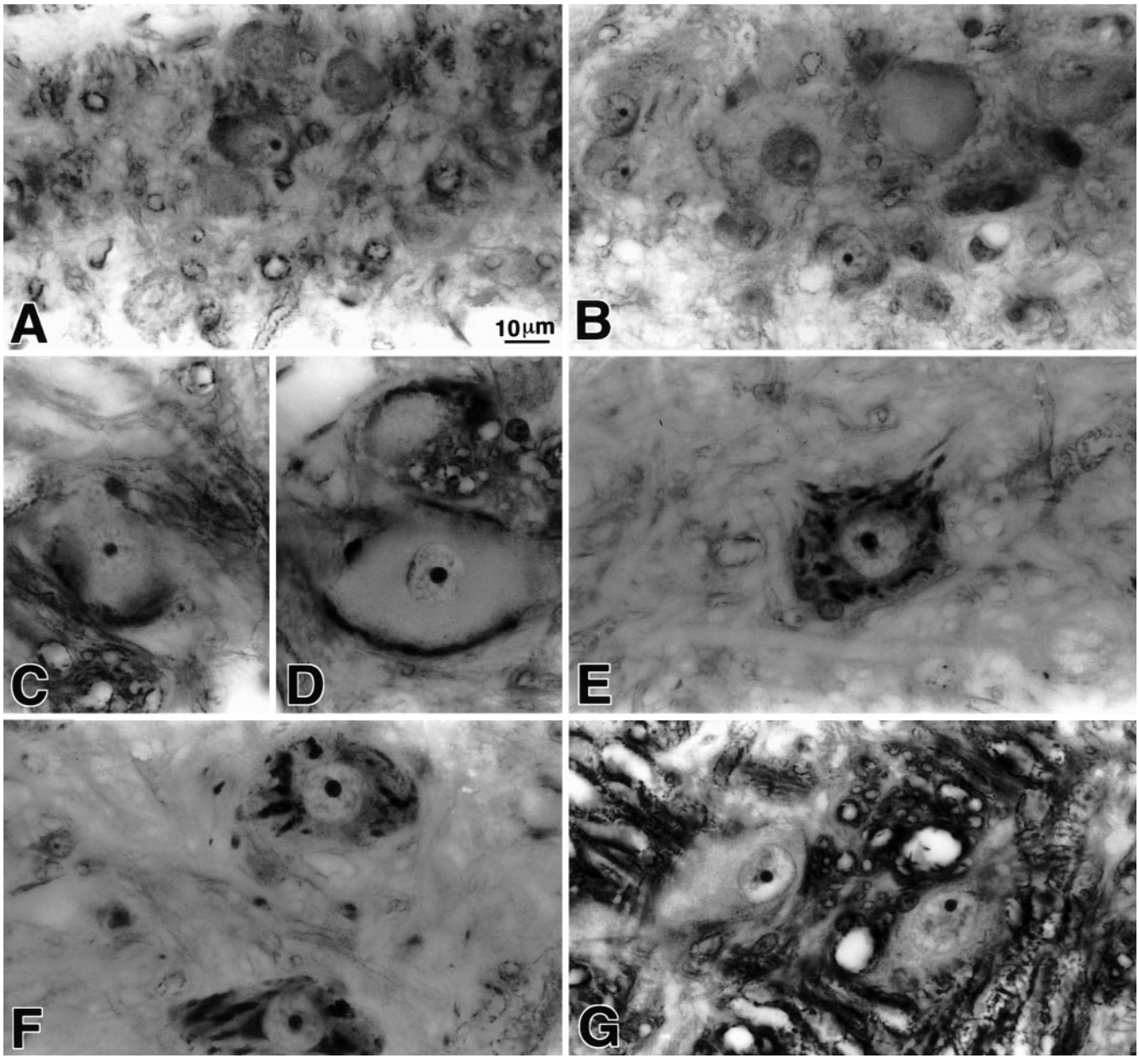

Fig. 5A–G Affected neurons revealing central chromatolysis among neurons of the medullary perihypoglossal nuclei (PHN) following the administration of the antimalarial drug arteether (24 mg/kg per day, 14-day). Affected neurons in panels **A**, **B** are from *n. intercalatus of Staderini* (Ic); in panels **C**, **D** from the *nucleus of Roller* (Ro). Unaffected motor neurons of the n. nervi hypoglossi are seen in panels **E**, **F**. Affected *n. interfascicularis hypoglossi* (Ifh) neurons are illustrated in **G**. Klüver-Barrera method. *Bar* 10 μm

els in 14d AE-treated monkeys. No evidence of neuronal injury was observed in either the 7-day arteether-treated high (24 mg/kg per day DA-387) or low (8 mg/kg per day DA-356) dose monkeys. Similarly, no evidence of injury within the lateral reticular subnuclear groups was observed in either the 14-day (DA-504, DA-505) nor the 7-day vehicle treated (DA-386) monkeys.

The numerical data in the 14-day arteether-treated monkeys revealed that the accessory nucleus (PRNacc) appeared to sustain a particularly high level of injury with 100%, 75%, and 88% of its neuronal population affected at the high, middle, and low dose levels, respectively. Similarly large injuries were seen in the PRNd (83–91%) and PRNv (84–85%) at the high and middle dose levels. Fewer paramedian neurons were affected at the low dose level with injury at 16% for the PRNd, and 23% for PRNv neurons.

The perihypoglossal nuclei

The perihypoglossal nuclear complex is located in the medulla oblongata. The nuclei derive their collective designation from their close proximity to the hypoglossal

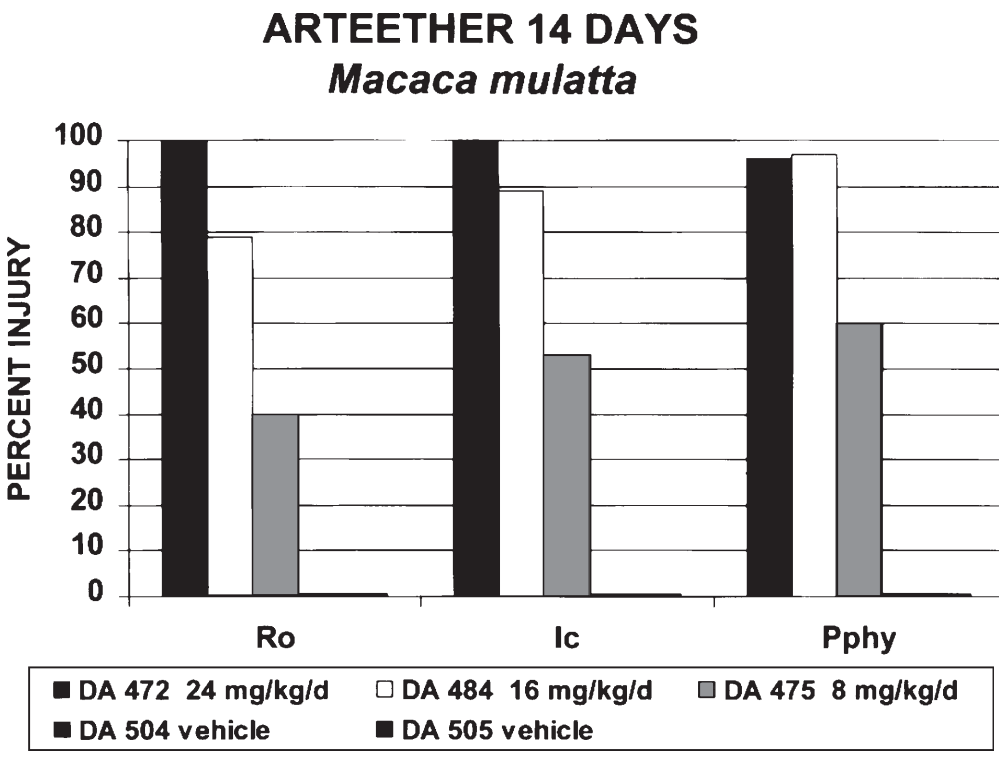

Fig. 6 Bar graph illustrating the percent of injured neurons present in the perihypoglossal nuclei: *nucleus intercalatus of Staderini* (Ic), *nucleus of Roller* (Ro), and *nucleus prepositus hypoglossi* (Pphy) following 14-day treatment with arteether. No evidence of injury was observed in the vehicle control cases

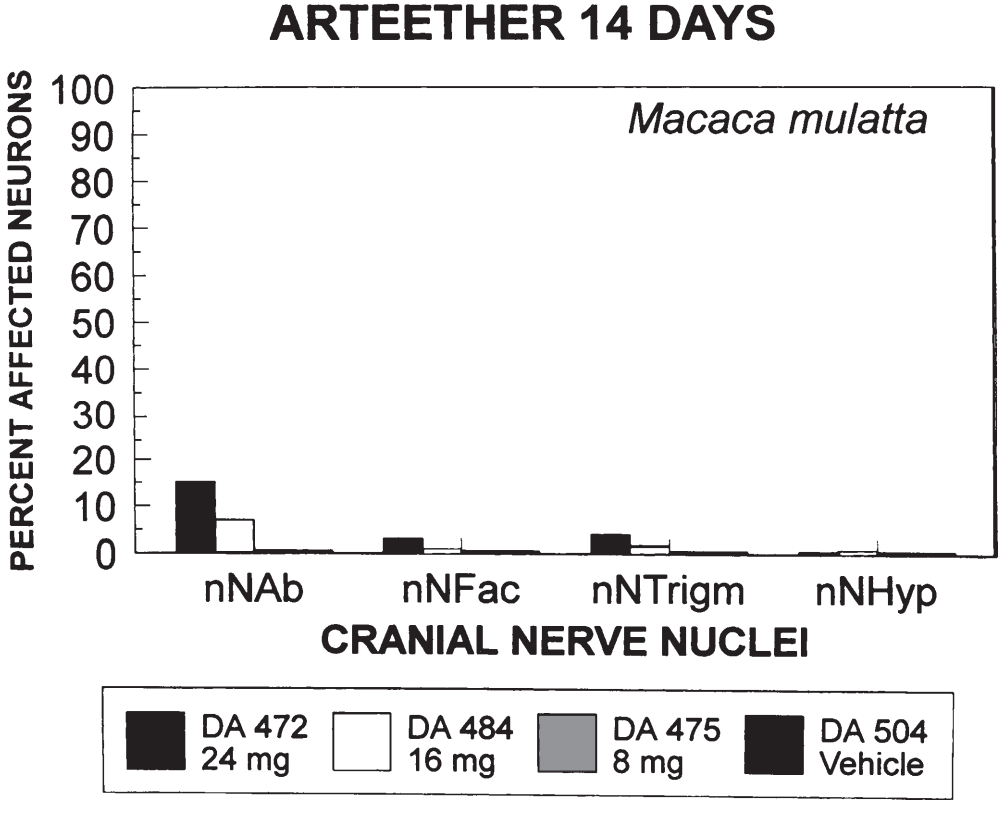

Fig. 7 Bar graph indicating the level of injury, or lack of injury, in the abducens (nNAb), facial (nNFac), trigeminal (nNTrigm), and hypoglossal (nNHyp) cranial nerve nuclei following 14-day treatment with arteether. No evidence of injury was observed in the vehicle control cases

nuclei. Three nuclear groups are distinguished (Brodal 1952; Brodal 1983): (1) *nucleus intercalatus of Staderini* located between the dorsal motor nucleus of the vagus (cranial nerve X), and the large motor neurons of the hypoglossal nucleus (cranial nerve XII); (2) *nucleus of Roller* located on the ventral border of the hypoglossal nucleus; (3) the much larger *nucleus prepositus hypoglossi* starting at the cranial border of the hypoglossal nucleus and extending cranially to the medial border of the medial vestibular nucleus.

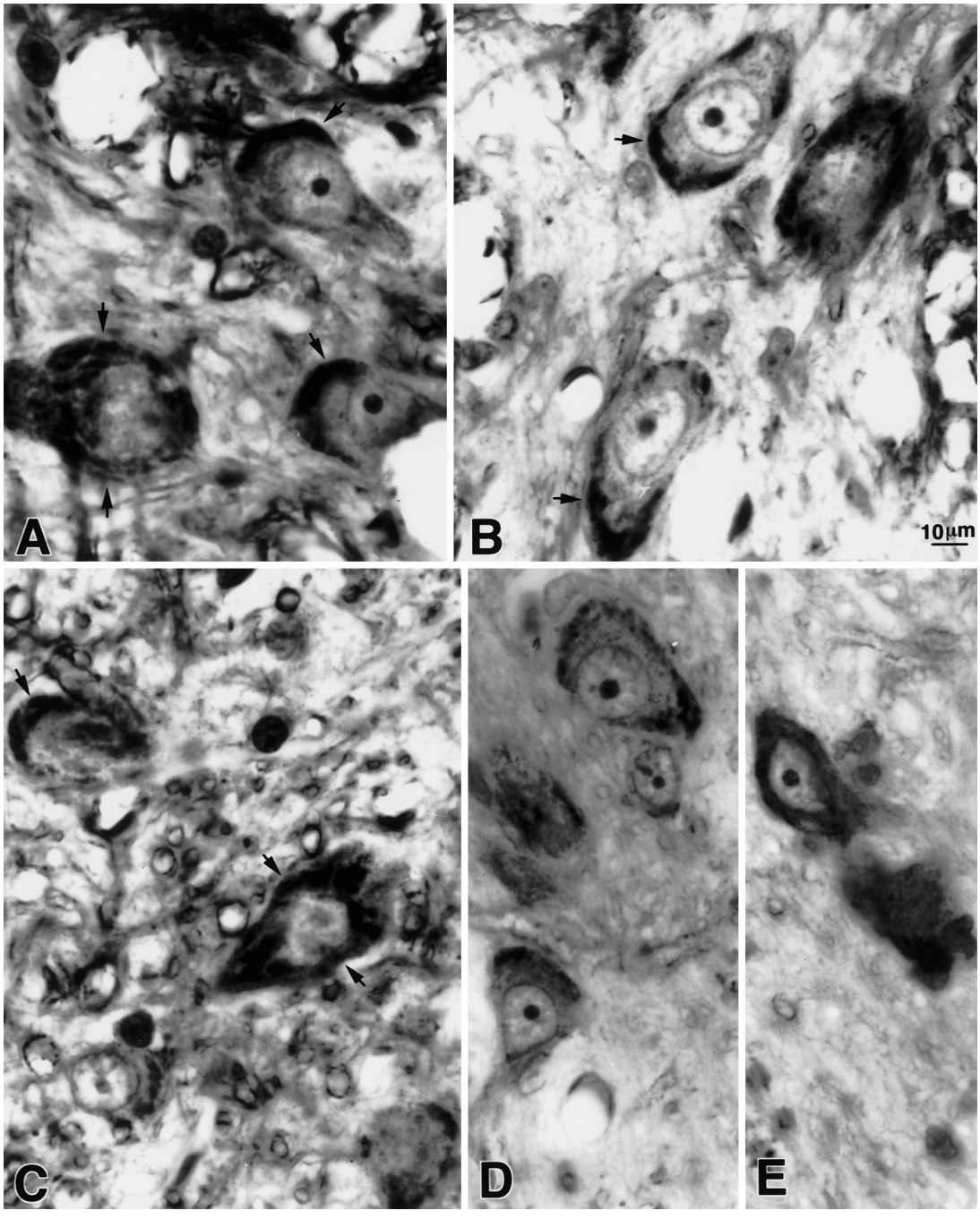

Fig. 8A–E These photomicrographs are from a Rhesus monkey (control case) given IM sesame oil only for 14 day. They illustrate the normal appearance of neurons in the *n. reticularis paramedianus, pars dorsalis* (**A**) and *pars ventralis* (**B**); the *n. prepositus hypoglossi* (**C**); *n. intercalatus of Staderini* (**D**); and *n. intercalatus* (**E**). The *single arrows* in **A**, **B**, **C** point to crescent profiles at the periphery of the cell body. These areas of the cytosol are actually composed of discrete, large clumps of Nissl substance. Three-dimensionally they overlap each other, with the result that they falsely appear as a single mass. Within the interior of the cytosol, finely granular Nissl substance is present in these neurons. In **B**, **C** this same photographic print artifact occurs to some degree in the cytosol of large neurons of the *n. reticularis paramedianus, pars dorsalis* (**A**) and *pars ventralis* (**B**) and the *n. prepositus hypo-glossi* (**C**). These cells are characterized by a filling of the cytosol with large clumps of Nissl substance. Klüver-Barrera method. *Bar* 10 μm

Bilateral neuronal injury was observed at all dose levels in 14d AE-treated monkeys and was found in each of the perihypoglossal nuclei: *n. intercalatus of Staderini* (Ic); *n. of Roller* (Ro); and *n. prepositus hypoglossi* (Pphy) at all three dose levels. Severe neuronal chromatolysis was typical at the high and middle dose levels. The numerical data revealed that at the high dose level, 96% of the neurons of the Pphy were affected, while all neurons (100%) of the nIc and nRo were affected. At the middle dose levels the number of affected neurons remained high with 97%, 89%, and 79% of prepositus, intercalatus, and Roller neurons affected, respectively. Injury remained substantial at the low dose level with

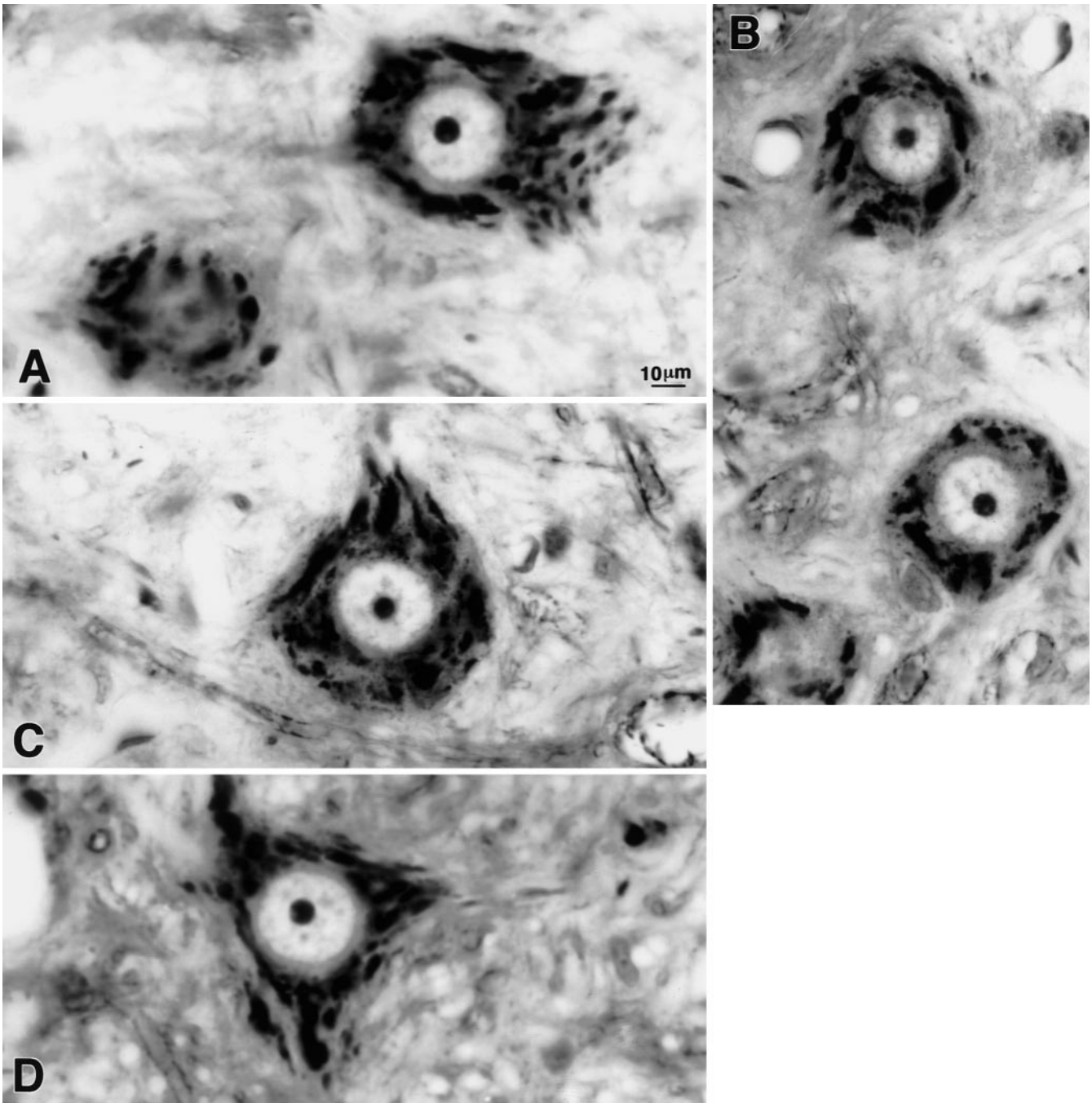

Fig. 9A–D These photomicrographs, from a control Rhesus monkey given IM sesame oil for 14 days illustrate the appearance of unaffected neurons located in the *n. nervi facialis* (*panels* **A**, **B**–**C** and *n. nervi hypoglossi* (**D**). Klüver-BaRrera method. *Bar* 10 μm

60% of prepositus, 53% of intercalatus, and 40% of Roller neurons affected. No evidence of injury was found in any of the perihypoglossal nuclei in either 7-day AE-treated or in the 14-day (DA-504, DA-505) and 7-day (DA-386) vehicle-treated monkeys.

Cranial nerve nuclei

The cranial nerve nuclei were studied in all 14 day and 7 day AE-treated monkeys and in all 14 day and 7 day control monkeys receiving the USP sesame oil vehicle. The nuclei studied included: (1) two general somatic efferent nuclei, *n. nervi hypoglossi*, and *n. nervi abducentis*; (2) special visceral efferent neurons, which include the *n. nervi trigemini motorius*, and *n. nervi facialis*. In all 14d AE-treated monkeys, the number of affected neurons varied between 0 and 3% except in the case of the abducens nerve nucleus in which a score of 16% was determined. Generally, a score of 1–3% may be considered

to occur in normative brain sections. No evidence of injury was found in any of the cranial nerve nuclei studied in 7-day AE-treated monkeys or in the 14-day and 7-day sesame oil-vehicle control monkeys.

Statistical analysis

Figure 2 shows percent injury for the arteether-treated (14-day and 7-day) and vehicle treated (14-day and 7-day) monkeys in the parvicellular, magnocellular, and subtrigeminal subnuclei of the lateral reticular nuclei. Mean percent injury was larger for the arteether-treated monkeys, $F(1,3)=14.4$, $P<0.033$ and within all nuclei; percent injury was significantly greater for the arteether treated monkeys than for the vehicle treated monkeys, $P<0.05$.

Within the paramedian reticular nuclei, Fig. 4 shows percent injury in the dorsal, ventral, and accessory subnuclei for individual monkeys. Treatment with arteether (14 day) resulted in significant neuronal injury, $F(1,3)=13.7$, $P<0.035$, and within all nuclei, mean percent injury for the arteether-treated monkeys was significantly larger than mean percent injury for the vehicle treated monkeys, $P<0.05$. The finding that neuronal injury was greatest in the accessory subnucleus at the low dose level suggests a far greater vulnerability of its neurons.

Figure 6 shows percent injury in the perihypoglossal nuclei for the vehicle-treated monkeys and the monkeys treated with arteether for 14 days. Again, mean percent injury for the arteether-treated monkeys was significantly larger than mean percent injury for those monkeys treated with vehicle, $F(1,3)=18$, $P<0.025$, and mean percent injury was significantly larger for the arteether-treated monkeys than for the vehicle-treated monkeys within Ro, Ic, and Pphy. Percent injury was most severe in Pphy for those monkeys treated with 8 and 16 mg/kg per day of arteether and was least severe in the Ro, although even in this nucleus nearly 80% of the cells were injured at 16 mg/kg per day and 40% of the cells were injured at 8 mg/kg per day.

Discussion

Neurology

We reported previously (Petras et al. 1997) that no gross neurological findings were observed in monkeys treated with arteether for 7 days, however, one monkey receiving 24 mg/kg per day for 7 days showed mild lethargy after day 4. Mild, sporadic anorexia was noted in all animals by day 14, and a single animal showed diffuse piloerection on day 14. Signs of tremor, nystagmus, and piloerection became evident at 12–14 days in monkeys treated for 14 days. Furthermore, the neuropathological data established that by the end of 14 days of treatment a severe brainstem neurotoxicity has occurred, and appears to simultaneously injure neurons of the medial and lateral ponto-medullary tegmentum, the vestibular nuclei, and the auditory nuclei. This report continues and extends the analysis of arteether-induced brain injury by describing the pathology that also occurs in the medullary precerebellar nuclei.

Neither the 7-day or 14-day arteether-treated monkeys were physiologically monitored or behaviorally tested for the early appearance of neurological or behavioral dysfunction. We do not know, therefore, if behavioral testing of monkeys would reveal progressive neurological impairments or decrements in behavioral performance. Rats administered daily doses of arteether continued to perform behavioral tasks up to the point where they too had sustained brainstem injury (Genovese et al. 1995). The continuance of animal experiments where low dose administration is coupled with long-term post-treatment survival periods, is central to the problem of clarifying if artemisinin compounds induce a reversible or irreversible brain injury. Arteether given at high and middle dose levels produced multiple systems injury throughout the hindbrain (pontine and medullary medial reticular formation nuclei, vestibular nuclei, auditory nuclei, and medullary precerebellar nuclei). The neuroanatomical distribution of the injury affects nuclear groups that subserve alerting reactions, posture and gait, cardiac and vasopressor regulation, auditory discrimination, and eye movements. The arteether-induced ponto-medullary neurotoxicity was pervasive. This resulted in a generalized debilitation, consequently, incremental neurological deficits did not occur.

The lateral reticular nuclei

The lateral reticular nuclei subserve a multiplicity of somatosensory and autonomic functions (Henry and Calaresu 1974a, b, c; Thomas et al. 1977) and are able to supply the cerebellum with information from spinal cord (Brodal 1943, 1949; Mehler et al. 1960; Morin et al. 1966; Mehler 1969; Menétrey et al. 1983; Rajakumar et al. 1992), brainstem, pyramidal (Kuypers 1958a, b, c; Walberg 1958; Brodal et al. 1967; Kunzel and Wiesendanger 1974; Corvaja et al. 1977a, b; Shokunbi et al. 1986; Rajakumar et al. 1992) and extrapyramidal (Walberg 1958; Hinman and Carpenter 1959; Mizuno et al. 1973) levels. We will summarize their anatomical relationships briefly with the view of demonstrating their importance should they be damaged by chemotherapeutic intervention during the treatment of malaria.

Neurological impairments following lesions of the lateral reticular nucleus – neurotoxicological implications

The experimental literature illustrates the possible consequence of neurotoxic injury to the lateral reticular nucleus. Experimentally placed *unilateral* lesions of the LRN of the cat (Corvaja et al. 1977b) result in (1) *ipsilateral*

hypertonia with limb abduction and extension, (2) *contralateral hypotonia* of limb extensor muscles accompanied by some tonic flexor muscle contractions; (3) loss of *ipsilateral tactile placing reflex*; (4) transient loss of ipsilateral *proprioceptive limb placing reaction*; (5) *contralateral diagonal lateropulsion* accompanied by frequent *falling*; (6) *contralateral head tilt.* Forelimb deficits were the most pronounced. Standing and walking were absent and cats remained in ipsilateral lateral recumbancy during the immediate postoperative period. The onset of righting reactions was followed by attempts at standing by the 3rd postoperative day; however, falling onto the contralateral side was typical. Improvement, but not full recovery, in posture appeared within 1–3 weeks. Neurological deficits of the tactile placing reflex, proprioceptive placing reaction, and of gait persisted over a period of 154 days. Microscopic examination of the brainstem revealed that lesions of the magnocellular subnucleus (dorsomedial sector) were correlated with ipsilateral forelimb hypertonia and contralateral forelimb hypotonia. Combined lesions of the magnocellular and the parvicellular subnuclei produced both forelimb and hind limb deficits.

Following arteether administration in the Rhesus monkey, neuropathologic lesions among LRN neurons was present among all subnuclear groups (magnocellular, parvicellular, and subtrigeminal). Had the lesions been focused upon the LRN we would expect postural and ambulatory deficits by the withdrawal of ascending afferent information originating in cutaneous receptors of the forelimbs and hind limbs and of inputs from the macula. Dysautonomia may have been manifested by alterations of systemic arterial pressure and cardiac rate (cardioacceleratory acitivity) by altering the communication between LRN neurons and the spinal preganglionic sympathetic neurons of the lateral horn. Experiments utilizing lower dose levels while monitoring cardiovascular functions would be advised. Responses to cerebral ischemia may be affected also. This is purely speculative since the brainstem lesions at high and middle dose levels in the arteether-treated 14-day cases was topographically widespread as witnessed by our arteehter-treated monkeys with lesions of the medial reticular formation, vestibular nuclei, as well as the LRN.

The paramedian reticular nuclei

The functional properties of the PRN are not as well studied as the LRN; nevertheless, the neuroanatomical data indicates their role in postural and locomotor functions. The PRN receives fibers from the spinal cord (Brodal and Gogstad 1957; Mehler et al. 1960; Mehler 1969), dorsal column nuclei (Brodal and Gostad 1957), vestibular nuclei (Ladpli and Brodal 1968), fastigial nuclei of the cerebellum (Thomas et al. 1956; Walberg et al. 1962); somatosensory cerebral cortex (Brodal and Gogstad 1957; Sousa-Pinto 1970), and the macula (Ghelarducci et al. 1974). All subnuclei of the PRN project to the cranial and caudal vermis of the cerebellum (Brodal and Torvik 1954; Somana and Walberg 1978). The neurological impairments ensuing from arteether induced injury could not be determined from our experiments. The connections of the PRN with the face region of the cerebral cortex, however, may indicate the possibility of somatosensory and somatomotor dysfunction following injury. The PRN has been implicated also in the control of eye movements and has been assigned to a system of median or paramedian nuclear groups associated with the median longitudinal fasciculus (MLF). The MLF is a prominent median fiber system flanking the brainstem raphe and serves as a pathway linking many motor and premotor nuclear groups with vestibular neurons participating in eye movement functions.

The perihypoglossal nuclei

The perihypoglossal nuclei (PHN), and their medullary precerebellar counterparts, the lateral reticular nuclei (LRN) and paramedian reticular nuclei (PRN), receive afferent fibers from: (1) the spinal cord (Brodal 1952; Mehler et al. 1960; Mehler 1969), (2) the vestibular nuclei (Baker et al. 1976; Mergner et al. 1977), (3) the cerebral cortex (face area in particular, Sousa-Pinto 1970), (4) the cerebellum. Two anatomical attributes anatomically and functionally differentiate the perihypoglossal nuclear complex from the LRN, and in part from the PRN: (1) the main source of cerebellar afferents stems from the flocculus and nodulus (Anguat and Brodal 1967), and (2) they are functionally linked with brainstem cell groups participating in the control of eye movements.

Eye movements

Motor neurons of the oculomotor, trochlear, and abducens nuclei innervate the extraocular muscles that move the eyeballs. A number of median and paramedian mesencephalic, pontine, and medullary nuclear groups supply afferent monosynaptic or polysynaptic connections to these motor nuclei and are collectively termed the *ocular premotor nuclei*. These cell groups link tegmental, macular, cerebellar, visual nuclei, pretectal nuclei, and vestibular nuclei for the coordinated control of eye movements (Büttner-Ennever and Büttner 1988; Büttner and Büttner-Ennever 1988; Büttner-Ennever et al. 1989; Büttner-Ennever and Büttner 1992).

Functionally, the rostral interstitial nucleus of the medial longitudinal fasciculus (rostral iMLF) contains burst neurons affecting horizontal and torsional saccades. It receives afferents from omnipause neurons in the PPRF, a region also characterized by excitatory burst neurons for horizontal saccades. An efferent trajectory from the riMLF terminates upon oculomotor and trochlear neurons. The medial reticular formation also contains ocular premotor units and these appear in the *n. reticularis pontis caudalis* as excitatory burst neurons for horizontal sac-

cades, and the *n. paragigantocellularis dorsalis* with inhibitory burst neurons for horizontal saccades. The paramedian tract cell groups all project to the cerebellar flocculus (Blanks et al. 1983; Langer et al. 1985a, b, 1986). This projection may permit the cerebellum to monitor eye position and stabilize gaze (Büttner-Ennever et al. 1989; Büttner-Ennever and Büttner 1992). Our earlier analysis of arteether-induced reticular formation injury (Petras et al. 1997) revealed affected neurons in the *n. reticularis pontis caudalis* and the *n. paragigantocellularis dorsalis* (J.M. Petras, unpublished work). Although the arteether injury data cannot reveal the functional properties of the affected neurons, the injury can be so widespread as to suggest injury to either excitatory or inhibitory burst neurons within the affected tegmental nuclei.

The interconnections of the perihypoglossal nuclei with ocular premotor nuclei exemplifies their role in the regulation of eye movements. All three nuclear groups receive afferent connections from the PPRF (Büttner-Ennever and Büttner 1988). The n. prepositus hypoglossi. The *n. prepositus hypoglossi* is reciprocally connected with the extraocular muscle nuclei (abducens, trochlear, and oculomotor). A projection, showing some rostrocaudal organization within the oculomotor nucleus, arises from the *ipsilateral* periphypoglossal complex, and other afferents originate from the underlying medullary reticular formation (Steiger, and Büttner-Ennever 1979). Further reciprocal connections are found with: (1) the n. intercalatus of Staderini; (2) the n. of Roller; (3) the medial, descending, lateral, and superior vestibular nn.; (4) the cerebellum; (5) the reticular formation (paramedian medullary and pontine cell groups); and (6) several nuclear groups of the visual system that includes the superior colliculi, nucleus of the optic tract, and the ventral lateral geniculate body (McCrea and Baker 1985a, b). The *n. prepositus hypoglossi* receives afferents from a number of eye movement premotor nuclear groups including: (1) the *interstitial n. of Cajal*; (2) the rostral iMLF; (3) the nucleus of the posterior commissure, and (4) the *n. reticularis tegmenti pontis* of von Bechterev. A prepositus-inferior olivary-cerebellar connection has been described (McCrea and Baker 1985a, b). The *n. intercalatus of Staderini receives* afferent connections from (1) neurons of the medial vestibular nn, which have been implicated in eye movements (Baker, Gresty, and Berthoz 1976; Mergner et al. 1977); (2) from reticulospinal neurons located within the PPRF (McCrea et al. 1987a, b); and (3) from the *n. prepositus hypoglossi* (McCrea and Baker 1985b; McCrea 1988). The *n. of Roller* receives projections from vestibular neurons that are active during eye movements (McCrea and Baker 1985b; Mcrea 1988) and from neurons of the PPRF. It emits efferent fibers to the *n. prepositus hypoglossi* (McCrea 1988a, b).

Conclusions

Neurological impairments of posture, gait, cardiovascular functions and eye movements may result from lesions of the precerebellar nuclei. At our low dose level, the precerebellar nuclei of *Macaca mulatta* are characterized, together with the medial and descending vestibular nuclei, by higher levels of injury than that found for the nuclei of the medial reticular formation, the superior olivary nuclear complex, and the nuclei of the trapezoid body (Petras et al. 1997). For example, injury levels of 47–53% were found in the LRN, 88% for the accessory paramedian reticular nucleus; 53% and 60% respectively, for the *n. intercalatus* and *n. prepositus hypoglossi* for the perihypoglossal nuclear complex; 56% and 65% of the spinal (descending) and medial vestibular nuclei, compared with reduced levels of injury sustained by either the pontine or medullary reticular nuclei with a range of 5–23%; and no evidence of injury in the superior and lateral vestibular nuclei (Petras et al. 1997).

The simian medullary precerebellar nuclei, and the medial and descending vestibular nuclei appear to have a high degree of vulnerability when arteether is given for 14 days at dose levels between 8 mg/kg per day and 24 mg/kg per day. The neurological consequences of this treatment may profoundly impair posture, gait, autonomic regulation, and could result in eye movement disorders.

Acknowledgements We thank Mr. Curtis King for his expert neuroanatomical and neuropathological support, Division of Neurosciences, WRAIR; Ms Michelle Sperry, Division of Experimental Theapeutics, WRAIR; Messrs. Suvit Boonkali, Chalerm Jeckseang, Arvuth Kaewsupo, Bamrung Chaikwang, Komdej Kongsunarat, Niyom Sornchand, Phongsak Maneerat, Ms Pranee Hansukjariya, the staff of the Department of Veterinary Medicine, AFRIMS. This investigation was funded in part by the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases (TDR) and the U.S. Army Medical Research and Material Command.

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