Differential Time-Course Decreases in Nonselective, μ-, δ-, and κ-Opioid Receptors After Focal Cerebral Ischemia in Mice

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Background and Purpose—Neuroprotection studies have demonstrated the involvement of opioids in ischemia, and we have previously reported the neuroprotective role of opioids in ischemia, and we have demonstrated alterations in Bmax of opioidergic receptors after 2 post-MCAO time points in mice.

Methods—In the present study, we have investigated in a detailed manner the postischemic time course of variations in [3H]diprenorphine (nonselective), [3H]DAMGO (μ), [3H]DADLE (δ), and [3H]U69593 (κ) relative binding densities after focal cerebral ischemia (0 to 48 hours) in mice.

Results—In frontoparietal cortices, our results demonstrate decreases in (1) δ receptor densities at 1 to 3 hours after MCAO, (2) μ and nonselective binding sites at 6 to 12 hours after MCAO, and (3) κ receptor densities between 6 and 24 hours after MCAO. In the rostral part of the infarct border zone, a decrease in δ-receptors was found concomitant with the extension of the infarct core; conversely, the decrease in δ-receptors appeared before (6 to 12 hours) macroscopic histological damage, which occurred between 12 hours and 24 hours after MCAO in the caudal part of this area. In this frontier, μ- and especially κ-binding sites were decreased later (12 to 48 hours after MCAO).

Conclusions—These differential alterations in opioidergic receptors could be due to the selective sublocalization of receptors, postsynaptically on cortical interneurons for μ- and δ-receptors versus presynaptically on cortical afferent pathways for the κ subtype. Further, our results suggest that δ- and μ-opioidergic receptors could be markers of infarct extension and neuronal death; the study of [3H]diprenorphine and selective binding sites argues in favor of the use of receptor-specific ligands. Finally, the relative preservation of κ-receptors might be correlated with the neuroprotective role of κ-agonists, as previously reported. (Stroke. 1999;30:1271-1277.)

Key Words: autoradiography ■ cerebral infarction ■ receptors, opioid ■ mice

Naloxone has been advanced as a potential neuroprotective, though much-debated, agent in experimental and clinical investigations; on the basis of these findings, the implication of the opioidergic system in cerebral ischemia has been considerably studied. Several investigations have unequivocally reported the neuroprotective role of κ-receptor agonists in models of global and focal cerebral ischemia (for review, see Reference 9). Despite these numerous studies, few investigations have been carried out to analyze with precision the evolution of the opioidergic system following cerebral ischemia (for review, see Reference 9). Moreover, the existing studies have been performed essentially with models of global cerebral ischemia, and/or with ligands that do not discriminate between the 3 subtypes of opioidergic receptors, namely, μ, δ, and κ.

We have recently reported differential alterations in μ, δ, and κ maximal binding capacities (Bmax), rather than affinities (Kd), not only in infarcted cortices but also in penumbral areas, 6 hours and 24 hours after permanent middle cerebral artery occlusion (MCAO) in the mouse. In this previous study, we demonstrated that the μ and δ Bmax values were decreased earlier than κ Bmax values in infarcted cortices. Likewise, μ and δ Bmax were significantly decreased at 6 hours after ischemia in the ipsilateral temporal auditory cortex, which is typically a penumbral cortex, since the consolidated infarct progressively recruits this adjacent tissue between 6 hours and 24 hours after MCAO. However, one criticism of our previous study is that it was performed at only 2 time points following the induction of ischemia; a more precise analysis of the time-related evolution of opioidergic receptor densities following stroke is necessary, notably for the determination of the therapeutic window for opioidergic drugs. Consequent to our previous study in which we reported only alterations in the Bmax values after cerebral ischemia in mice, we have quantified in the present study the relative binding densities of the total population of opioidergic

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receptors with a nonselective ligand (diprenorphine) in comparison with \( \mu \), \( \delta \), and \( \kappa \)-opioidergic receptors labeled with 3 selective ligands at 0 hours, 1 hour, 3 hours, 6 hours, 12 hours, 24 hours, and 48 hours after MCAO in mice. We report here the evolution of the 4 populations of receptors not only in the infarct core but also in the penumbral areas in parallel with the growth of the ischemic lesion.

Materials and Methods

Middle Cerebral Artery Occlusion

All studies were conducted in accordance with the French legislation and European directives. Eighty OF mice (body weight, 17±2 grams, mean±SD, Iffa Credo, France) were kept in thermoregulated (22±2°C), humidity-controlled (55±10%) facilities under a 12-hour light/12-hour dark cycle (light on between 8 AM and 8 PM) and were allowed free access to food and water. Permanent focal cerebral ischemia was achieved by MCAO as described by Welsh et al.,14 with the following modifications. Mice were anesthetized with chloral hydrate (500 mg/kg, IP), and maintained normothermic (37.5±0.5°C) by means of a heating blanket (Homeothermic Blanket Control Unit, Harvard Apparatus Limited). Under an operating microscope (Leica M715), the scalp was incised between the eye and ear; the temporal muscle was electrocoagulated (Aesculap TB50) and partially removed. The left middle cerebral artery (MCA) was identified, a craniotomy was carried out with a high-speed drill (Technobox-810), and the dura was incised. The left middle cerebral artery (MCA) was occluded, a craniotomy was carried out with a high-speed drill (Technobox-810), and the dura was incised. The MCA was occluded by bipolar electrocoagulation, and the scalp was sutured. For the sham-operated animals, the experimental procedures were similar, except that the MCA was not coagulated.

Autoradiography

Euthanasia was performed by vertebral dislocation 24 hours after surgery for the sham-operated group (n=12), or at 0 hours (control group, n=8), 1 hour (n=10), 3 hours (n=9), 6 hours (n=9), 24 hours (n=10), and 48 hours (n=10) after MCAO. The brains were rapidly removed, frozen in isopentane at −80°C, and stored at −80°C. Coronal brain sections (15 μm) were obtained with a cryomicrotome (Kryostat 1720 Digital, Leitz) and mounted onto gelatin-coated slides. Autoradiographic experiments were performed with [\(^{3}H\)]diprenorphine (Amersham), [\(^{3}H\)]DAMGO, [\(^{3}H\)]DADLE and [\(^{3}H\)]U69,593 (DuPont NEN) (specific activities of 31, 53, 32, and 46 Ci/mmol, respectively) for nonselective, \( \mu \), \( \delta \), and \( \kappa \) binding sites, respectively. Based on their respective affinities, [\(^{3}H\)]diprenorphine, 2 nmol/L for [\(^{3}H\)]DAMGO, and 1 nmol/L for [\(^{3}H\)]DADLE and [\(^{3}H\)]U69,593 in appropriate buffers and according to protocols described elsewhere.15-17 Nonspecific binding was determined by incubation of adjacent brain slices in the presence of 10 μmol/L levorphanol (Sigma). Brain sections were coexposed with standards ([\(^{3}H\)]-microscales, Amersham) on tritium-sensitive films (Hyperfilm, Amersham) for 8 weeks at −20°C. Thereafter, the films were developed (LX 24, Kodak), rinsed with water, and fixed (AL 24, Kodak). Relative densities of receptors (expressed in femtomoles per milligram of tissue equivalent) were quantified with a computer-assisted image analyzer (BIOCOM RAG 200) using a 5-order polynomial relationship between optical density and radioactivity.

Histological Analysis

Macroscopic histological analysis was performed on 12 brain sections per animal, adjacent to those used for autoradiography, and stained by the hematoxylin-eosin technique. Infarcted areas were delineated by the relative paleness of histological staining in the ischemic tissue. Regions of interest were determined through the use of a stereotaxic atlas of the mouse brain,19 and infarcted volumes were calculated by the integration of infarcted areas as quantified with a computer-assisted image analyzer (BIOCOM RAG 200) on each brain slice.

Statistical Analyses

For each ligand, a multiparametric analysis of variance (MANOVA) was first undertaken (Statview 4.5, Abacus Concepts Inc) with 3 factors: brain region, side (ipsilateral versus contralateral, repeated factor) and time after MCAO. Because such an analysis revealed a highly significant (P<0.001) principal effect for 1 or more of these 3 factors and interactions between them, a second-order analysis was performed to determine the individual effect of each parameter, the last stage of this segmentation being obtained by a 1-way ANOVA with time after MCAO as the principal factor, followed by post hoc multicomparison tests (Fisher protected least significant difference [PLSD] test), for each ipsilateral and contralateral region of interest. Infarct volumes, calculated for each post-MCAO time, were compared between groups through the use of a 1-way ANOVA and the post hoc Fisher PLSD test. For all statistical analyses, the significance level was accepted to be P<0.05.

Results

Histological Alterations

No histological alterations were seen in control mice (0 hours); the sham-operated group was not significantly different from the control group (P=0.69, Fisher PLSD test; Table 1). Histological analysis revealed that the infarct core corresponded essentially to the motor and somatosensory frontal-parietal cortices, which were totally infarcted at 1 to 3 hours after MCAO. At this time, a limited volume of peri-infarcted tissue was situated in the anterior motor frontal and temporal auditory cortices. The temporal auditory cortex was progressively recruited by the infarct between 6 hours and 24 hours after MCAO (Figure 1). Selective necroses and swelling appeared to increase between 1 hour and 24 hours after MCAO, especially in the border zone in which the extension of infarct occurs. At these later time points, the infarcted volume reached a maximal value (21±4 mm³) (Table 1). In other regions, such as the entorhinal and cingular cortices, no alterations could be observed.

Opioid Receptor Alterations

For each ligand, the MANOVA revealed a highly significant (P<0.001) effect for the region of interest, side, and post-MCAO time factors; interactions between these factors were also revealed. The signal-to-noise ratio was measured to
evaluate any putative influence on the variations that had been detected. For each ligand, noise was low (m, 3 ± 1%; d, 3 ± 0.4%; and k, 7 ± 2%; mean ± SD, in percent of the total signal measured in ipsilateral and contralateral cortices of the sham and control groups), and thus background noise was unlikely to significantly influence the quantification of opioid receptors, and in turn, the variations observed consequently to focal cerebral ischemia.

**Alterations of Opioid Binding Sites in the Infarct Core**

In the cortices infarcted early, namely, the motor and somatosensory frontoparietal cortices, the number of [3H]diprenorphine binding sites was significantly decreased (≈−25% to −50% versus sham and control groups) at intermediate time points (≈6 to 12 hours), except in layers I-II-III of the anterior somatosensory frontoparietal cortex, where the decrease was observed as soon as 1 hour after MCAO (−21% and −24% versus sham and control groups, respectively) (Figures 2 and 3). These decreases of binding sites in the superficial layers of the cortex were not dependent on the rostrocaudal level and appeared at intermediate time points (between 6 and 24 hours after MCAO; Figure 1), when compared with δ- and k-receptors (Figures 2 and 3).

μ-Opioid receptor densities were significantly decreased (−30% to −60%, versus sham and control groups) in all layers of the frontoparietal cortices between 6 and 12 hours after MCAO (Figures 2 and 3). The δ-opioid receptor...
population was significantly decreased as early as at 1 hour after MCAO in all layers of the intermediate frontoparietal cortex (−24% to −36% versus control group), and in layers I-II-III (−32% and −51% versus sham and control groups, respectively) and IV (−39% versus control group) of the anterior somatosensory frontoparietal cortices. In the deep layers (V-VI) of anterior (−35%, 3 to 6 hours, versus sham and control groups), and in all layers of the posterior somatosensory frontoparietal cortices (−26% to −58%, 3 to 6 hours, versus sham and control groups), δ-receptor decreases were evidenced between 3 and 6 hours after MCAO.

In the anterior and posterior motor frontoparietal cortices, significant decreases in δ-binding densities appeared at 3 hours after MCAO in superficial layers (I-II-III, −32% to −38%; IV, −23% to −34%, versus sham and control groups), and at 1 and 6 hours in deep layers of the anterior (−21% versus control group) and posterior (−30% to −38% versus sham and control groups) cortices, respectively. In infarcted areas, κ-receptor densities were not decreased at the early time points (1 to 6 hours), except in layers I-II-III of the intermediate (−24% and −48%, 6 hours versus sham and control groups, respectively) and posterior (−24%, 1 hour versus control group) somatosensory frontoparietal cortices.

After 12 hours of ischemia, the decreases in δ-receptor densities were highly significant in the motor (P<0.001; −46% to −73%; μ, −39% to −58%; δ, −49% to −74%; and κ, −21% to −45%, 24 to 48 hours, versus sham and control groups) appeared in these cortices, with a similar profile of binding changes for all cortical layers (Figure 2).

In the frontoparietal cortices, the multicomparison test showed that μ-, and especially δ-, opioidergic receptors were decreased earlier (6 to 12 hours and 1 to 6 hours after MCAO, respectively) than κ-opioidergic receptors (6 to 24 hours after MCAO) (eg, Figure 3). Furthermore, the decreases in μ-receptors displayed comparable temporal profiles after 6 to 12 hours MCAO in the motor and the somatosensory cortices, and the profile was independent of the rostrocaudal level studied (Figures 2 and 3). In contrast, the δ-receptors subtypes were first decreased (1 hour after MCAO) in the anterior and intermediate levels of somatosensory frontoparietal cortices (Figures 2 and 3) and thereafter in the anterior and posterior motor frontoparietal cortices and all rostrocaudal levels of the somatosensory frontoparietal cortices (3 hours after MCAO) (Figures 2 and 3). Thus, the extension of the δ-receptor decreases followed a ventrodorsal and rostrocaudal progression which corresponds well with the extension of histological damage.

Alterations of Opioidergic Binding Sites in the Infarct Border Zone
In peri-infarcted areas, such as anterior frontal motor cortices, δ-binding sites were first decreased in layers I-II-III and V-VI (3 hours, −40% versus sham and control groups; and 6 hours,
237% versus control group, respectively), and thereafter in layer IV (12 hours, \(\sim -50\%\) versus control group). [\(^3\)H]Diprenorphine, \(\mu\)- and \(\kappa\)-binding sites were also decreased but only after 12 hours in the superficial layers (\(\sim -59\%\) versus sham and control groups; \(\sim -32\%\) versus control group, respectively) in the same region (Figure 2). The decreases in \(\delta\)-receptor densities, observed between 3 and 12 hours after MCAO, were found to be concomitant with the early extension of the histological damage noted in this area. In contrast, \(\delta\)-receptor densities were decreased 6 hours after MCAO in layers I-II-III (\(\sim -37\%\) versus control group) and 12 hours after MCAO in both layers IV (\(\sim -50\%\) versus sham and control groups) and V-VI (\(\sim -44\%\) versus control group) of the temporal auditory cortex and in striatal cortex (\(\sim -40\%\) versus sham and control groups) (Figures 2 and 4); such decreases were evidenced before the appearance of macroscopic histological damage, which was seen only between 12 and 24 hours after MCAO in these areas (Figure 1). Similarly, [\(^3\)H]Diprenorphine and \(\mu\)-binding sites were decreased at 12 hours after MCAO in layers I-II-III (\(\sim -44\%\) and \(\sim -30\%\) versus sham and control groups, for nonselective and \(\mu\)-receptors, respectively) and IV (\(\sim -40\%\) and \(\sim -27\%\) versus sham or control animals, for nonselective and \(\mu\)-receptors, respectively). \(\kappa\)-Receptor densities were not significantly decreased in the temporal auditory cortex, except an early decrease (\(\sim -23\%\) versus control group, 3 hours after MCAO) in layer IV and a delayed decrease in layers V-VI of this cortical area (\(\sim -45\%\) versus control group, 48 hours after MCAO) (Figures 2 and 4). No alteration of any of the opioidergic binding sites were observed in other ipsilateral cortical areas, such as the cingular, retrosplenial, and entorhinal cortices, or in cortical regions of the contralateral hemisphere.

**Discussion**

The present study highlights significant alterations in opioidergic binding site densities, not only in the infarct core but also in penumbral tissues, after focal cerebral ischemia in the mouse. Moreover, when compared with the 2 post-MCAO time-point analyses performed previously,\(^{10}\) the extensive analysis of opioidergic binding sites after MCAO demonstrates that, in fact, (1) \(\mu\) and \(\delta\) subtypes have differential time-related decreases in the infarct core, (2) time-related decreases in \(\delta\)-opioidergic receptors occur along with the appearance of the growing infarct (1 to 3 hours after MCAO), and (3) \(\kappa\)-receptors show little decrease before 24 hours after MCAO in infarcted cortices. Furthermore, a time course was observed for the nonselective binding sites that differs from those of \(\mu\)-, \(\delta\)-, and \(\kappa\)-receptors.

**Alterations of Opioidergic Binding Sites in Infarct Core**

In infarcted areas, the early decreases of not just \(\delta\)- but also \(\mu\)- and nonselective opioid binding sites are in accordance with our previous report in which we demonstrated a marked decrease in \(\delta\) and \(\mu\) \(B_{\text{max}}\) values 6 hours after MCAO in the mouse brain.\(^{10}\) As previously postulated by many authors,\(^{16-18}\) we attribute these changes to the irreversible...
neuronal damage observed in the frontoparietal cortices at early time points.19 Because apoptotic and DNA-damaged neurons have been observed in the acute stage after focal cerebral ischemia,19–22 phenomena that evolve toward necrosis23–25 and complete disappearance of neurons at delayed time points,19,23 one might hypothesize that the early decrease in opioid receptor densities is due to such progressive neuronal damage and cell death. Our results demonstrate selective alterations with the 3 subtypes of opioidergic receptors; k-receptors were decreased later than μ- and δ-receptors, in accordance with our previous study.10 This delayed alteration in k binding sites could be related to a cell-specific localization of opioidergic receptors. As previously reported for other specific presynaptic and postsynaptic proteins such as synaptophysin and MAP-2,26–28 one could hypothesize that μ- and δ-receptors, classically described as neuronal postsynaptic receptors29,30 (although some reports have described μ-, δ-, and k-receptors as both presynaptic and postsynaptic receptors as a function of the brain region studied and neurotransmitter coexpressed with the opioids),31–33 are localized on cortical interneurons and are more sensitive to cerebral ischemia than subcortical afferent pathways on which k-receptors are localized presynaptically.34 A second hypothesis, in which the varied alterations described above could be due to an upregulation of the k-opioid receptors localized on glial cells, seems unlikely, because many studies have demonstrated, through the use of immunocytochemistry and electron microscopy, that only a few opioidergic receptors (<20%) are expressed by glial cells.34–36 Based on our present observations, the precocious alteration in μ-, and especially δ-, receptors could constitute markers of neuronal death and infarct extension through the use of specific ligands. The relative preservation of k-receptor densities during the first hours after MCAO (until 6 hours after MCAO), which is similar to the therapeutic window of opportunity for k-agonists (significant decrease of infarct volume with treatment initiation delayed until 6 hours after MCAO;7,8 for review, see Reference 9), supports the use of k-agents as neuroprotective drugs.

Alterations of Opioidergic Binding Sites in Infarct Border Zone

The anterior frontal motor and temporal auditory cortices have been defined as a border zone (or penumbra) because they are not infarcted at early time points (1 to 6 hours) but are progressively recruited by the expansion of the ischemic lesion. In the anterior frontal motor cortex as well as in frontoparietal cortices, the decreases in δ-opioidergic receptor densities were concomitant with the appearance of histological damage, as seen with the hematoxylin-eosin staining technique. Furthermore, the decreases in [3H]diprenorphine, μ-, and k-binding sites, which occur 12 hours after MCAO, could delineate the neuronal loss in this cortical area. Conversely, in the temporal auditory cortex, the decreases in δ (at 6 hours after MCAO, layers I-II-III; at 12 hours after MCAO, layers IV and V-VI and striatal cortex), μ (layers I-II-III and V-VI) and [3H]diprenorphine binding densities (layers I-II-III and IV) at 12 hours after MCAO, occurred just before (6 hours after MCAO) or concomitant with (12 hours after MCAO; Figure 2) the macroscopically evident histological changes. The changes in δ relative binding densities noted in the present study occurred at the same post-MCAO time point as decreases in δ Bmax values (6 hours) observed in our previous study10 that examined only 2 post-MCAO time points (6 and 24 hours). In contrast, the decrease in μ relative binding densities appeared slightly later (12 to 24 hours versus 6 hours) than decreases in μ Bmax values observed previously10; this difference between the present result and our previous report10 illustrates the precise evolution of opioid receptors that could not be demonstrated with an experimental paradigm with only 2 time points. Two nonexclusive hypotheses could be advanced to explain the precocious alterations in opioidergic receptor densities: (1) a selective neuronal loss, as reported in the hippocampus after global cerebral ischemia16,18 and in the infarct border zone after focal ischemia in the mouse,29 or (2) downregulation mechanisms linked to neuronal dysfunction in hypoxic neurons37 or in the infarct border zone after focal cerebral ischemia.38,39 Moreover, it seems that in the temporal auditory cortex, the alteration in μ- and [3H]diprenorphine receptors were relatively similar, and occurred at intermediate time points (12 to 24 hours after MCAO). This similarity could be explained by (1) the heterogeneity of decreases in the 3 subtypes of opioidergic receptor as a function of post-MCAO time (early or intermediate for δ- and μ-receptor decreases and delayed or nonsignificant for the k subtype) and (2) the important proportion (∼30% in the cortex) of μ-receptors with regard to the overall population of opioidergic receptors.40–42 The nonselective decreases in opioidergic binding sites observed at later time points are likely due to a neuronal death throughout the infarcted cortices. An exception would be the temporal auditory cortex, in which the density of k-receptors was not altered in layers I-II-III, and only at 48 hours after MCAO in layers V-VI; this finding is of interest, because the pharmacological strategy for neuroprotection principally targets penumbral tissue, in which there remain potentially viable cells.

Conclusion

In summary, our results demonstrate an involvement of the opioidergic system in focal ischemic processes. The rapid decrease in δ-binding sites indicates that this receptor subtype is particularly susceptible in the acute phase of cerebral ischemia, and later on, μ- and all other opioidergic receptors are decreased in chronically infarcted tissues. The selective alteration in δ-receptors argue for the in vivo use of specific δ-receptor ligands as markers of early neuronal death in opposition to nonselective ligands, such as diprenorphine, which are less-sensitive markers of the infarct extension. The relative preservation of k-binding densities might be correlated with the neuroprotective effect of k-agonists, known to be effective even when the treatment is induced 6 hours after MCAO.7–9 Our present observation would thus further speak in favor of the use of k agents as neuroprotective agents.

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References


The use of opioid-based drugs as neuroprotectants has been of interest for many years. Of the various subclasses, the \( \kappa \)-receptor agonists appear to hold the most promise for cerebral ischemia. However, the window of opportunity during which opioid receptor subtype ligands can bind in cortex during focal ischemia has not been well defined.

In the accompanying article, Boutin et al performed a highly systematic investigation of the temporal sequence and topology of the loss of opioid receptor ligand binding during permanent focal ischemia in the mouse. Ligand binding for \( \mu \)-, \( \delta \)-, and \( \kappa \)-opioid receptors as well as a nonselective opioid ligand was quantified at 1, 3, 6, 12, 24, and 48 hours of permanent occlusion of the distal middle cerebral artery, which restricts injury to cerebral cortex. In general, significant reductions of \( \delta \)-ligand binding were detected at shorter ischemic durations than reductions of \( \mu \)-ligand binding, which in turn preceded reductions of \( \kappa \)-ligand binding. In the ischemic border region where infarction developed more slowly, the same temporal sequence of subtype ligand binding tended to be preserved, although the decrease in binding was delayed by several hours and the loss of \( \kappa \)-ligand binding was less prominent than that for the \( \delta \)-ligand. The authors conclude that \( \delta \)-ligand binding may be an early marker of neuronal injury and that neuroprotection shown by others with \( \kappa \)-agonists theoretically may have a longer therapeutic window.

It is speculated that the better preservation of \( \kappa \)-ligand binding may be related to an enrichment in presynaptic membranes. It is also possible that neurons expressing \( \kappa \)-receptors are less vulnerable to ischemia because of activation of these receptors during ischemia by endogenous agonists. Because exogenously administered agonists provide protection, further work is needed to discern whether neurons expressing \( \kappa \)-receptors are inherently less vulnerable to ischemia, or whether \( \kappa \)-agonists act by modulating neurotransmitter release and thereby protect postsynaptic neurons. In either case, the present findings that loss of \( \kappa \)-receptor is delayed 12 to 24 hours after the onset of ischemia is encouraging because it suggests a relatively long therapeutic window.

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