Stroke

L-Kynurenine/Aryl Hydrocarbon Receptor Pathway Mediates Brain Damage After Experimental Stroke

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Background—Aryl hydrocarbon receptor (AhR) is a transcription factor that belongs to the basic helix-loop-helix PAS (Per-Arnt-Sim homology domain) family known to mediate the toxic and carcinogenic effects of xenobiotics. Interestingly, AhR is widely expressed in the central nervous system, but its physiological and pathological roles are still unclear.

Methods and Results—To define the role of AhR in stroke, we used middle cerebral artery occlusion in mice and oxygen-glucose deprivation in rat cortical neurons. The results presented here show that the ischemic insult increases total and nuclear AhR levels and AhR transcriptional activity in neurons in vivo and in vitro. We also show that AhR has a causal role in acute ischemic damage because pharmacological or genetic loss-of-function approaches result in neuroprotection. Inhibition of cAMP response element-binding protein–dependent signaling may participate in the deleterious actions of AhR. Finally, we have also found that L-kynurenine, a tryptophan metabolite with AhR agonistic properties, is an endogenous ligand that mediates AhR activation in the brain after middle cerebral artery occlusion.

Conclusions—Our data demonstrate that an L-kynurenine/AhR pathway mediates acute brain damage after stroke and open new possibilities for the diagnosis and treatment of this pathology. (Circulation. 2014;130:2040-2051.)

Key Words: L-kynurenine ■ aryl hydrocarbon receptor ■ stroke

Ischemic stroke, which results from cerebral arterial occlusion, is becoming a major cause of morbidity and mortality in today’s society and affects millions of people every year. Currently, the only approved treatment for the acute phase of stroke is the recombinant thrombolytic tissue-type plasminogen activator.1 Identifying molecules that contribute to the ischemic damage may help to elucidate potential therapeutic targets.

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The aryl hydrocarbon receptor (AhR) or dioxin receptor is a ligand-activated transcription factor that belongs to the basic helix-loop-helix/Per-Arnt-Sim of highly conserved proteins.2 One of its main functions is to participate in the metabolism of xenobiotics from environmental pollutants such as halogenated and polycyclic aromatic hydrocarbons and polychlorinated biphenyls.3 On AhR activation by xenobiotics, AhR translocates into the nucleus and dimerizes with the AhR nuclear translocator (ARNT); the heterodimer then binds to dioxin-responsive elements located upstream of target genes such as Cyp1a1, Cyp1b1, and AhRR, leading to a wide variety of toxic responses, including severe thymic involution, wasting syndrome, chloracne, immune suppression, inflammation, reduced fertility, hepatotoxicity, tumor promotion, and death.2,4 Furthermore, AhR is postulated to play important roles in normal cell physiology and function, as demonstrated by the phenotype observed in the AhR knockout mice5 and by different studies that link AhR activation by endogenous or naturally occurring ligands to vascular and cardiac homeostasis,6 immune system function7 and tumor development.8 Although AhR and its target genes are widely expressed in different brain regions, including cerebellum, ventrolateral medulla, hippocampus, and cortex,9-11 little is known about its role in brain physiology and pathology. Of note, AhR inhibition has been shown to protect against N-methyl-d-aspartate excitotoxicity12 and to decrease apoptosis in cortical neurons,13 suggesting its potential involvement in neurological insults such as stroke.

In this study, we investigated the role of AhR in acute ischemic brain injury by using in vivo and in vitro models of stroke induced either by middle cerebral artery occlusion (MCAO) in mouse or by exposure of cultured rat cortical neurons to oxygen-glucose deprivation (OGD), respectively. Our data show that AhR activation by its endogenous ligand L-kynurenine (L-Kyn) has a deleterious role in cerebral
ischemia and identify the L-Kyn/AhR pathway as a potential therapeutic target for stroke.

Methods

Reagents

The AhR antagonists 6,2'-4'-trimethoxyflavone14 (TMF) and CH2339115 (CH), the putative AhR agonist L-Kyn,4,16–18 and the specific tryptophan 2,3-dioxygenase (TDO) inhibitor 680C91 were from Tocris Bioscience. The AhR agonist benzo[a]pyrene and 1-methyl-d-tryptophan, a specific indoleamine-2,3-dioxygenase (IDO) inhibitor, were from Sigma. The AhR agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was from Accustandard.

Animals

Experiments were performed in male C57Bl/6 mice at 10 to 12 weeks of age obtained from The Jackson Laboratories and in wild-type (WT) control (C57Bl/6) and AhR−/− heterozygous mice (C57Bl/6) described elsewhere.19 WT and AhR−/− knockout mice (C57Bl/6) were obtained from Taconic. Animals were kept in a room with controlled temperature and a 12-hour dark/light cycle and fed with standard food and water ad libitum. All experimental protocols adhered to the regulations of the Animal Welfare Committee of the Universidad Complutense (following EU directives 86/609/CEE and 2003/65/CE).

In Vivo Experimental Groups

All experiments were performed and quantified in a randomized fashion by investigators blinded to specific treatments. Mice were subjected to a distal permanent or transient MCAO by ligation (online-only Data Supplement). To evaluate the contribution of AhR, several groups were used for the determination of infarct outcome in mice 24 to 48 hours after ischemia. AhR+/− and AhR−/− mice and their WT littermates were subjected to MCAO. Furthermore, C57Bl/6 mice received an intraperitoneal administration of either vehicle (dimethyl sulfoxide), the AhR antagonist TMF (5 mg/kg), or the AhR antagonist CH (10 mg/kg). Another group received either vehicle or the AhR agonist benzo[a]pyrene (40 mg/kg). In another set of experiments, C57Bl/6 mice were treated with either vehicle or TMF±10 mg/kg L-Kyn. To study the AhR-dependent effects of TMF, benzo[a]pyrene, and L-Kyn, AhR−/− mice were treated with these compounds. Finally, mice were treated with vehicle, a TDO inhibitor (680C91; 10 mg/kg), or an IDO inhibitor (1-methyl-d-tryptophan; 10 mg/kg). In several experiments, coadministration of the TDO inhibitor (10 mg/kg) plus L-Kyn (10 mg/kg) was also performed. All treatments were administered 10 minutes after MCAO except for the TDO and IDO inhibitors, which were administered 10 minutes and 8 hours after ischemia, after preliminary experiments in which we failed to find a significant effect when these inhibitors were administered only 10 minutes after ischemia.

Infarct Volume Measurement and Assessment of Functional Deficits

Twenty-four and 48 hours after MCAO, infarct outcome was assessed by 2,3,5-triphenyltetrazolium chloride (TTC) staining or magnetic resonance imaging. Functional deficits were graded by use of the grip and a 12-hour dark/light cycle and fed with standard food and water ad libitum. All experimental protocols adhered to the regulations of the Animal Welfare Committee of the Universidad Complutense (following EU directives 86/609/CEE and 2003/65/CE).

In Vitro Experimental Groups

OGD in rat cortical neurons was performed as described in the online-only Data Supplement. In some experiments, TMF (3–10 μmol/L) in the absence or presence of L-Kyn (50 μmol/L) was included 24 hours before, during, and after OGD. In another set of experiments, TMF (10 μmol/L) with or without L-Kyn (50 μmol/L) was included only during and after OGD. In another group, neurons were treated with the TDO inhibitor 680C91 (10 μmol/L) after OGD. In several experiments, TCDD (20 nmol/L) was used as a positive control of AhR activation. Medium and cells were collected 24 hours after OGD for viability assessment. Cells were collected at 0.5, 1, 3, 5, 8, and 24 hours after OGD for mRNA and protein assays, nuclear/cytoplasmic extracts, and immunocytochemistry.

Measurement of Cell Viability After OGD

Lactate dehydrogenase release from damaged cells and morphological analysis were used to determine neuronal cell death after OGD (online-only Data Supplement).

Statistical Analysis

Results are expressed as mean±SEM for the indicated number of experiments. Data were tested for homoscedasticity with the Bartlett test. Statistical significance was determined by use of a nonparametric, 2-tailed Mann-Whitney t test; a nonparametric, 1-way Kruskal-Wallis ANOVA test followed by a Dunn post hoc testing; or a nonparametric, 2-way ANOVA followed by Bonferroni post hoc testing. Correlation analysis was performed by use of a nonparametric Spearman correlation, and a linear regression of the data is displayed. Values of P<0.05 were considered statistically significant. In each figure, the mean value of every group labeled with a specific symbol is significantly different (P<0.05) from the mean value of the reference group, which is indicated in each case. All statistical analyses were performed with Prism version 5.0 (GraphPad Software, Inc).

Results

AhR Expression Is Increased in the Postischemic Brain

Western blot analysis showed low levels of AhR expression in sham-operated mice (n=4; P>0.05; Figure 1A). After ischemia, AhR levels increased in the peri-infarct and core regions. In the peri-infarct, AhR upregulation was observed at 18 hours, peaked at 24 to 48 hours, and started to decrease at 72 hours. In the core, AhR expression reached a plateau at 5 hours and returned to baseline at 7 days. AhR immunostaining showed similar results (n=3–4; Figure 1B). AhR nuclear translocator, the heterodimer partner of AhR, was also increased in both peri-infarct and core regions (Figure IA and IB in the online-only Data Supplement).

To determine the cell type responsible for the increased AhR expression after ischemia, we used immunofluorescence staining and confocal analysis 24 hours after MCAO (n=3–4; Figure 1C). Sham animals showed some diffuse AhR immunoreactivity in neurons at the ipsilateral and contralateral sides. Exposure to ischemia increased AhR expression mainly in neurons (AhR and NeuN colocalization) in the peri-infarct area but also in the core at different time points after MCAO (II/III layers of neocortex; Figure 1C and Figure II in the online-only Data Supplement). AhR immunoreactivity was rarely observed in astrocytes (glial fibrillary acidic protein positive; Figure 1C) and was not detectable in microglial cells (Iba-1 positive; Figure 1C).

Exposure to MCAO Induces AhR Nuclear Translocation and Transcriptional Activity in Mouse Brain

Because the activity of AhR as a transcription factor is regulated by shuttling from the cytoplasm to the nucleus,20 MCAO-induced changes in AhR subcellular location were assessed by protein analysis in nuclear/cytoplasmic fractions and immunofluorescence. Western blot analysis demonstrated an initial 2-fold increase in AhR nuclear/cytoplasmic ratio followed by
a second 2.4-fold increase, at 1 and 18 hours after ischemia, respectively, in brains of MCAO-exposed animals compared with the sham group, returning to basal levels after 7 days (n=5; \( P < 0.05 \); Figure 1D). AhR immunoreactivity showed a diffuse cytoplasmic and nuclear pattern in sham mice (Figure 1E); in contrast, 18 hours after MCAO, nuclear translocation of AhR in neurons in peri-infarct and core regions became evident (n=4; \( P < 0.05 \); Figure 1E). At this time, AhR nuclear translocator was also expressed in neurons (Figure 1B in the online-only Data Supplement), allowing AhR transcriptional activity. Indeed, analysis by reverse transcriptase–polymerase chain reaction confirmed the induction of the AhR target genes \( Cyp1a1 \) and \( AhRR \) 18 hours after MCAO compared with the sham group (n=5; \( P < 0.05 \); Figure 1F). These data indicate that in vivo cerebral ischemia induces AhR nuclear translocation and transcriptional activity.

**AhR Participates in Acute Ischemic Damage After MCAO in Mice**

We next examined whether AhR participates in ischemic brain injury. First, we used a pharmacological approach to inhibit or to activate AhR. Mice were exposed to MCAO and, 10 minutes later, were treated with vehicle or the AhR antagonists TMF or CH (Figure 2A–2E). Administration of TMF (5 mg/kg) dramatically decreased the infarct size after permanent (MCAO; n=10–11; \( P < 0.05 \); Figure 2B) and transient ischemia (transient MCAO; Figure IIIB in the online-only Data Supplement),...
determined either by magnetic resonance imaging at 24 hours or by TTC staining at 48 hours. Animals treated with TMF also presented a lower modified Neurological Severity Score 48 hours after MCAO (Figure IIIA in the online-only Data Supplement). This improvement was also seen with another AhR antagonist, CH (10 mg/kg; n=7; P<0.05; Figure 2C).

Finally, AhR activation by the prototypical AhR agonist benzo[a]pyrene increased infarct volume compared with the vehicle group (40 mg/kg; n=7; P<0.05; Figure 2D); therefore, AhR inhibition or activation results in neuroprotection or potentiation, respectively, of brain damage after stroke.

To confirm these results, WT, AhR+/−, and AhR−/− mice were used (Figure 2E and Figure IVA–IVC in the online-only Data Supplement). First, haploinsufficiency in AhR+/− mice, in which the expression levels of AhR and the AhR target gene AhRR after MCAO were lower than in the WT group (n=5; P<0.05), was also neuroprotective, showing a reduction in infarct volume (n=7–9; P<0.05; Figure IVD in the online-only Data Supplement and Figure IVF in the online-only Data Supplement) compared with their WT littermates. Complete loss of AhR in AhR−/− mice also resulted in neuroprotection after MCAO (n=7–11; P<0.05; Figure 2E) compared with the AhR+/+ group. Finally, AhR−/− mice were treated with either TMF or benzo[a]pyrene after MCAO to demonstrate their AhR-dependent effects. Two-way ANOVA demonstrated a significant effect of AhR genotype (P<0.05), treatment (P<0.05), and AhR genotype–by–treatment interaction (P<0.05). Whereas treatment of AhR+/+ animals with the AhR antagonist TMF or the AhR agonist benzo[a]...
pyrrole caused a reduction or an increase in the infarct volume, respectively (n=7–11; P<0.05 versus AhR+/− vehicle; Figure 2E), these effects were lost in AhR−/− mice (n=7–11; P>0.05 versus AhR+/− vehicle; Figure 2E), demonstrating that the neuroprotective or harmful actions of TMF and benzo[a] pyrrole, respectively, are entirely AhR dependent. Together, these results demonstrate a detrimental role of AhR in the acute phase of ischemic injury in vivo.

**AhR Regulates cAMP Response Element-Binding Protein Survival/Death Signaling After Ischemia: Effect of AhR Pharmacological Modulation**

AhR has been implicated in numerous physiological and pathological processes.† The absence of changes in the inflammatory milieu after AhR inhibition (Figure V in the online-only Data Supplement) and the specific neuronal expression of AhR in our in vivo model suggest that AhR exerts its main functions in neurons after cerebral ischemia. Indeed, our findings in rat cortical neurons exposed to OGD (Figure VI in the online-only Data Supplement) corroborate the results observed in vivo, inasmuch as AhR expression and activation were increased after OGD in cortical rat neurons and AhR inhibition protected neurons against ischemic damage.

cAMP response element (CRE)–binding protein (CREB) is one of the most important neuronal transcription factors known by its implication in the expression of survival and antiapoptotic genes such as brain-derived neurotrophic factor (BDNF) on binding to CRE.21,22 Previous studies suggested that AhR might impair CREB-mediated neuronal BDNF gene expression.23 Therefore, we explored BDNF expression after MCAO or AhR inhibition. The AhR antagonist TMF increased cortical BDNF levels in sham-operated mice (n=6; P<0.05; Figure 3A) 24 hours after surgery. After MCAO, BDNF levels increased, showing a positive correlation with lesion size (n=5; Spearman r=0.7062, P=0.0182; Figure 3B). As a result, BDNF values were normalized for infarct volumes, and thus, we found that animals treated with TMF had higher normalized BDNF levels compared with the vehicle group (n=5; P<0.05; Figure 3B), suggesting an inhibitory effect of AhR on CREB-mediated BDNF expression.

Because CREB signaling leads to inhibition of apoptotic cascades,23 we reasoned that AhR could modulate proapoptotic and antiapoptotic proteins after MCAO. Indeed, the AhR antagonist TMF reduced proapoptotic proteins p53 and Puma and increased the antiapoptotic Bcl-X (n=5–6; P<0.05; Figure 3C–3E). No changes were observed in Bax, Bad, and Bim (data not shown). Furthermore, cleaved caspase 3 and terminal deoxynucleotidyl transferase dUTP nick-end labeling staining showed a decreased number of apoptotic cells in the peri-infarct region of MCAO animals treated with TMF compared with the vehicle group (n=3; Figure 3F and 3G).

Thus, our data suggest that AhR activation after cerebral ischemia inhibits CREB signaling and subsequent CREB–induced prosurvival pathways. Of note, CREB and phosphorylated CREB (ser133) levels were increased in vivo and in vitro after administration of the AhR antagonist TMF (n=5–6; P<0.05; Figure 3H and Figure VIIA in the online-only Data Supplement). Accordingly, nucleofection of cortical neurons with a CRE-responsive luciferase construct demonstrated that AhR inhibition by TMF increases CREB transcriptional activity in control and OGD neurons (n=12; P<0.05; Figure 3I). The increase in mRNA levels of Bdnf and Npas4, another CREB target gene, in TMF–treated cortical neurons exposed to OGD (Figure VIIIB in the online-only Data Supplement) further supports that AhR inhibits CREB transcriptional activity.

Finally, AhR coimmunoprecipitation after in vivo MCAO (n=3–4; P<0.05; Figure 3J) or in vitro OGD (n=3–4; P<0.05; Figure VIIIC in the online-only Data Supplement) showed an increased interaction of AhR with CREB-binding protein (CBP) and with CREB, suggesting that the AhR inhibitory effect on CREB signaling could be due, at least in part, to a direct interaction with the CREB/CBP complex. Consistently, AhR inhibition by TMF significantly reduced receptor association to both CBP and CREB.

**L-Kyn Is an Endogenous AhR Ligand in Cerebral Ischemia**

A variety of endogenous ligands have been demonstrated to activate AhR in different models.9 Our findings strongly suggest that AhR is activated by an endogenous agonist generated by cerebral ischemic damage. L-Kyn, a key metabolite of the kynurenine pathway (Figure 4A), has recently been identified as an in vivo AhR agonist.8 Because this pathway is activated after stroke,24 we explored whether L-Kyn could account for endogenous AhR activation in cerebral ischemia.

First, levels of L-Kyn and its precursor, L-tryptophan (L-Trp), were determined in the ipsilateral cortex and in plasma (Figure 4B–4E). Two-way ANOVA showed a significant effect of surgery (P<0.05), time (P<0.05), and surgery–by-time interaction (P<0.05) in brain L-Kyn and L-Trp levels but not in plasma. Brain L-Kyn levels increased as early as 3 hours after MCAO and remained elevated 24 hours after (n=7–10; P<0.05; Figure 4B). This increase was associated with an initial increase and a subsequent reduction in L-Trp levels from 3 to 24 hours after MCAO (n=7–10; P<0.05; Figure 4C). After MCAO, no changes were observed in plasma L-Kyn and L-Trp levels (n=7–14; Figure 4D and 4E).

The specific increase in L-Kyn after MCAO in brain but not in plasma made us reason that local L-Kyn biosynthesis could be induced after ischemia. Consequently, we tested brain mRNA expression of Ido and Tdo, the 2 key enzymes in the initial step of the kynurenine pathway (Figure 4A).25 Whereas no changes were detected in Ido1 and Ido2 mRNA levels (n=5; Figure 4F), ischemia increased the expression of Tdo compared with sham animals (n=5; P<0.05; Figure 4F). This result was confirmed by immunofluorescence (n=3; Figure 4G): Whereas sham animals presented low TDO levels in the ipsilateral cortex, MCAO increased TDO expression in the core and peri-infarct regions 5 and 24 hours after the occlusion. Colocalization experiments demonstrated that TDO is located mainly in neurons and by some astrocytes (n=3; Figure 4H). Ischemia-induced TDO upregulation indicates that, in addition to the augmented peripheral L-Kyn synthesis after stroke,26 local L-Kyn production is increased and is expected to contribute to brain L-Kyn levels after MCAO.

Hence, L-Kyn could be the endogenous AhR agonist in this setting. Thus, we decided to examine the effects of L-Kyn in cultured cortical neurons. First, L-Kyn activated AhR
in neurons 0.5 to 1 hour after the treatment as shown by an increased nuclear accumulation of AhR, comparable to that induced by TCDD (Figure VIII A and VIII B in the online-only Data Supplement). Moreover, L-Kyn increased the expression of the AhR target genes Cyp1a1 and Cyp1b1 mRNA in cortical neurons, an effect blocked by TMF (Figure VIII C and VIII D in the online-only Data Supplement). Finally, L-Kyn decreased CRE-mediated transcription in neurons, demonstrated by a reduction in both Bdnf and Npas4 mRNA expression and in the CRE-luciferase reporter assay (Figure IXA and IX B in the online-only Data Supplement). Together, these data indicate that L-Kyn is a specific AhR agonist in neurons that...
could mediate AhR-dependent inhibition of CREB signaling after experimental stroke.

**L-Kyn Plays a Deleterious Role in Cerebral Ischemia Through AhR Activation**

The neuroprotection achieved by AhR inhibition in in vivo and in vitro ischemia implies a deleterious role of L-Kyn through AhR activation. Indeed, L-Kyn aggravated OGD-induced neuronal damage, assessed by morphological analysis and lactate dehydrogenase release determination (n=24; P<0.05; Figure 5A and 5B), an effect reversed by TMF, indicating that L-Kyn–induced deleterious effects after OGD depend on AhR activation. In vivo, intraperitoneal administration of L-Kyn (10 mg/kg) after MCAO increased brain and plasma

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**Figure 4.** L-kynurenine (L-Kyn) levels are increased in the ischemic brain. A, Schematic diagram of L-Kyn synthesis and metabolism through the kynurenine pathway. Cerebral (B and C) and plasma (D and E) L-Kyn and L-tryptophan (L-Trp) levels in sham and middle cerebral artery occlusion (MCAO) animals 3, 16, and 24 hours after surgery (n=7 for brain samples and n=14 for plasma samples). F, Expression of indoleamine-2,3-dioxygenase (IDO) 1, IDO2, and tryptophan 2,3-dioxygenase (TDO) mRNA in the ipsilateral cortex of sham and MCAO animals 5 hours after surgery; β-actin levels were used for normalization (n=5). G, Representative immunofluorescence images of TDO (green) in the cortex of sham and MCAO animals 5 and 24 hours after ligation. White arrows indicate TDO immunoreactive cells (n=4 for each group except for sham group, which was n=3; scale bar=50 μm). The nuclear marker TOPRO is in blue. H, Colocalization analysis of TDO (green) with the neuronal marker NeuN, the astrocytic marker glial fibrillary acidic protein (GFAP), or the microglial marker Iba1 in the brain of MCAO mice 24 hours after ligation (n=4 for each group except for sham group, which was n=3; scale bar, 20 μm). Data were compared by using a nonparametric 2-way ANOVA followed by Bonferroni post hoc testing (B–E, *P<0.05 vs t=0 hours, #P<0.05 vs sham) or a nonparametric 2-tailed Mann-Whitney test (F, *P<0.05 vs sham). Two-way ANOVA shows a significant effect of surgery \[F(3, 65)=26.20; P<0.0001\], time \[F(3, 65)=4.32; P=0.0077\], and surgery-by-time interaction \[F(3, 65)=4.86; P<0.001\] in B and a significant effect of surgery \[F(3, 78)=38.28; P<0.0001\], time \[F(3, 78)=14.09; P<0.0001\], and surgery-by-time interaction \[F(3, 78)=13.73; P<0.0001\] in C.
L-Kyn levels (n=5–10; P<0.05; Figure 5C and 5D) concomitantly with an increase in infarct volume (n=7–10; P<0.05; Figure 5E and 5F) compared with the vehicle group, an effect not observed at a lower dose of L-Kyn (5 mg/kg; n=7; P>0.05; Figure 5E). Moreover, the L-Kyn–induced increase in infarct volume was AhR dependent; it was absent after coadministration of the AhR antagonist TMF or in AhR−/− mice (n=7; P<0.05; Figure 5E and 5F).

Having demonstrated that exogenous L-Kyn has an AhR-dependent, detrimental role after ischemia, we finally explored whether inhibition of L-Kyn biosynthesis was neuroprotective in this setting. The TDO inhibitor 680C91 reduced infarct volume compared with the vehicle-treated group (n=10; P<0.05; Figure 6A). However, no differences were observed between vehicle and the IDO inhibitor 1-methyl-d-tryptophan (n=7; Figure 6B), supporting that brain TDO activity accounts for L-Kyn biosynthesis after stroke. Coadministration of L-Kyn abolished the neuroprotective actions of the TDO inhibitor (n=7; P<0.05; Figure 6C). In addition, TDO inhibition after MCAO abolished the postischemic brain L-Kyn augmentation while producing an accumulation of L-Trp (n=5–7; P<0.05; Figure 6D and 6E), reinforcing that neuroprotective actions of TDO inhibition are mediated by a reduction in L-Kyn synthesis after stroke.

![Figure 5. Deleterious, aryl hydrocarbon receptor (AhR)-dependent effect of L-kynurenine (L-Kyn) in in vitro and in vivo models of cerebral ischemia. A and B, AhR-dependent effect of L-Kyn (50 μmol/L) on neuronal viability after oxygen-glucose deprivation (OGD), determined by morphological analysis (A) and lactate dehydrogenase (LDH) release (B), in neurons treated with vehicle, 6,2′,4′-trimethoxyflavone (TMF; 10 μmol/L), L-Kyn, and TMF+L-Kyn. Data in B are expressed as percent of vehicle-treated neurons (n=48; 3 independent experiments). C and D, Cerebral (C) and plasma (D) L-Kyn and L-tryptophan (L-Trp) levels determined by high-performance liquid chromatography in naïve, sham, and middle cerebral artery occlusion (MCAO) animals treated with vehicle or L-Kyn (10 mg/kg) at 3 and 16 hours after surgery (n=5 for brain samples, n=10 for plasma samples). E, Effect of in vivo L-Kyn administration (5–10 mg/kg) on infarct volume and its dependence on AhR, determined 48 hours after MCAO; right: representative brain sections (n=11 for each group except for AhR−/− vehicle group; n=7). Data were compared by using a nonparametric, 1-way Kruskal-Wallis ANOVA with Dunn post hoc testing (B, *P<0.05 vs vehicle, #P<0.05 vs L-Kyn; C and D, *P<0.05 vs naive, #P<0.05 vs sham, $P<0.05 vs MCAO vehicle at 3 hours; E, *P<0.05 vs vehicle, $P<0.05 vs L-Kyn 10 mg/kg, #P<0.05 vs L-Kyn 5 mg/kg) or a nonparametric 2-way ANOVA followed by Bonferroni post hoc testing (F, *P<0.05 vs AhR−/− vehicle, #P<0.05 vs AhR−/− L-Kyn 10 mg/kg). Two-way ANOVA showed a significant effect of AhR genotype [F(1,28)=41.50, P<0.0001], treatment [F(1, 28)=16.42, P<0.0004], and AhR genotype–by–treatment interaction [F(1, 28)=5.13, P=0.0314]. HV indicates hemisphere volume.](http://circ.ahajournals.org/doi/abs/10.1161/CIRCRESAHA.117.313192)
Finally, we investigated whether TDO inhibition, by diminishing L-Kyn levels, might decrease AhR activation. Indeed, the TDO inhibitor decreased AhR activation after in vivo (n=7; P<0.05; Figure 6F) and in vitro (n=5; P<0.05; Figure 6G–6I) cerebral ischemia. Together, our results demonstrate that inhibition of TDO-mediated L-Kyn biosynthesis decreases AhR activation and its subsequent deleterious effects after experimental stroke.

Discussion

AhR or dioxin receptor is a transcription factor with important roles in xenobiotic-induced toxicity and carcinogenesis but also in some physiological functions such as reproduction, organ homeostasis, and adaptive immunity.27–29 Now, the results presented here show that cerebral ischemia induces AhR overexpression and activation in neurons, which participate in ischemic brain damage concomitantly with the downregulation of CREB prosurvival pathways. We have also found that cerebral ischemia induces the synthesis of L-Kyn, an L-Trp metabolite with AhR agonistic properties that may account for the endogenous activation of AhR in this setting. These data are the first to demonstrate a pathophysiological role of this receptor in the context of stroke, a high-incidence cerebrovascular pathology with limited therapeutic treatment.

We have found that AhR is overexpressed in mouse brain after experimental stroke, mainly in neurons located at the peri-infarct but also at the ischemic core. Likewise, AhR expression is found in cultured rat cortical neurons, as previously reported,12,13,27 and increases after in vitro experimental ischemia. To the best of our knowledge, this is the first evidence showing that AhR signaling pathways are induced in neurons after a deleterious stimulus such as cerebral ischemia. The early profiles of AhR activation and transcriptional activity strongly point to a damaging role of this receptor in stroke physiopathology.

Indeed, using pharmacological and genetic loss-of-function approaches, we have demonstrated the detrimental role of AhR in the ischemic brain. After in vivo experimental stroke by permanent MCAO, 2 different AhR antagonists, TMF14 and CH₃,15 were neuroprotective. The antagonism of AhR (with TMF) was also beneficial when ischemia was followed by recanalization (transient MCAO). Conversely, AhR activation by the prototypical AhR agonist benzo[a]pyrene aggravated stroke outcome. In addition, either AhR haploinsufficiency or total deletion decreased ischemic damage and improved neurological scores. The ligands used failed to affect stroke outcome in AhR−/− mice, showing that their actions are AhR dependent. The deleterious effects of in vivo AhR activation were confirmed in cortical neurons exposed to OGD. All these data together demonstrate that AhR participates in acute cerebral damage after ischemic stroke and that specific antagonists of this receptor such as TMF may be useful for inhibiting the detrimental actions of this receptor after cerebral ischemia.

Several studies have demonstrated that AhR inhibition reduces neuronal apoptosis27,28 and neurotoxicity concomitant with an increase in BDNF.12,13 In this context, CREB is a critical transcription factor against ischemic injury because of its target genes that include the neuroprotective neurotrophin BDNF and the so-called activity-regulated inhibitor of death genes or by antagonizing apoptotic death signals.29–31 Indeed, our results show that the AhR antagonist TMF increased normalized BDNF levels and decreased apoptosis by affecting apoptosis-related genes after MCAO. In vitro studies confirmed that AhR inhibits CREB transcriptional activity, as revealed by an upregulation of BDNF and NPAS4 and by an increase in CRE-responsive luciferase activity in cultured neurons exposed to OGD and AhR inhibition. These data strongly suggest that inhibition of CREB signaling participates in the detrimental role of AhR after stroke. Because in vivo and in vitro coinmunoprecipitation experiments of AhR demonstrate its direct interaction with CBP and CREB, our results support that cerebral ischemia increases AhR recruitment to CBP/CREB complexes playing an inhibitory role in CREB survival genes.

Supporting that AhR is an important modulator of physiological functions, several endogenous AhR ligands have been described. Our study demonstrates that one of them, L-Kyn,8,16–18 an L-Trp derivative through the so-called kynurenine pathway, is a key AhR ligand in neurons. Interestingly, in both animals and humans, the kynurenine pathway is increased after stroke and is associated with stroke severity and poor prognosis.24,32 Consistently, we found that brain L-Kyn levels increased as early as 3 hours and remained elevated 24 hours after MCAO, coinciding with the maximal nuclear AhR expression. In vitro, L-Kyn induced AhR nuclear translocation and upregulation of AhR target genes in a manner similar to the classic AhR agonist TCDD, effects that were inhibited by the AhR antagonist TMF or in AhR−/− mice. All these results support that, in the ischemic setting, L-Kyn is the endogenous AhR ligand responsible for neuronal AhR activation.

In normal brain, 60% of L-Kyn is taken up from blood,33 and 40% is generated locally from L-Trp, which is transported across the blood–brain barrier. The initial step of the brain kynurenine pathway is carried out mostly by 2 key enzymes, IDO and TDO, which metabolize L-Trp into different metabolites, including L-Kyn. TDO is expressed in mainly liver but is also present in brain,34–36 suggesting a role in brain function. Supporting this, we demonstrate that TDO, but not IDO, is upregulated predominantly in neurons of the ipsilateral cortex of the ischemic brain. In addition, L-Trp, an amino acid that regulates TDO function,37 is significantly increased in brain after ischemia. All these data point toward L-Kyn synthesis by TDO as the main pathway for the augmented L-Kyn concentration in the ischemic brain. Importantly, TDO inhibition abolished, at early times, both the L-Kyn increase and AhR nuclear translocation in the ischemic brain, as well as AhR activation and transcriptional activity after OGD in cultured neurons, strongly suggesting that, in vivo, AhR is activated by L-Kyn very rapidly after the ischemic occlusion and is thus causally involved in brain damage at those crucial times at which lesion is expanding after the arrest of the blood flow in the affected area. We cannot disregard that some cerebral L-Kyn may derive from the periphery because large amounts of L-Kyn are produced in the liver through L-Trp degradation by TDO.38 In addition, local synthesis of L-Kyn by microglia, astrocytes, or macrophages in the ischemic brain could also occur in an inflammatory microenvironment.39 Of note, both AhR and TDO are coexpressed by neurons placed in the infarct border (data not shown), suggesting both a paracrine and an autocrine model for AhR activation by L-Kyn.
Figure 6. The inhibition of endogenous L-kynurenine (L-Kyn) biosynthesis is neuroprotective concomitantly with reduced aryl hydrocarbon receptor (AhR) activation. **A** through **C**, Infarct volume after administration of the tryptophan 2,3-dioxygenase (TDO) inhibitor 680C91 (10 mg/kg; n=10; **A**), the indoleamine-2,3-dioxygenase (IDO) inhibitor 1-methyl-d-tryptophan (10 mg/kg; n=6; **B**), and the TDO inhibitor+L-Kyn (10 mg/kg; n=10 for each group except for vehicle group, which was n=6; **C**). Infarct volume was determined 48 hours after middle cerebral artery occlusion (MCAO) by 2,3,5-triphenyltetrazolium chloride (TTC) staining. Data are represented as percent of total infarcted hemisphere or areas along the anterior-posterior axis. Representative brain sections from each group are shown. **D** and **E**, Cerebral (**D**) and plasma (**E**) L-Kyn and L-tryptophan (L-Trp) levels determined by high-performance liquid chromatography in sham and MCAO animals treated with vehicle or the TDO inhibitor 680C91 (10 mg/kg) at 3 and 16 hours after ligation (n=5 for brain samples, n=10 for plasma samples). **F**, AhR Western blots of cytoplasmic and nuclear fractions of MCAO treated with vehicle or the TDO inhibitor 680C91 (10 mg/kg) 4 and 16 hours after occlusion. **G**, AhR nuclear translocation (green) in oxygen-glucose deprivation (OGD)–exposed neurons after 24 hours of treatment with vehicle or the TDO inhibitor 680C91 (10 μmol/L). TOPRO was used as nuclear marker (blue). Scale bar, 20 μm. **H**, Nuclear mean intensity (Int) of AhR in OGD neurons after 24 hours of vehicle or TDO inhibitor treatment, represented as in Figure VIII in the online-only Data Supplement (n=50 cells; 3 independent experiments). **I**, Cyp1b1 mRNA expression in OGD neurons after 24 hours of vehicle or TDO inhibitor treatment (n=5). Data were compared by using a nonparametric 2-tailed Mann–Whitney test (**A** and **B**, *P*<0.05 vs vehicle; **F**, $#P$<0.05 vs vehicle at 16 hours; **H** and **I**, *$P$*<0.05 vs vehicle) or a nonparametric, 1-way Kruskal–Wallis ANOVA followed by Dunn post hoc testing (**C**, *$P$*<0.05 vs vehicle, *$P$*<0.05 vs TDO inhibitor; **D** and **E**, *$P$*<0.05 vs naive, *#$P$*<0.05 vs sham, $#P$<0.05 vs MCAO vehicle).
Therefore, apart from AhR, we have identified TDO as an important therapeutic target because its inhibition drastically reduced brain ischemic damage concomitantly with a decrease in brain L-Kyn concentration and with an inhibition of AhR activation. From our data, we can conclude that a cascade of events, initiated by the early activation and overexpression of AhR and TDO-mediated L-Kyn biosynthesis followed by the subsequent downregulation of endogenous neuroprotective or antiapoptotic pathways, participates in brain damage (lesion size and neurological function) after ischemic stroke.

The kynurenine pathway is becoming recognized as a key player in the mechanisms of neuronal damage in several neurodegenerative disorders (for a review, see Vécezi et al40). L-Kyn has a central role on this pathway because its degradation by different enzymes generates neuroactive metabolites such as quinolinic acid and kynurenic acid, which show agonistic and antagonistic N-methyl-d-aspartate receptor properties, respectively. Accordingly, studies on the effect of L-Kyn administration before hypoxia/ischemia and reperfusion demonstrated a novel mechanistic insight for previously described deleterious actions of L-Kyn in the central nervous system. Here that they do not play a role in our setting. To the best of our knowledge, our present data are the first to prove that the neurotoxic actions of L-Kyn are AhR dependent, thus providing a novel mechanistic insight for previously described deleterious actions of L-Kyn in the central nervous system.

Conclusions

Our study links both L-Kyn and AhR signaling routes in ischemic brain injury and thus recognizes the L-Kyn/AhR pathway as a potential therapeutic target in this setting. Our findings also demonstrate that this pathway may be therapeutically targeted at different levels, L-Kyn biosynthesis by TDO and AhR activation, posing novel possibilities for the treatment of acute stroke. Furthermore, our results open new lines of investigation into the effects of the interference of this pathway at later time points or when the occluded blood vessel is recanalized by recombinant tissue-type plasminogen activator, as in clinical practice, or the consequences that polymorphisms of this receptor may have on stroke outcome. In addition, considering the well-described role of AhR in xenobiotic-induced toxicity and carcinogenesis, further studies are required to determine whether exposure to environmental contaminants such as dioxin could affect susceptibility to stroke damage in patients at risk or even the development of idiopathic neurodegenerative diseases.

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Disclosures

None.

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**CLINICAL PERSPECTIVE**

The aryl hydrocarbon receptor (AhR) or dioxin receptor is a ligand-activated transcription factor, classically defined as a transcriptional regulator involved in adaptive xenobiotic response, but with increasing importance in cellular physiology and tumor development. However, its actions in models of acute injury by circulatory pathologies such as stroke have not been illustrated. Therefore, we studied AhR expression and function in in vivo and in vitro experimental stroke. Here, we show for the first time that AhR is upregulated in the ischemic brain and plays a causal role in acute ischemic damage. Inhibition of cAMP response element-binding protein–dependent signaling may be involved in the deleterious actions of AhR. Moreover, we have also found that L-kynurenine, a tryptophan metabolite, is elevated in brain and plasma after experimental stroke and is an endogenous ligand that mediates AhR activation in the brain in this setting. In summary, our study recognizes the L-Kyn/AhR pathway as a potential therapeutic target in this setting, that may be therapeutically interfered with at 2 different levels, L-Kyn biosynthesis by tryptophan-2,3-dioxygenase and AhR activation, thus posing novel possibilities for the treatment of acute ischemic stroke. Furthermore, our results open new lines of investigation on the consequences that polymorphisms of this receptor may have on stroke outcome. In addition, considering the well-described role of AhR in xenobiotic-induced toxicity and carcinogenesis, further studies are required to determine whether exposure to environmental contaminants such as dioxin could affect susceptibility to stroke damage in patients at risk, or even the development of idiopathic neurodegenerative diseases.
L-Kynurenine/Aryl Hydrocarbon Receptor Pathway Mediates Brain Damage After Experimental Stroke
María I. Cuartero, Iván Ballesteros, Juan de la Parra, Andrew L. Harkin, Aine Abautret-Daly, Eoin Sherwin, Pedro Fernández-Salguero, Ángel L. Corbí, Ignacio Lizasoain and María A. Moro

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SUPPLEMENTARY INFORMATION

Supplementary Methods

Middle cerebral artery occlusion (MCAO) in mice

The surgical procedure was a variant of that described by Chen et al. (1986) and Liu et al. (1989). Mice were anesthetized with isoflurane 1.5-2% in a mixture of 80% air/20% oxygen, and body temperature was maintained at physiological levels with a heating pad during the surgical procedure and anesthesia recovery. Mice were subjected to permanent focal cerebral ischemia (MCAO) through the distal occlusion of middle cerebral artery (MCA) by ligature of the trunk just before its bifurcation between the frontal and parietal branches with a 9-0 suture, in combination with the occlusion of the ipsilateral common carotid artery. For transient MCAO (tMCAO), mice were reperfused 60 minutes after the occlusion. Mice in which the MCA was exposed but not occluded served as sham-operated controls (sham). Following surgery, individual animals were returned to their cages with free access to water and food. All the groups were performed and quantified in a randomized fashion by investigators blinded to treatment groups. Physiological parameters were not significantly different among the different groups studied.

Infarct volume measurement and assessment of functional deficits

Brain was removed and cut into 1-mm thick coronal slices and stained with 2,3,5-triphenyltetrazolium chloride (1% TTC in 0.1M phosphate buffer). Infarct size was determined as follows: infarct volumes were measured by sampling stained sections with a digital camera (Nikon Coolpix 990, Nikon Corporation, Tokyo, Japan) and the image of each section was analyzed using ImageJ 1.44I (NIH, Bethesda, MD, USA). The digitalized image was displayed on a video monitor. With the observer masked to the experimental conditions, the areas of the infarcted tissue (InfArea), the whole ipsilesional hemisphere (IpsArea) and the whole contralesional hemisphere (ContrArea) are delineated for each slice. Then, infarct volume, expressed as % of the
hemisphere that is infarcted (%IH), is calculated using the formula, as it has been previously described\(^3\):

\[
%IH = \frac{\text{InfVol}}{\text{ContrVol}} \times 100
\]

where

\[
\begin{align*}
\text{InfVol} &= \text{(Infarcted Tissue Volume)} = \sum \frac{\text{InfArea}}{\text{SwellingIndex}}, \\
\text{ContrVol} &= \text{(Contralesional Hemisphere Volume)} = \sum \frac{\text{ContrArea}}{\text{SwellingIndex}}, \\
\text{SwellingIndex} &= \frac{\text{IpsArea}}{\text{ContrArea}}.
\end{align*}
\]

In addition, infarct size was determined by magnetic resonance imaging 24 hours after MCAO using a BIOSPEC BMT 47/40 (Bruker, Ettlingen, Germany). Infarct volume was calculated using the ImageJ software (NIH, USA) from the T2-weighted images.

The functional deficits produced by the stroke were graded 48h after MCAO using the grip test, in which mice are placed on a suspended wire and latency to fall is measured, and the modified Neurological Severity Score (mNSS), as previously described\(^4\)-\(^5\). This neurological score evaluates motor symptoms (hemiparesis and gait), balance and reflexes.

**Primary cultures of rat cortical neurons**

Rat cortical neuronal cultures were performed as described\(^6\). Studies were performed at 14 days in vitro (DIV), time at which the cultures consisted of 94±6% neurons, as determined by flow cytometry\(^7\).

**Exposure of cortical neurons to oxygen-glucose deprivation (OGD)**

As an \textit{in vitro} cerebral ischemia model OGD followed by reoxygenation was used. This model is a commonly used \textit{in vitro} stroke model that recapitulates both permanent and transient cerebral ischemia as it produces both necrotic and apoptotic cell phenotype\(^7\)-\(^8\). Exposure of cortical neurons to oxygen-glucose deprivation (OGD) was performed as described previously\(^6\)-\(^8\)-\(^9\). Culture medium was replaced by a solution
containing (in mM): NaCl (130), KCl (5.4), CaCl₂ (1.8), NaHCO₃ (26), MgCl₂ (0.8), NaH₂PO₄ (1.18) and 2% horse serum (HS) bubbled with 95%N₂/5% CO₂, humidified at 37°C, and maintained at a constant pressure of 0.15 bars. Time of exposure to OGD was 150 minutes. OGD was terminated by replacing the exposure medium with oxygenated MEM containing 0.6% glucose, 0.029% glutamine, 50 IU/ml penicillin, 50µg/ml streptomycin, 2% HS (reperfusion medium) and returned to the normoxic incubator. Control cultures in a solution identical to OGD solution but containing glucose (33mM; control solution) were kept in the normoxic incubator for the same time period as the OGD, incubation solution was then replaced with reperfusion buffer and cultures were returned to the normoxic incubator until the end of the experiment.

**Measurement of cell viability after OGD**

As a marker of necrotic tissue damage LDH activity from damaged neurons was determined. Culture medium and 1% Triton X-100/PBS cell lysate were collected 24h after OGD. LDH activity was measured spectrophotometrically at 340 nm by following the oxidation of NADH (decrease in absorbance) in the presence of pyruvate¹⁰ using a Sunrise micro-plate reader (TECAN). LDH was measured in at least six different wells per experiment, and the experiment was repeated at least three times. LDH release to medium was calculated as percentage of total LDH (determined in cell lysate) and expressed as percentage of OGD-induced LDH. To correct background due to HS or phenol-red medium, an internal control (culture medium) was included. Basal LDH was 6±1% for control OGD neurons. Changes in the morphology of neurons treated with different drugs and exposed to OGD were observed using a phase-contrast microscope (Olympus). Neurons with fragmented neurites and vacuolated soma were considered non-viable, whereas neurons with intact neurites and a cell body that was smooth and round-to-oval in shape were considered viable.
**Quantitative RT-PCR**

Total RNA was extracted using TRIzol® reagent (Invitrogen, USA) from brain homogenates or cell cultures (n=5-7 per group). RNA (1 µg) was reverse-transcribed using the iScript cDNA Synthesis kit (BioRad, Germany) and quantitative real-time PCR was performed in triplicate using a BioRad iQ5 Thermocycler. The mRNA expression was normalized to that of actin and expressed as the fold difference relative to the controls. Specific primers were designed using Primer Express software and are indicated in Supplementary Table 1.

**Nucleofection and reporter gene assay**

A luciferase reporter plasmid with a multimerized CREB binding consensus sequence located upstream of a minimal promoter was used. The CRE luciferase construct serves as an indicator of CREB activity. Cultured primary cortical neurons of 7DIV were nucleofected using the AD1 Primary Cell 4D-Nucleofector Y kit (Lonza) with 6µg CRE luciferase plasmid, following manufacturer’s instructions. Seven days after nucleofection, cortical neurons were subjected to OGD and treated with either vehicle, the AhR antagonist TMF, the AhR agonist TCDD or L-Kyn (n=8; 3 different experiments). Six hours after reperfusion, luciferase quantification was performed with the Luciferase Assay System (Promega). Each sample was assayed in triplicate and data were presented as the % of control neurons treated with vehicle.

**TUNEL (Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling) Assay**

TUNEL assay was performed using In Situ Cell Death Detection Kit, TMR red (Roche) following manufacturer’s instructions.

**Nuclear extracts**

Brain tissue from C57Bl/6 mice collected from ipsilateral cortex after MCAO (n=4) or primary cortical cultures (n=5) were used for nuclei isolation. Nuclear extracts
were prepared crushing tissue or incubating the cell suspension of the culture in 10mM HEPES (pH 7.9), 1.5mM MgCl₂, 10mM KCl, and 0.5% Nonidet P40. Nuclei were pelleted at 12,000g for 1 min at 4°C and lysed in 20mM HEPES (pH 7.9), 15mM MgCl₂, 420mM NaCl and 0.2mM EDTA. After centrifugation (12,000g for 5 min at 4°C), the pellet was discarded and 50 µg of enriched nuclear protein supernatant were used for SDS-PAGE and western blot.

**SDS-PAGE and Western blot**

For determination of total protein levels, brain tissue was collected from the peri-infarct and infarct areas of mice (n=4-5) killed 5, 18, 24, 48 and 72h and 7days after MCAO. Samples were homogenized by sonication using lysis buffer: 10mM Tris–HCl pH 8.0, 420mM NaCl, 1mM EDTA and 0.5% NP-40 with protease inhibitor cocktail (Roche Diagnostics). Equal amounts of total protein (30µg) were resolved by SDS-PAGE and transferred to PVDF membranes (HybondTM-P; Amersham Biosciences Europe GmbH). The membranes were blocked with 5% nonfat milk in TBS-T (0.05% Tween 20 in TBS) and incubated with rabbit anti-AhR (Enzo Life Sciences, 1:1000), rabbit anti-ARNT (1:500, Thermo Scientific), mouse Anti-CREB (Santa Cruz Biotechnology; 1:500), mouse anti-pCREB (Cell Signaling; 1:1000), Rabbit Anti-CBP (Santa Cruz Biothecnology; 1:500), Rabbit Anti-p53 (Cell Signaling; 1:500), Rabbit Anti-Puma (Santa Cruz Biothecnology; 1:250) and Rabbit Anti-Bcl-x (Cell Signaling; 1:500). Anti mouse β-actin (Sigma; 1:10000) was included to ensure equal protein loading and Sp1 to determine the purity of cytoplasmic/nuclear extracts. Immunoreactive bands were visualized using the corresponding horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnology) and subsequent enhanced chemiluminescence detection (PerkinElmer Life and Analytical Sciences). The chemiluminiscent signal was acquired with a CCD camera (Syngene) and quantified with Gene Tools software (Syngene).
Analysis of L-tryptophan and L-kynurenine by HPLC

Sham and MCAO animals were sacrificed at 3, 16 and 24 after MCAO and brain and plasma were collected to determine L-tryptophan (L-Trp) and L-kynurenine (L-Kyn) levels. After brain extraction, core and peri-infarct areas from ischemic mice (or a similar region for sham and naive animals) were dissected out from the brains under a microscope, weighed and snap-frozen. Due to the sensitivity of the assay, for brain kynurenine determination, brain tissue from 2 animals per condition was pooled. Plasma was taken from each single animal used for brain tissue isolation. Therefore, each brain kynurenine determination corresponds to brain tissue from two animals and each plasma kynurenine determination corresponds to one single animal.

Cortex samples were added to a zinc acetate mobile phase (50nM glacial acetic acid, 100mM zinc acetate and 3% acetonitrile dissolved in double-distilled NANO pure water, pH 4.9) containing 6% perchloric acid (for protein precipitation) and 200ng/20µl internal standard (N-methyl-serotonin). The samples were then sonicated (approximately 10s), vortexed vigorously, and centrifuged at 4°C and 14,000 rpm for 20 minutes. The supernatants were then transferred into new eppendorf tubes, using a syringe fitted with a 0.45µm filter (Phenomenex). Serum samples were defrosted on ice and added to an equal volume of the mobile phase containing 6% perchloric acid and 200ng/20µl internal standard (N-methyl-serotonin). The samples were then vortexed vigorously, and centrifuged at 4°C and 14,000 rpm for 20 minutes. The supernatants were processed as described above.

L-Trp and L-Kyn were analyzed using the Shimadzu ADVP system, with injection volumes of 20µl. A Reverse-Phase analytical column (Kinetex™ Core Shell Technology column with specific area of 100mm x 4.6mm and particle size of 2.6µm, Phenomenex), fitted with a guard column is used, coupled to a PDA-UV detector (Shimadzu SPD-M10A VP) (L-Kyn, L-Trp), calibrated to integrate at 230nm, 250nm,
and 365nm, as well as a fluorescent detector (Shimadzu RF-20A XS prominence fluorescence detector) (internal standard), set to excitation wavelength 254nm and emission wavelength 404nm. The flow rate was 0.8ml/min and the acquisition time was 18 minutes. The signal to noise ratio is 3:1. Peak height was used to determine unknown sample concentration. Basal levels were estimated in naïve animals (t=0h). Data were represented as ng of L-Trp and L-Kyn per g of tissue in brain samples or per ml in plasma samples.

Co-immunoprecipitation

Ipsilateral cortices of mice were homogenized in ice-cold lysis buffer containing 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA-Na, 1% NP-40, 0.02% sodium azide, 0.1% SDS, 0.5% sodium deoxycholate and protease inhibitor cocktail (Roche Diagnostics). After lysis for 15 min, samples were centrifuged at 20,000 × g for 15 min. Neurons were washed twice in PBS, and then lysed in 400µl of the previously described buffer; lysates were centrifuged at 12,000 × g for 15 min at 4°C. The supernatants were pre-incubated for 1 h at 4°C with 0.025 ml of protein G-sepharose beads (Sigma-Aldrich), and then centrifuged to remove nonspecifically-adhered proteins and obtain the target supernatant for the subsequent IP experiment. The cleared supernatants were incubated with Rabbit anti-AhR (Enzo life Sciences) and Rabbit anti-CBP (SantaCruz Biothecnology) antibodies overnight. The antibodies-conjugated tissue sample and the protein G-sepharose beads were added for incubation during 4 hours. Immune complexes were isolated by centrifugation, and bound proteins were eluted by heating at 100°C in loading buffer. Proteins were analyzed by western blot.

Cerebral BDNF levels determination

BDNF levels were determined with BDNF Emax™ ImmunoAssay System, (Promega) according to manufacturer's instructions. The tissue-containing tubes were
weighed, and 100 µl of pre-chilled lysis buffer (20 mM Tris buffer, pH 7.4, 137 mM NaCl, 1% Nonidet-P40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM sodium vanadate, 10 µM aprotinin, 10 µM actinomycin, and 100 µM leupeptin) was added to each tube, followed by sonication. BDNF levels were calculated from the standard curve prepared for each plate, using a Sunrise microplate reader (TECAN). The standard curves were linear within the range used (0–500 pg/ml) and the quantities of BDNF in experimental samples were always within the linear range of the standard curve.

**Immunofluorescence**

For AhR detection after brain ischemia mice were anesthetized with sodium pentobarbital (120 mg/kg) 5, 18, 24, 48 hours, and 3 and 7 days after ligation (n=4/group) and perfused transcardiacally with 0.1M phosphate buffer containing 5ml of heparin sulfate (1000 U/ml) followed by 4% p-formaldehyde in 0.1M phosphate buffer (pH 7.4). Sham-operated mice served as controls (n=4/group). The brains were post-fixed overnight and placed in 30% sucrose for 2 days. Coronal series sections (30µm) were cut on a freezing microtome (Leica SM2000R, Leica Microsystems GmbH, Wetzlar, Germany) and stored in cryoprotective solution. Double-label immunofluorescence was performed on free-floating sections and tissue sections were incubated overnight at 4°C with rabbit anti mouse AhR (1:250, Enzo Life Sciences), mouse anti-mouse AhR (1:250, Enzo Life Sciences), mouse anti-mouse NeuN (1:500, Millipore Bioscience Research Reagents), mouse anti-mouse GFAP (1:500, BD Biosciences), rabbit anti-mouse Iba1 (1:500, Wako), and anti-goat TDO (1:100; SantaCruz).

For AhR immunodetection in rat neuronal cultures, control and OGD neurons were fixed with 4% paraformaldehyde and probed with rabbit polyclonal antibody against AhR (1:250; Enzo Life Sciences) and with mouse monoclonal antibody anti-NeuN (1:250; Millipore Bioscience Research Reagents).
The secondary antibodies used were goat anti-rabbit biotin, goat anti-mouse biotin and donkey anti-rat biotin (Vector laboratories) in combination with Alexa488 streptavidin (Molecular Probes), donkey Cy3 anti-rat (Jackson Immunoresearch) and donkey Cy3 anti-mouse (Vector Laboratories). For DNA staining we used TOPRO3 (Invitrogen). Controls performed in parallel without primary antibodies showed very low levels of nonspecific staining.

Image acquisition was performed with a laser-scanning confocal imaging system (Zeiss LSM710) and image analysis was performed with the ZEN2009 software (Zeiss). All co-localization images shown were confirmed by orthogonal projection of z-stack files. To quantify the nuclear fluorescent intensities, the TOPRO signal was used to draw manually around individual neuronal nucleus assessed by NeuN staining. On average, 50-200 cells were analyzed per coverslip (4/group, 2-3 different experiments). All quantification was performed with ImageJ Software (National Institutes of Health).

**Protein determination by cytometric bead array (CBA)**

Protein homogenates from brain infarcted tissue obtained 24h after MCAO were used to measure the protein levels of IL-10, TNF-α, IL-6, MCP-1 and IL-12/IL-23p40 by a BD™ Cytometric Bead Array (CBA). This assay allows for the discrimination of different particles on the basis of size and fluorescence. Capture Antibodies (Abs) were covalently coupled to microspheres (beads) according to the manufacturer’s instructions (BD Bioscience). Samples or standards were added to 75mm tubes containing 50μl of mixed capture beads followed by 1-hour incubation at room temperature. Then, 50μl of phycoerythrin (PE) detection reagent (BD Bioscience) was added to each sample or standard tube and left 1 hour at RT. After the incubation, samples and standards were washed with the kit buffer and centrifuged 5 min at 200g. Finally, the supernatant was discarded and another 300μl of wash buffer were added to each tube.
Four-color flow cytometric analysis was performed using a FACSCalibur® flow cytometer (BD). Data were acquired with the BD CellquestTM PRO and analyzed using the FCAP ArrayTM software. FSC vs. SSC gating was employed to exclude any sample particles other than the 7.5-µm polystyrene beads. Data were displayed as two-color dot plots (FL-2 vs. FL-4) such that the eight discrete FL-4 microparticle dye intensities were distributed along the Y-axis. Standard curves were plotted [cytokine calibrator concentration vs. FL-2 mean fluorescence intensity (MFI)] using a four-parameter logistic curve-fitting model. Cytokine concentrations were determined from these standard curves. When a sample had a cytokine concentration below the detection limit for the assay, a value of 0 was assigned for that particular cytokine concentration.

Supplementary references


## Supplementary Table 1

<table>
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<tr>
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<th>Reverse 3′ Primer</th>
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Supplementary Figure Legends

Supplementary Figure 1. The transcriptional AhR partner ARNT is up-regulated in the ischemic cortex. (A-B) Temporal expression of ARNT in peri-infarct (A) and core (B) regions after MCAO determined by western blot (n=4). B-actin levels were used as loading control. (C) Double immunofluorescence of ARNT (green) in combination with the neuronal marker NeuN in the peri-infarct region of MCAO mice 24 hours after occlusion (n=3; scale bar=50µm). Data were compared by a non-parametric, one-way Kruskal-Wallis ANOVA with Dunn’s post-hoc testing (A: *P<0.05 vs. sham group).

Supplementary Figure 2. Schematic overview of neuronal AhR expression in the ipsilesional cortex after MCAO.

Supplementary Figure 3. Effect of the AhR antagonist TMF on infarct outcome after permanent or transient MCAO. Effect of the AhR antagonist 6,2’,4´-trimethoxyflavone (TMF; 5 mg/Kg) on the infarct volume, after permanent (A; MCAO) or transient MCAO (B; tMCAO), determined by MRI or TTC staining respectively. Data are shown as % of total infarcted hemisphere (n=7 for each group except for MCAO vehicle group, n=5). (C) Neurological deficits assessed by the mNSS 24 hours after permanent occlusion. Data were compared by non-parametric 2-tailed Mann-Whitney tests (A-C: *P<0.05 vs. MCAO Veh).

Supplementary Figure 4. Effect of AhR haploinsufficiency on infarct volume. (A) Western blot of AhR in WT (AhR+/+) and AhR+/− mice 24 hours after MCAO (n=7). (B) Representative double immunofluorescence of AhR (green) with NeuN (red) in the ipsilateral cortex of WT and AhR+/− mice 24 hours after MCAO (n=4 for MCAO WT and n=5 for MCAO AhR+/−; scale bar=50µm). (C) Expression of AhRR mRNA in WT and AhR+/− mice 18 hours after MCAO (n=5). (D-F) Infarct outcome in WT and AhR+/− determined as infarct volume 48 hours after MCAO (D) or neurological deficits using
the mNSS scale (E) or the Grip Test (F). Data in D are shown as % of total infarcted hemisphere (left) or as % of infarcted hemisphere along anterior-posterior axis (right) (n=9 for MCAO WT and n=11 for MCAO AhR+/−). Data were compared by non-parametric 2-tailed Mann-Whitney tests (A, C and D-F: *P<0.05 vs. MCAO WT (AhR+/−)).

**Supplementary Figure 5. Effect of the AhR antagonist TMF on brain protein levels of inflammatory mediators after permanent middle cerebral artery occlusion (MCAO).** Protein levels of IL-6 (A), IL-10 (B), TNF-α (C), MCP-1 (D) and IL-12p70 (E) were determined 24 hours after MCAO by a cytomeric bead array in vehicle and TMF (5 mg/Kg)-treated mice (n=6 for each group except for sham vehicle group, n=5). Samples were tested in duplicate. Data were compared by a non-parametric two-way ANOVA followed by Bonferroni post-hoc testing (A-E: *P<0.05 vs. Sham Vehicle). Two-way ANOVA for IL-6, IL-10 and MCP1 showed a significant effect of surgery F (1,14) =17.82; P=0.0009), F(1,14)=22.48; P=0.0003) and F(1,14)=21.09; P=0.0004, respectively, but not of treatment or surgery x treatment interaction. No effects were detected for TNF-α and IL-12p70.

**Supplementary Figure 6. Characterization of AhR in rat cortical neurons after OGD.** (A) AhR western blot in control and OGD-exposed neurons 24 hours after the insult. β-actin levels were used as loading controls (n=6). (B) Representative double immunofluorescence of AhR (green) with neuronal marker NeuN (red) in control and OGD-exposed neurons 24 hours after the ischemic insult. (C) AhR western blots of cytoplasmic (c) and nuclear (n) fractions of control and OGD neurons 3 hours after OGD exposure. Bottom plot summarizes AhR nuclear/cytoplasmic ratio (n=7). (D) Representative images of nuclear translocation of AhR (green) in neurons, 3 (left) and 24 hours (right) after OGD exposure. TOPRO was used as nuclear marker (blue). Bottom plots summarize mean nuclear intensity of AhR in control and OGD neurons, where data distribution is represented by box plots, showing the 25th and 75th centiles.
together with the median, and whiskers represent the 10th and 90th centiles, respectively (n=200 cells/group; 3 independent experiments). (E-F) Effect of pre-treatment with the AhR antagonist 6,2’,4’-trimethoxyflavone (TMF; 3, 5 and 10µM; F) or post-treatment (TMF; 10µM) (G) on neuronal viability after OGD, determined by LDH activity. Experimental design is shown in the upper panel. Data are expressed as % of LDH release vs. control neurons treated with vehicle (n=24; 3 independent experiments). Data were compared by non-parametric 2-tailed Mann-Whitney tests (A and C-D: *P<0.05 vs. Control; F: *P<0.05 vs. Vehicle) or a non-parametric, one-way Kruskal-Wallis ANOVA followed by Dunn’s post-hoc testing (E: *P<0.05 vs. Vehicle).

Supplementary Figure 7. AhR modulates CREB signaling in cortical neurons after OGD. (A) Temporal expression of CREB and p-CREB in OGD neurons. Actin was used as loading control (n=3). Right plots: western blot quantification. (B) Expression of BDNF and NPAS4 mRNA in OGD neurons treated with vehicle or TMF (10µM) 5 hours after OGD. GADPH levels were used to normalize mRNA expression (n=7). (C) Co-immunoprecipitation (IP) of AhR in control and OGD neurons treated with vehicle or TMF (10µM) for 6 hours after OGD exposure. Immune complexes were analyzed by western blot with AhR, CBP and CREB antibodies. Quantification is shown in the right plots. Data are represented as % of CBP/AhR or % of CREB/AhR of control neurons treated with vehicle (n=3). Data were compared by non-parametric 2-tailed Mann-Whitney tests (A-B: *P<0.05 vs. Vehicle) or a non-parametric two-way ANOVA followed by Bonferroni post-hoc testing (C: *P<0.05 vs. Control Vehicle, #P<0.05 vs. OGD Vehicle). Two-way ANOVA for CBP/AhR interaction demonstrated a significant effect of OGD (F(1,12)= 4.59; P=0.048), of treatment (F(1,12)= 78.06; P<0.0001) and OGD x treatment interaction (F(1,12)= 9.68; P=0.009). Two-way ANOVA for CREB/AhR interaction showed a significant effect of OGD (F(1,12)=17.09; P=0.0014), of treatment (F(1,12)=66.15; P<0.0001) and OGD x treatment interaction (F(1,12)=5.42; P=0.0382).
Supplementary Figure 8. *In vitro* characterization of L-kynurenine as an AhR ligand in cortical neurons. (A) Representative images of nuclear translocation of AhR (green) in control neurons after 1h treatment with vehicle, L-Kyn (50µM) or TCDD (10nM). TOPRO was used as nuclear marker (blue). Right: mean nuclear AhR intensity in neurons after 0.5 or 1h of treatment, represented by box plots showing the 25th and 75th centiles together with the median; whiskers represent the 10th and 90th centiles, respectively (n=200 cells/group; 3 independent experiments; scale bar=20µm). (B) Representative western blots of cytoplasmic and nuclear AhR 1h after the treatment with vehicle, L-Kyn (50µM) or TCDD (20nM). Right: AhR western blot quantification (n=4). (C-D) Cyp1b1 (C) and Cyp1a1 (D) mRNA expression in neurons after 5h-treatment with vehicle, TMF (10µm), L-Kyn (50µM), TMF+L-Kyn, TCDD (10nM) or TMF+TCDD (n=5). Data were compared by a non-parametric one-way Kruskal-Wallis ANOVA followed by Dunn’s post-hoc testing (A-B: *P<0.05 vs. Vehicle; C-D: *P<0.05 vs. control vehicle, #P<0.05 vs. control L-Kyn, $P<0.05 vs. control TCDD).

Supplementary Figure 9. AhR activation by L-Kyn modulates CREB transcriptional activity in cortical neurons. (A) Expression of BDNF and NPAS4 mRNA in neurons 5 hours after vehicle or L-Kyn (50µM) treatment. GADPH levels were used to normalize mRNA expression (n=7). (B) Luciferase CREB reporter assay in neurons after 6 h-treatment with vehicle or the AhR agonists TCDD (20nM) or L-Kyn (50µM). Data are expressed as % of vehicle (n=3). Data were compared by a non-parametric 2-tailed Mann-Whitney test (A: *P<0.05 vs. vehicle) or a non-parametric, one-way Kruskal-Wallis ANOVA with Dunn’s post-hoc testing (B: *P<0.05 vs. vehicle).
Supplementary figure 1
Supplementary figure 2
Supplementary figure 3
Supplementary figure 5
Supplementary figure 6
Supplementary figure 7
A

1 hour after treatment

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1 hour

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1 hour after treatment

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AhR nuclear / cytoplasmic ratio

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Supplementary figure 8
Supplementary figure 9