Behavioral/Systems/Cognitive

Hypoxia/Reoxygenation Impairs Memory Formation via Adenosine-Dependent Activation of Caspase 1

Gabriel S. Chiu,[1,2] Diptaman Chatterjee,[2] Patrick T. Darmody,[2] John P. Walsh,[2] Daryl D. Meling,[2] Rodney W. Johnson,[3] and Gregory G. Freund[1,2,3]

[1]Division of Nutritional Sciences, [2]Department of Pathology, Program in Integrative Immunology and Behavior, and [3]Department of Animal Sciences, University of Illinois, Urbana Illinois 61801

After hypoxia, a critical adverse outcome is the inability to create new memories. How anterograde amnesia develops or resolves remains elusive, but a link to brain-based IL-1 is suggested due to the vital role of IL-1 in both learning and brain injury. We examined memory formation in mice exposed to acute hypoxia. After reoxygenation, memory recall recovered faster than memory formation, impacting novel object recognition and cued fear conditioning but not spatially cued Y-maze performance. The ability of mice to form new memories after hypoxia/reoxygenation was accelerated in IL-1 receptor 1 knockout (IL-1R1 KO) mice, in mice receiving IL-1 receptor antagonist (IL-1RA), and in mice given the caspase 1 inhibitor Ac-YVAD-CMK. Mechanistically, hypoxia/reoxygenation more than doubled caspase 1 activity in the brain, which was localized to the amygdala compared to the hippocampus. This reoxygenation-dependent activation of caspase 1 was prevented by broad-spectrum adenosine receptor (AR) antagonism with caffeine and by targeted A1/A2A AR antagonism with 8-cyclopentyl-1,3-dipropylxanthine plus 3,7-dimethyl-1-propargylxanthine. Additionally, perfusion of adenosine activated caspase 1 in the brain, while caffeine blocked this action by adenosine. Finally, resolution of anterograde amnesia was improved by both caffeine and by targeted A1/A2A AR antagonism. These findings indicate that amygdala-based anterograde amnesia after hypoxia/reoxygenation is sustained by IL-1β generated through adenosine-dependent activation of caspase 1 after reoxygenation.

Introduction

Injury to the brain through loss of oxygen triggers memory loss and causes learning deficiencies (Shukitt-Hale et al., 1996), including anterograde amnesia (Beatty et al., 1987). Importantly, acute hypoxia activates the neuroimmune system, especially its IL-1 arm (Johnson et al., 2007). Brain-based IL-1 regulates cognitive function (Dantzer et al., 2008), and excess IL-1 in the brain is congruous with memory loss and impaired learning (Pugh et al., 2001). While neuroimmune system-generated IL-1 can cause brain injury (Ma et al., 2003), the mechanism by which IL-1 is produced in the brain during reduced oxygen conditions is not understood. IL-1α and IL-1β are both present in the brain, and each is implicated in complications related to hypoxia and ischemia (Touzani et al., 1999). Previously, we demonstrated that delayed recovery from acute hypoxia, as measured by social withdrawal in mice, was reliant on IL-1β because inhibition of the inflammatory caspase, caspase 1, dramatically shortened recuperation time (Johnson et al., 2007). As a member of the cysteine–aspartic acid protease family, caspase 1 exists intracellularly as an inactive proenzyme (Damiano et al., 2004) until it is proteolytically processed by Nod-like receptor (NLR)-containing multiprotein inflammasomes (Miao et al., 2011). Activated caspase 1 enzymatically processes pro-IL-1β to a secretable mature form (Bauernfeind et al., 2009). Inflammasome activation is elicited by a variety of microbe- and host-associated bioactives (Schroder et al., 2010), including endogenous danger signals generated during reoxygenation such as reactive oxygen species (ROS) (Tschopp et al., 2010), uric acid (Lamkanfi et al., 2007), and ATP (Di Virgilio, 2007).

The restoration of oxygen after hypoxia is required for recovery but can, itself, cause tissue damage (González-Correa et al., 2007). Reoxygenation is frequently described in conjunction with reperfusion as occurs in ischemic injuries like myocardial infarction (Galaris et al., 1989) and stroke (Kostulas et al., 1999). Recently, reoxygenation unassociated with reperfusion has been linked to neural injury and cognitive dysfunction associated with sleep apnea (Gozal et al., 2001). Hypoxia/reoxygenation can precipitate endoplasmic reticulum stress (Bi et al., 2005), cell death (Saikumar et al., 1998), and inflammation (Johnson et al., 2007), but the means by which hypoxia/reoxygenation triggers these sequelae is not clear. Currently, hypoxia/reoxygenation-dependent generation of ROS is a favored causative to reoxygenation injury (Li and Jackson, 2002), but membrane destabilization is also a consequence of hypoxia/reoxygenation (Bickler and Hansen, 1994; Calabresi et al., 1995). Importantly, membrane damage causes increases in extracellular concentrations of ATP, ADP, and adenosine (Guinzberg et al., 2006).

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Correspondence should be addressed to Dr. Gregory G. Freund, Department of Pathology, College of Medicine, 506 South Mathews Avenue, University of Illinois at Urbana-Champaign, Urbana, IL 61801. E-mail: freun@illinois.edu.

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Intracellular adenosine concentrations rapidly increase during states of negative energy balance when ATP hydrolysis outstrips ATP synthesis (Bruns, 1991; Fredholm et al., 1999). In contrast, extracellular adenosine is primarily derived from enzymatic phosphohydrolysis of ATP in the interstitial space (Hart et al., 2008). During hypoxia and ischemia, the extracellular concentration of adenosine can increase from 30–300 nM (Rudolphi and Schubert, 1997) to 10–50 μM (Hagberg et al., 1987). In addition, extracellular AMP derived from intracellular ATP and ADP can be phosphohydrolyzed by CD73 to adenosine (Kobie et al., 2006). G protein-coupled adenosine receptors (ARs), which are divided into the subclasses A1, A2A, A2B, and A3, all recognize extracellular adenosine (Fredholm et al., 2001) and are blocked by the nonselective lipophilic competitive antagonist and nutraceutical, caffeine (Fredholm et al., 1999), as well as specific pharmacologic inhibitors. Therefore, in this study we sought to show that memory formation after acute hypoxia is adversely impacted by brain IL-1β through a mechanism reliant on AR-dependent activation of caspase 1.

Materials and Methods

Materials. All reagents and chemicals were purchased from Sigma-Aldrich except as noted. All primers were purchased from Applied Biosystems.

Animals. Animal use was conducted in accordance with Institutional Animal Care and Use Committee-approved protocols at the University of Illinois (Urbana, IL). C57BL/6J male animals were purchased from The Jackson Laboratory at 7 weeks of age. C57BL/6J IL-1 receptor 1 knock-out (IL-1R1 KO) mice were bred in-house. Mice were group housed (×8 cage) in standard shoebox cages (length, 46.9 cm; width, 25.4 cm; height, 12.5 cm) and allowed water and food *ad libitum*. Housing temperature (72 °F) and humidity (45–55%) were controlled as was a 12/12 h reversed dark-light cycle (2200–1000 h). Video recording of animal behavior was performed under red light using a night shot-capable video camera (Sony HDR-XR500V). Except for locomotor activity, which was performed as a repeated measure, all treatments at all time points represent separate cohorts of mice. The total number of mice used was 630.

Intracerebroventricular cannulation. As we have described previously (Johnson et al., 2007), mice were anesthetized intraperitoneally with a sodium ketamine hydrochloride/xylazine hydrochloride solution delivering 80 mg/kg ketamine and 12 mg/kg xylazine. Animals were placed in a Kopf stereotaxic instrument (David Kopf Instruments), and mouse-specific brain infusion cannulas (Plastics One) were placed using the coordinates 0.6 mm posterior, 1.5 mm lateral to the bregma, and 2.5 mm ventral from the surface of the skull. Cannulas were fixed to the skull with cyanoacrylate gel adhesive (Plastics One) and protected by a plastic guard. Mice were allowed 7 days to recover.

Hypoxia/reoxygenation. As we have described previously (Sherry et al., 2009a,b), mice ($n = 16$/episode) were transferred from their home cages to the BioSperix ProOx/A-Chamber Biological Atmosphere System (Biospherix) and subjected to either a 6% oxygen and 94% nitrogen environment (hypoxia) or an atmospheric air environment (normoxia) for 2 h. After exposure, mice were returned to their home cages.

Injectables. As we have described previously (Johnson et al., 2007), the caspase 1 inhibitor Ac-YVAD-CMK (Bachem) was administered intracerebroventricularly at a dose of 50 ng/μl/mouse immediately before hypoxia. Kineret, a recombinant IL-1 receptor antagonist (IL-1RA) (AmGen), was administered intraperitoneally at a dose of 1.4 mg/kg/mouse 30 min before hypoxia. CafCit (caffeine citrate) (Bedford Laboratories) was administered intraperitoneally at a dose of 100 mg/kg/mouse immediately before hypoxia. 8-cyclopentyl-1,3-dipropylxanthine [A1 AR antagonist (2.5 mg/kg/mouse)] plus 3,7-dimethyl-1-propargylxanthine [A2A AR antagonist (2.5 mg/kg/mouse)] (Chen et al., 2001) were administered as an intraperitoneal cocktail 1 h before hypoxia. *N*-acetyl cysteine (NAC) (Hospira) was administered intraperitoneally at a dose of 50 mg/kg/mouse immediately before hypoxia. For all studies, vehicle/control injection was phosphate-buffered saline (PBS) or normal saline (saline), as indicated, except for targeted AR inhibition, which was 1:5 DMSO/castor oil.

Novel object recognition. Novel object recognition was performed as we have described previously (Lavin et al., 2011; York et al., 2012b). In studies examining retrograde amnesia, mice (1 h before hypoxia) were individually transferred from their home cage to a home cage-sized memory arena containing two identical objects placed 10 cm apart at the short-side wall end for 5 min (training). Mice were then subjected to hypoxia or normoxia. At the time points indicated post-hypoxia, mice (individually) were transferred back to the memory arena now containing one familiar object and one unfamiliar object (novel object) (testing). Investigative behavior of the objects was video recorded for 5 min and evaluated using EthoVision XT 7 (Noldus Information Technology) video tracking software. Percent investigation was calculated by dividing the time spent examining each object by the total time spent investigating both objects. In studies examining anterograde amnesia, mice were transferred for training at the time points indicated post-hypoxia. Mice were then returned to their home cage for 55 min. As above, testing was initiated by returning mice to the memory arena with one familiar object and one novel object. Investigative behavior of the objects and time spent examining each object were performed as described above.

Locomotion. Spontaneous locomotor activity was measured as we have described previously (Lavin et al., 2011, York et al., 2012a). At the times indicated, mice were video recorded in their home cage for 5 min. Distance moved was quantified using EthoVision XT 7.

Cued fear conditioning. Cued fear conditioning was performed as we have described previously (York et al., 2012b). Four hours post-hypoxia, mice were placed in a Lafayette Instruments Cued and Contextual Fear Test Chamber. After a 30 s acclimatization period, mice were exposed to a white light (~23000 lux) for 2 s, followed by a 2 s foot shock (60 V, 1 mA). After a 30 s wait, mice were re-exposed to the light plus foot shock cycle as described above (training). Mice were then returned to their home cage. At the time points indicated, mice were reintroduced to the testing apparatus and allowed to acclimate for 30 s. Mice then underwent two cycles of light without foot shock similar in parameters to the above (testing). All freezing behavior was evaluated via an integrated infrared photo beam array. Data were analyzed using the Motor Monitor Host Software (Lafayette Instruments).

Alternation. Spatially cued spontaneous alternations were performed as we have described previously (Lavin et al., 2011, York et al., 2012b). In brief, mice were placed in a symmetrical three-arm, clear Plexiglas Y-maze (40 cm length × 9 cm width × 16 cm height per arm with an arm angle of 120°) with side walls decorated with black triangles, black circles, or black diagonal lines. Mice were randomly placed in one of the arms. Movement was recorded for 5 min and mouse exploration was evaluated from the video record. Mice were tested at 4, 52, and 76 h after hypoxia. Results are presented as the ratio of perfect alternations to total arm entrances. Perfect alternations were defined as exploration of two novel arms sequentially before a return to the start arm independent of a right or left arm choice at initiation. To have entered an arm, the mouse was required to have all four legs in that arm.

Quantitative PCR. As we have described previously (York et al., 2012a), RNA was isolated from the hippocampus dissected from PBS-perfused whole brains. RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (PN 4368813; Applied Biosystems). The TaqMan Gene Expression primers used were glial fibrillary acidic protein (GFAP) (Mm01253033_m1), aquaporin 4 (Mm000802131_m1), CD11b (Mm00434455_m1), F4/80 (Mm00802529_m1), and the peripheral benzodiazepine receptor (PBR) (Mm00437828_m1). Quantitative PCR (qPCR) was performed on a 7900 HT Fast Real-Time PCR System (Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems). To compare gene expression, a parallel amplification of endogenous RPS3 (Mm00656272_m1) was performed. Reactions with no reverse transcription and no template were included as negative controls. Relative quantitative evaluation of target gene to RPS3 was performed by comparing ΔCts, where Ct is the threshold concentration.

Caspase 1 activity. PBS-perfused whole brains, brain regions (as indicated), and livers were frozen in liquid nitrogen and then freeze fractured in reaction buffer containing 50 mM NaCl (Fisher Scientific),

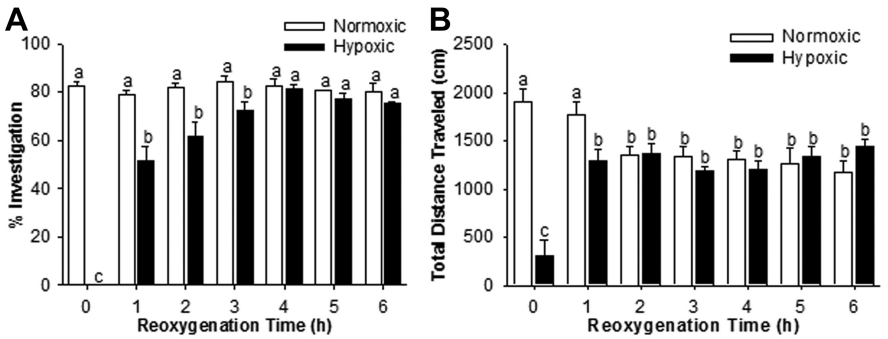

Figure 1. Restoration of memory recall after acute hypoxia. ***A***, Wild-type mice were trained in memory formation using novel object recognition 1 h before hypoxia. Mice were then exposed to normoxia or hypoxia for 2 h. Memory recall (percent investigation) was measured at the reoxygenation time points indicated. Results are expressed as means ± SEM; $n = 8$. Bars without a common superscript are different ($p < 0.05$). ***B***, Mice were treated as in ***A***, and spontaneous locomotor activity (total distance traveled) was measured at the reoxygenation time points indicated. Results are expressed as means ± SEM; $n = 6$. Bars without a common superscript are different ($p < 0.05$).

10% glycerol, 1 mM DTT, 1 mM EDTA, 1 mM bestatin, 1 mM pepstatin (EMD4Bioscience), 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, and 50 mM HEPES, pH 7.4 (USB Corporation) using the TissueLyser II (Qiagen) at a rotational frequency of 30/s for 2 min. Lysates were clarified at 16,000 × *g* for 15 min at 4°C, and the supernatant protein concentrations were determined using the DC Protein Assay (Bio-Rad) and a ELx800 Absorbance Microplate Reader (BioTek Instrument). Supernatant protein concentrations were normalized to 10 mg/ml (whole brain and liver) or 2.5 mg/ml (brain regions) with reaction buffer. Caspase 1 activity was determined colorimetrically in the clarified lysates using the caspase 1 substrate Ac-YVAD-p-nitroaniline (p-NA) (Enzo Life Science) at a final concentration of 4 mM. Substrate incubation was at 37°C for the times indicated. Moles of p-NA liberated were determined by a standard curve ranging from 0.075 mM to 0.3 mM p-NA (Enzo Life Science). Caspase 1 activity was calculated as (Δ[p-NA]/Δ time)/(total protein).

Adenosine perfusion. Mice were killed via CO_2 asphyxiation, and the heart was immediately exposed using straight 11.5 cm scissors (Fine Science Tools). The left ventricle was pieced with a 23 gauge, 1.25 inch needle (BD) attached to a BD 30 ml syringe. Mice were perfused (as indicated) with 10, 30, 50, or 100 μM adenosine, 500 μM NAC, 500 μM caffeine, 50 μM adenosine plus 500 μM caffeine, or 50 μM adenosine plus 500 μM NAC in PBS, pH 7.4, or PBS alone, pH 7.4.

Glutathione. Similar to methods we have described previously (Godbout et al., 2002), glutathione (GSH) and glutathione disulfide (GSSG) were measured using the Glutathione Assay Kit (Sigma-Aldrich). As above, PBS perfused whole brains were frozen in liquid nitrogen and then freeze fractured in the kit-provided assay buffer using TissueLyser II. After brain homogenization, GSH/GSSG was quantified spectrophotometrically following the instructions of the manufacturer and an ELx800 Absorbance Microplate Reader (BioTek Instruments).

Phospho-ERK 1/2, phospho-p38 MAPK, and phospho-JNK. Similar to methods we have described previously (Sherry et al., 2007), whole brains were frozen in liquid nitrogen and then freeze fractured as above in a homogenization buffer containing 50 mM NaCl, 10% glycerol, 1 mM DTT, 2 mM sodium orthovanadate, 250 nM okadaic acid, 1:200 Protease Inhibitor Cocktail III (Calbiochem), and 50 mM HEPES, pH 7.4, using the TissueLyser II (Qiagen). Lysates were clarified at 16,000 × *g* for 15 min at 4°C, and the supernatant protein concentrations were determined using the DC Protein Assay, as above. Phospho (p)-ERK 1/2, p-p38 MAPK, and p-JNK were measured in 50 μl of lysate using a Bio-Plex phosphoprotein assay (Bio-Rad) and a Luminex 100 System following the instructions of the manufacturer (Luminex). Results are expressed relative change in phosphorylation/total protein.

Immunohistochemistry. Similar to methods we have described previously (Johnson et al., 2007, Davis-Devine et al., 2003), mice were perfused with ice-cold, 10% neutral, buffered formalin. Brains were removed and, using a Zivic Mouse Brain Slicer (Zivic Instruments), coronal sections ranging from the bregma to −3.0 mm from the bregma were generated. These slices were fixed in 10% neutral buffered formalin for 24 h and then paraffin embedded and sectioned. A 4 μm section at −1.7 mm from the bregma was immunostained for GFAP using a rabbit anti-GFAP antibody (DAKO) at a dilution of 1:2000 at room temperature for 30 min. Detection was performed using the Rabbit Link/SS Label detection kit (Biogenex) in conjunction with the Biogenex i6000 Automated Staining System (incubation time was 15 min). After coverslipping, the entire slide was imaged at 40× with a NanoZoomer 2.0-HT (Hamamatsu).

Uric acid. Blood was collected via cardiac puncture using a 26 G × 3/8 inch needle (Becton Dickinson) and allowed to stand at room temperature for 30 min. Serum was generated by centrifuging samples at 10,000 × *g* for 15 min. Serum uric acid was determined on a AU680 analyzer (Beckman Coulter).

Statistics. All data are presented as mean ± SEM. Data were analyzed using SigmaPlot 11.2 (Systat Software). To test for statistical differences, a one-way or two-way ANOVA was used with or without repeated measurements where needed. Tukey's test was used for *post hoc*, pair-wise, multiple-comparison procedures. Where indicated, raw data were transformed using a log10 transformation to attain equal variance. All statistical analysis included testing for time point × treatment interactions. Statistical significance was denoted at $p < 0.05$.

Results

Restoration of memory recall after acute hypoxia

Figure 1*A* demonstrates that if memory formation (training) occurred 1 h before hypoxia, the ability of mice to recall that memory in the testing phase was restored after 4 h of reoxygenation (82.5 ± 2.1% vs 81.3 ± 2.1%, normoxia vs hypoxia). Immediately after hypoxia, mice did not explore either a familiar or novel object. After 1, 2, and 3 h of reoxygenation, mice explored a novel object as if it were a familiar object (51.8 ± 5.7%, 61.6 ± 6.2%, and 72.6 ± 3.6%, respectively). Main effects of hypoxia ($p < 0.001$) and time ($p < 0.001$) were as follows: 0 h time point, $p < 0.001$, normoxic versus hypoxic (82.5 ± 1.9% vs 0 ± 0%); 1 h time point, $p < 0.001$, normoxic versus hypoxic (79.2 ± 1.6% vs 51.8 ± 5.7%); 2 h time point, $p < 0.001$, normoxic versus hypoxic (81.9 ± 2.1% vs 61.6 ± 6.2%); 3 h time point, $p < 0.05$, normoxic versus hypoxic (84.5 ± 2.4% vs 72.6 ± 3.6%). After 4, 5, and 6 h of reoxygenation, mice preferably explored the novel object over the familiar (81.3% vs 18.7%, 77.5% vs 22.5%, and 75.7% vs 24.3%, respectively). Fig. 1*B* shows that hypoxic mouse spontaneous locomotor activity was comparable to that of normoxic mice after 2 h of reoxygenation. Main effects of hypoxia ($p < 0.05$) and time ($p < 0.05$) were as follows: 0 h time point, $p < 0.001$, normoxic versus hypoxic (1910.4 ± 128.9 cm vs 314.7 ± 159.1 cm); 1 h time point, $p < 0.05$, normoxic versus hypoxic (1772.5 ± 128.5 cm vs 1297.7 ± 115.3 cm).

Memory formation recovers more slowly than memory recall after acute hypoxia

Figure 2*A* illustrates that if memory formation occurs after hypoxia (training), the ability of mice to learn does not recover until 6 h of reoxygenation (66.2 ± 1.0% vs 76.3 ± 1.8%). At 3, 4, and 5 h of reoxygenation, mice exposed to hypoxia explored a novel object: 54.3 ± 0.8%, 57.0 ± 2.4%, and 55.9 ± 1.5%, respectively. Main effects of groups ($p < 0.001$) were as follows: normoxic

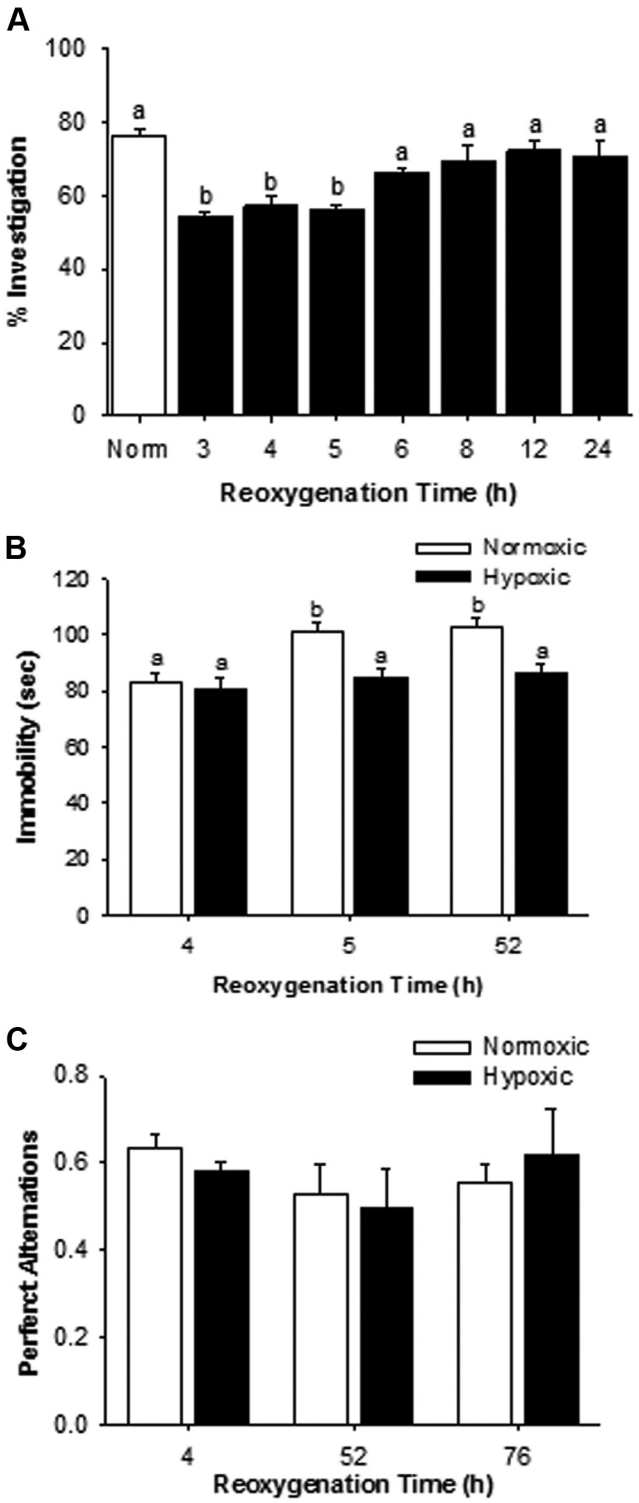

Figure 2. Memory formation recovers more slowly than memory recall after acute hypoxia. ***A***, Wild-type mice were exposed to normoxia (Norm) or hypoxia for 2 h. After hypoxia, mice were trained in memory formation using novel object recognition 1 h before the time points indicated. Memory recall (percent investigation) was measured at the reoxygenation time points indicated. Results are expressed as means ± SEM; $n = 6–8$. Bars without a common superscript are different ($p < 0.05$). ***B***, Mice were treated as in ***A***. Mice were trained in memory formation using cued fear conditioning after 4 h of reoxygenation. Memory recall (immobility) was measured after 5 and 52 h of reoxygenation. Results are expressed as means ± SEM; $n = 4$. Bars without a common superscript are different ($p < 0.05$). ***C***, Mice were treated as in ***A***. Perfect alternations were measured after 4, 52, and 76 h of reoxygenation. Results are expressed as means ± SEM; $n = 4$.

versus 3 h time point, $p < 0.001$ (75.3 ± 1.9% vs 54.3 ± 0.8%); normoxic versus 4 h time point, $p < 0.001$ (75.3 ± 1.9% vs 57.0 ± 2.47%); normoxic versus 5 h time point, $p < 0.001$ (75.3 ± 1.9% vs 55.9 ± 1.5%). Data were transformed. Fig. 2*B* confirms that memory formation after hypoxia is impaired. In the cued fear conditioning test, both normoxic and hypoxic mice (after 4 h of reoxygenation) had similar immobility when first exposed to cue/foot shock (training). When retested with just the cue (testing), after 5 and 52 h of reoxygenation, normoxic mice demonstrated a 121.9 and 123.8% increase in immobility, respectively, when compared to the initial cue/foot shock exposure (training). In contrast, hypoxic mice, when retested with just the cue at 5 and 52 h of reoxygenation, showed immobility comparable to that of the initial cue/foot shock exposure (training). Main effects of hypoxia ($p < 0.001$) and time ($p < 0.05$) were as follows: 4 h time point, $p < 0.659$, normoxic versus hypoxic (83.0 ± 3.7 s vs 80.7 ± 4.4 s); 5 h time point, $p < 0.05$, normoxic versus hypoxic (101.1 ± 3.3 s vs 84.8 ± 3.1 s); 52 h time point, $p < 0.05$, normoxic versus hypoxic (102.7 ± 3.3 s vs 86.2 ± 3.6 s). Figure 2*C* shows that perfect alternations in a spatially cued Y-maze were not affected by hypoxia at 4, 52, and 76 h after reoxygenation. Main effects of hypoxia and time were $p = 0.626$ and $p = 0.235$.

Knockout of IL-1R1 improves memory formation and locomotion while blunting activation of ERK1/2 and p38 MAPK

Figure 3*A* demonstrates that if memory formation (training) occurred 4 h after hypoxia, IL-1R1 KO mice had accelerated recovery of memory formation. At 5 h of reoxygenation, hypoxic IL-1R1 KO mice explored a novel object similar to normoxic wild-type (WT) mice (70.2 ± 7.4% vs 63.5 ± 3.1%). Hypoxic WT mice explored a novel object as if it were a familiar object (47.9 ± 4.7%). Main effects of genotype ($p < 0.05$) and hypoxia ($p = 0.235$) were as follows: normoxic WT versus hypoxic WT, $p < 0.05$ (63.5 ± 3.1% vs 47.9 ± 4.7%); normoxic IL-1R1 KO versus hypoxic IL-1R1 KO, $p = 0.645$ (66.9 ± 3.3% vs 70.2 ± 7.4%); hypoxic WT versus hypoxic IL-1R1 KO, $p < 0.05$ (47.9 ± 4.7% vs 70.2 ± 7.4%). Similar results were seen in mice administered IL-1 RA (Fig. 3*B*) [main effects of treatment ($p < 0.05$) and hypoxia ($p = 0.055$): saline normoxic vs saline hypoxic, $p < 0.05$ (66.9 ± 5.3% vs 53.2 ± 5.3%); IL-1RA normoxic vs IL-1RA hypoxic, $p = 0.457$ (72.1 ± 3.4% vs 67.2 ± 4.0%); saline hypoxic vs IL-1RA hypoxic, $p < 0.05$ (53.2 ± 5.3% vs 67.2 ± 4.0%)] and in mice administered Ac-YVAD-CMK (Fig. 3*C*) [main effects of treatment ($p < 0.05$) and hypoxia ($p = 0.5$): PBS normoxic vs PBS hypoxic, $p < 0.05$ (69.7 ± 3.5% vs 55.7 ± 4.2%); Ac-YVAD normoxic vs Ac-YVAD hypoxic, $p = 0.157$ (67.1 ± 3.3% vs 75.5 ± 5.0%); PBS hypoxic vs Ac-YVAD hypoxic, $p < 0.05$ (55.7 ± 4.2% vs 75.5 ± 5.0%)]. Fig. 3*D* illustrates that spontaneous locomotor activity of IL-1R1 KO mice was restored after 1 h of reoxygenation as opposed to 2 h in hypoxic WT mice. Main effects of hypoxia ($p < 0.001$), genotype ($p = 0.781$), and time ($p = 0.193$) were as follows: 0 h time point: normoxic WT versus hypoxic WT, $p < 0.001$ (2202.2 ± 109.2 cm vs 936.2 ± 267.6 cm); normoxic IL-1R1 KO versus hypoxic IL-1R1 KO, $p < 0.001$ (2068.5 ± 40.0 cm vs 535.0 ± 260.5 cm); normoxic WT versus normoxic IL-1R1 KO, $p = 0.695$ (2202.2 ± 109.2 cm vs 2068.5 ± 40.0 cm); hypoxic WT versus hypoxic IL-1R1 KO, $p = 0.165$ (936.2 ± 267.6 cm vs 535.0 ± 260.5 cm); 1 h time point: normoxic WT versus hypoxic WT, $p < 0.05$ (1989.1 ± 132.5 cm vs 1283.3 ± 132.1 cm); normoxic IL-1R1 KO versus hypoxic IL-1R1 KO, $p = 0.546$ (1417.1 ± 223.1 cm vs 1615.6 ± 140.1 cm); normoxic WT versus normoxic IL-1R1 KO, $p = 0.111$ (1989.1 ±

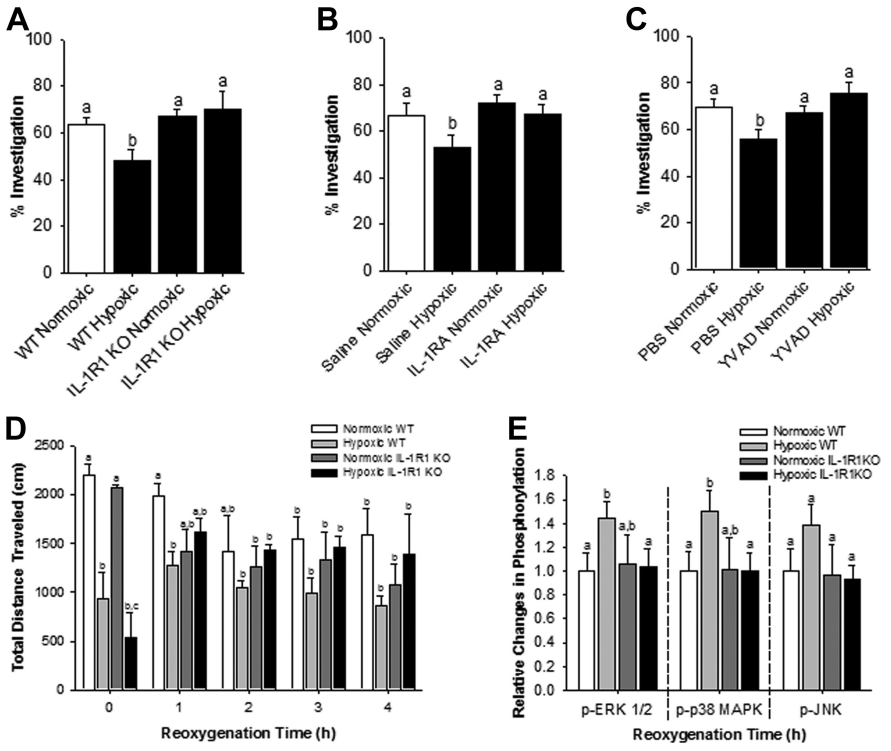

Figure 3. Knockout of IL-1R1 improves memory formation and locomotion while blunting activation of ERK1/2 and p38 MAPK. ***A***, WT or IL-1R1 KO mice were exposed to normoxia or hypoxia for 2 h. Mice were trained in memory formation using novel object recognition after 4 h of reoxygenation. Memory recall (percent investigation) was measured after 5 h of reoxygenation. Results are expressed as means ± SEM; $n = 4$. Bars without a common superscript are different ($p < 0.05$). ***B***, WT mice treated with/without intraperitoneal IL-1RA were exposed to normoxia or hypoxia as in ***A***, and memory was tested as in ***A***. Results are expressed as means ± SEM; $n = 6$. Bars without a common superscript are different ($p < 0.05$). ***C***, WT mice treated with/without intracerebroventricular Ac-YVAD-CMK were exposed to normoxia or hypoxia as in ***A***, and memory was tested as in ***A***. Results are expressed as means ± SEM; $n = 6$. Bars without a common superscript are different ($p < 0.05$). ***D***, WT or IL-1R1 KO mice were treated as in ***A***, and spontaneous locomotor activity (total distance traveled) was measured at the reoxygenation time points indicated. Results are expressed as means ± SEM; $n = 4$. Bars without a common superscript are different ($p < 0.05$). ***E***, WT and IL-1R1 KO mice were exposed to normoxia or hypoxia as in ***A***. Brain p-pERK1/2, p-p38 MAPK, and p-JNK were measured 1 h after hypoxia. Results are expressed as means ± SEM; $n = 6–9$. Bars without a common superscript are different ($p < 0.05$).

132.5 cm vs 1417.1 ± 223.1 cm); hypoxic WT versus hypoxic IL-1R1 KO, $p = 0.245$ (1283.3 ± 132.1 cm vs 1615.6 ± 140.1 cm). Fig. 3*E* shows that after 1 h of reoxygenation, ERK1/2 (normoxic vs hypoxic: 1.0 ± 0.2-fold vs 1.4 ± 0.1-fold; $p = 0.05$) and p38 MAPK (normoxic vs hypoxic: 1.0 ± 0.2-fold vs 1.5 ± 0.2-fold; $p = 0.05$) were activated in the brains of WT but not IL-1R1 KO mice ($p = 0.94$ and $p = 0.91$, respectively). In contrast, JNK was not significantly activated in either WT or IL-1R1 KO 1 h after reoxygenation.

AR blockade prevents hypoxia-dependent activation of caspase 1 in the brain

Figure 4*A* shows that after 1 h of reoxygenation, brain caspase 1 is 208.8% more active in hypoxic mice than in normoxic mice. Hypoxic mice not allowed to significantly reoxygenate did not demonstrate an increase in brain caspase 1 activity. In addition, after 6 h of reoxygenation, brain caspase 1 activity was similar to that of normoxic mice: 0 h time point: $p = 0.525$, normoxic versus hypoxic (1.0 ± 0.1-fold vs 1.1 ± 0.2-fold); 1 h time point: $p < 0.001$, normoxic versus hypoxic (1.0 ± 0.2-fold vs 2.1 ± 0.2-fold); 6 h time point: $p = 0.930$, normoxic versus hypoxic (1.0 ± 0.1-fold vs 1.0 ± 0.1-fold). Figure 4, *B* and *C*, illustrates that mice pretreated with either caffeine or an A1/A2A AR inhibitor cocktail did not upregulate caspase 1 activity in the brain after 1 h of reoxygenation [saline hypoxic vs caffeine hypoxic: $p < 0.001$ (2.6 ± 0.0-fold vs 1.0 ± 0.2-fold); vehicle hypoxic vs A1/A2A antagonist (antag) hypoxic: $p < 0.05$ (2.6 ± 0.3-fold vs 1.5 ± 0.1-fold)]. Table 1 demonstrates that after 1 h of reoxygenation, hypoxia lowers the brain GSH/GSSG ratio by 49% ($p < 0.05$) and that caffeine and NAC each prevented this decline. The GSH/GSSG ratio was unchanged if hypoxic mice were not afforded significant time to reoxygenate (1.00 ± 0.22-fold vs 1.02 ± 0.22-fold, normoxia vs hypoxia; $p = 0.832$). Importantly, NAC-pretreated mice did not lose the ability to upregulate brain caspase 1 activity after reoxygenation (Fig. 4*D*). Main effects of treatment ($p = 0.358$) and hypoxia ($p < 0.001$) were as follows: saline normoxic versus saline hypoxic: $p < 0.05$ (1.0 ± 0.0-fold vs 2.1 ± 0.5-fold); NAC normoxic versus NAC hypoxic: $p < 0.05$ (1.24 ± 0.2-fold vs 2.2 ± 0.4-fold); saline hypoxic versus NAC hypoxic: $p = 0.498$ (2.1 ± 0.5-fold vs 2.2 ± 0.4-fold). Fig. 4*E* shows that mice perfused with 30, 50, and 100 μM adenosine have a 167%, 225%, and 247% increase in brain caspase 1 activity, respectively. PBS perfused versus adenosine perfused. Main effect of treatment ($p < 0.001$) is as follows: 0 μM versus 10 μM, $p = 0.844$ (1.0 ± 0.1 vs 0.8 ± 0.1); 0 μM versus 30 μM, $p < 0.05$ (1.0 ± 0.1 vs 1.6 ± 0.2); 0 μM versus 50 μM, $p < 0.001$(1.0 ± 0.1 vs 2.3 ± 0.3); 0 μM versus 100 μM, $p < 0.001$ (1.0 ± 0.1 vs 2.5 ± 0.1); 10 μM versus 30 μM, $p = 0.01$ (0.8 ± 0.1 vs 1.6 ± 0.2); 10 μM versus 50 μM, $p < 0.001$(0.8 ± 0.1 vs 2.3 ± 0.3); 10 μM versus 100 μM, $p < 0.001$(0.8 ± 0.1 vs 2.5 ± 0.1); 30 μM versus 50 μM, $p = 0.075$ (1.6 ± 0.2 vs 2.3 ± 0.3); 30 μM versus 100 μM, $p = 0.023$ (1.6 ± 0.2 vs 2.5 ± 0.1); 50 μM versus 100 μM, $p = 0.940$ (2.3 ± 0.3 vs 2.5 ± 0.1). Figure 4, *F* and *G*, demonstrate that caffeine but not NAC inhibits adenosine-dependent activation of caspase 1 when adenosine is perfused into mice (Fig. 4*F*, main effect of treatment, $p < 0.001$) as follows: PBS versus adenosine, $p < 0.001$ (1.0 ± 0.1 vs 1.9 ± 0.1); adenosine versus caffeine, $p < 0.001$ (1.9 ± 0.1 vs 0.9 ± 0.1); adenosine versus adenosine plus caffeine, $p < 0.001$ (1.9 ± 0.1 vs 1.3 ± 0.1); adenosine plus caffeine versus caffeine, $p = 0.06$ (0.9 ± 0.1 vs 1.3 ± 0.1); PBS versus adenosine plus caffeine, $p = 0.267$ (1.0 ± 0.1 vs 1.3 ± 0.1); PBS versus caffeine, $p = 0.841$ (1.0 ± 0.1 vs 0.9 ± 0.1). In Figure 4*G*, the main effect of treatment ($p < 0.05$) is as follows: PBS versus adenosine, $p < 0.05$ (1.0 ± 0.0 vs 1.9 ± 0.1); adenosine versus NAC, $p < 0.05$ (1.9 ± 0.1 vs 1.2 ± 0.3); adenosine versus adenosine plus NAC, $p = 0.991$ (1.9 ± 0.1 vs 1.8 ± 0.1); adenosine plus NAC versus NAC, $p = 0.076$ (1.8 ± 0.1 vs 1.2 ± 0.3); PBS versus adenosine plus NAC, $p < 0.05$ (1.0 ± 0.0 vs 1.8 ± 0.1); PBS versus NAC: $p = 0.897$ (1.0 ± 0.0 vs 1.2 ± 0.3). Finally, to examine another potential activator of the inflammasome, uric acid was examined. After 1 h of reoxygenation, serum uric acid levels were similar in normoxic and hypoxic mice (3.3 ± 0.4 mg/dl vs 3. 1 ± 0.2 mg/dl).

Hypoxia induces brain region-specific activation of caspase 1

Figure 5*A* shows that 1 h after hypoxia/reoxygenation, caspase 1 activity in the amygdala compared to control was increased 180% (normoxic vs hypoxic, 1.0 ± 0.1 vs 1.8 ± 0.1, $p < 0.001$). Hippocampal caspase 1 activity was not impacted by hypoxia/reoxygenation (normoxic vs hypoxic, 1.0 ± 0.1 vs 0.7 ± 0.1, $p = 0.134$), and neither was prefrontal cortex or cerebellar caspase 1 activity (data not shown). To determine whether significant gliosis occurred after hypoxia, immunohistochemistry for GFAP was performed. Fig. 5*B* demonstrates no change in GFAP expression at 1 and 6 h of reoxygenation in both WT and IL-1R1 KO mice. Additionally, qPCR for GFAP and aquaporin 4 gene transcripts were examined in the amygdala and hippocampus at 1 and 6 h after reoxygenation in WT and IL-1R1 KO mice, revealing no impact of hypoxia (data not shown). To examine microglia infiltration/proliferation/activation, gene transcripts for CD11b, F4/80, and PBR were examined in the amygdala and hippocampus after 1 and 6 h of reoxygenation in WT and IL-1R1 KO mice. No differences were observed (data not shown).

AR blockade speeds recovery of memory formation after hypoxia

Figure 6*A* demonstrates that if memory formation (training) occurred 4 h after hypoxia, mice administered caffeine had accelerated recovery of memory formation. At 5 h of reoxygenation, hypoxic mice pretreated with caffeine explored a novel object similarly as normoxic mice (68.0 ± 3.6% vs 69.5 ± 5.6%). Hypoxic mice with a preadministered vehicle explored a novel object as if it were a familiar object (48.6 ± 2.4%). Main effects of treatment ($p < 0.05$) and hypoxia ($p < 0.05$) were as follows: saline normoxic versus saline hypoxic, $p < 0.05$ (69.5 ± 5.6% vs 48.6 ± 2.4%); caffeine normoxic versus caffeine hypoxic, $p = 0.838$ (69.2 ± 4.6% vs 68.0 ± 3.6%); saline hypoxic versus caffeine hypoxic, $p < 0.05$ (48.6 ± 2.4% vs 68.0 ± 3.6%). Fig. 6*B* demonstrates that if memory formation (training) occurred 4 h after hypoxia, mice administered 8-cyclopentyl-1,3-dipropylxanthine plus 3,7-dimethyl-1-propargylxanthine had accelerated recovery of memory formation. At 5 h of reoxygenation, hypoxic mice pretreated with 8-cyclopentyl-1,3-dipropylxanthine plus 3,7-dimethyl-1-propargylxanthine explored a novel object similarly as normoxic mice (76.1 ± 3.7% vs 73.5 ± 3.4%). Hypoxic mice preadministered vehicle explored a novel object as if it were a familiar object (62.4 ± 3.2%). Main

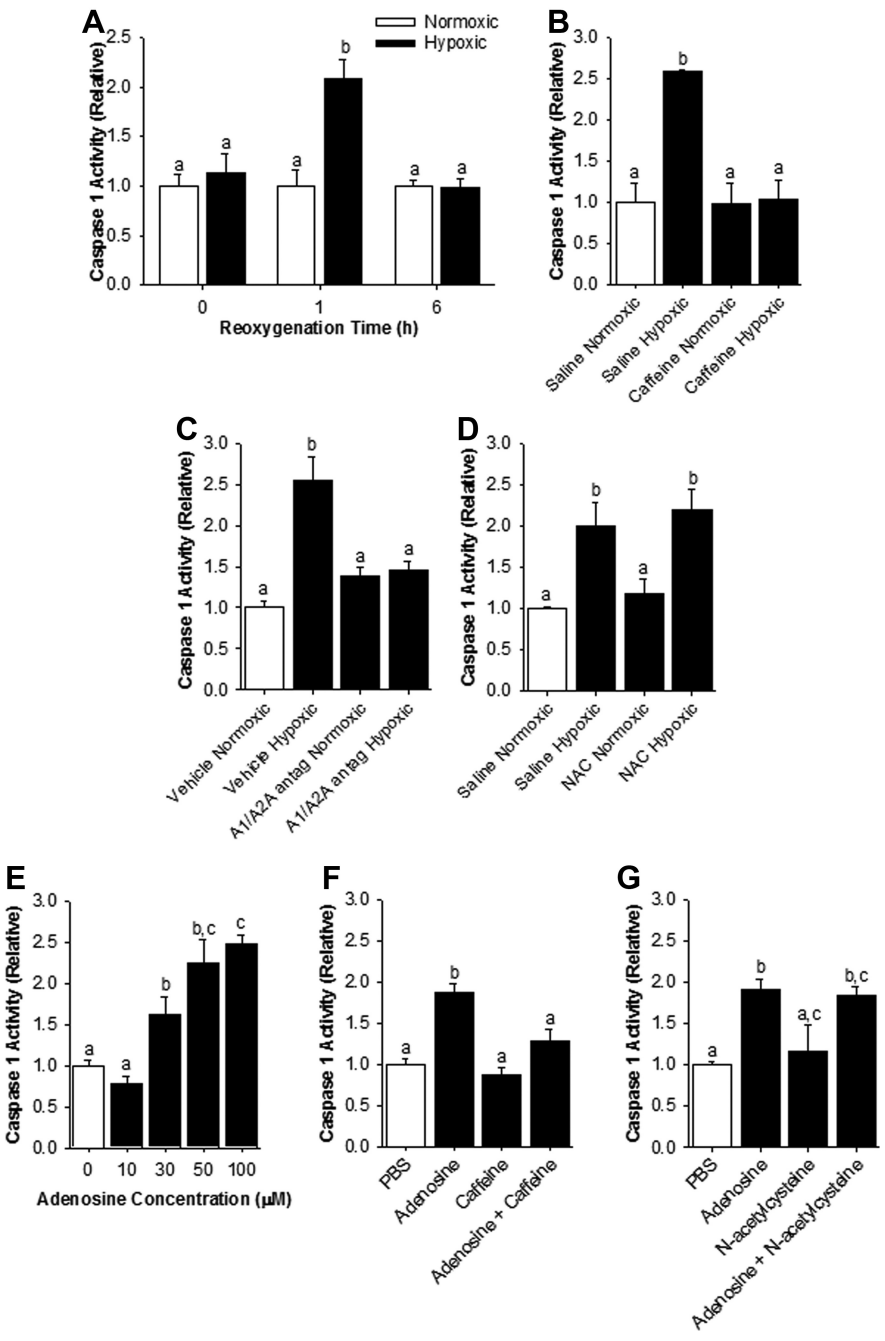

Figure 4. AR blockade prevents hypoxia-dependent activation of caspase 1 in the brain. ***A***, Wild-type mice were exposed to normoxia or hypoxia for 2 h. Caspase 1 activity was measured at the reoxygenation time points indicated. Results are expressed as means ± SEM; $n = 4$. Bars without a common superscript are different ($p < 0.05$). ***B***, Wild-type mice treated with/without caffeine were exposed to normoxia or hypoxia as in ***A***, and caspase 1 activity measured 1 h after reoxygenation. Results are expressed as means ± SEM; $n = 4$. Bars without a common superscript are different ($p < 0.05$). ***C***, Wild-type mice treated with/without 8-cyclopentyl-1,3-dipropylxanthine plus 3,7-dimethyl-1-propargylxanthine (A1/A2A antag) were exposed to normoxia or hypoxia as in ***A***, and caspase 1 activity was measured 1 h after reoxygenation. Results are expressed as means ± SEM; $n = 4$. Bars without a common superscript are different ($p < 0.05$). ***D***, Wild-type mice treated with/without NAC were exposed to normoxia or hypoxia as in ***A***, and caspase 1 activity was measured 1 h after reoxygenation. Results are expressed as means ± SEM; $n = 6$. Bars without a common superscript are different ($p < 0.05$). ***E***, Wild-type mice were perfused with the adenosine concentrations indicated. Caspase 1 activity was measured 1 h after perfusion. Results are expressed as means ± SEM; $n = 6$. Bars without a common superscript are different ($p < 0.05$). ***F***, Wild-type mice were perfused with/without 50 μM adenosine plus 500 μM caffeine. Caspase 1 activity was measured 1 h after perfusion. Results are expressed as means ± SEM; $n = 6$. Bars without a common superscript are different ($p < 0.05$). ***G***, Wild-type mice were perfused with/without 50 μM adenosine plus 500 μM NAC. Caspase 1 activity was measured 1 h after perfusion. Results are expressed as means ± SEM; $n = 6$. Bars without a common superscript are different ($p < 0.05$).

Table 1. Impact of reoxygenation on the glutathione to glutathione disulfide ratio in the brain

Treatment	Normoxic	Reoxygenation (1 h)
Saline	1.00 ± 0.21^a	0.49 ± 0.12^b
Caffeine	0.88 ± 0.15^a	0.87 ± 0.10^a
NAC	0.82 ± 0.02^a	1.03 ± 0.03^a

Wild type mice were exposed to normoxia or hypoxia for 2 h. Whole brains were harvested and analyzed for GSH and GSSG concentrations. Results are expressed as relative change in GSH/GSSG ratio, means ± SEM; $n = 4$. Results within individual rows without a common superscript are different ($p < 0.05$).

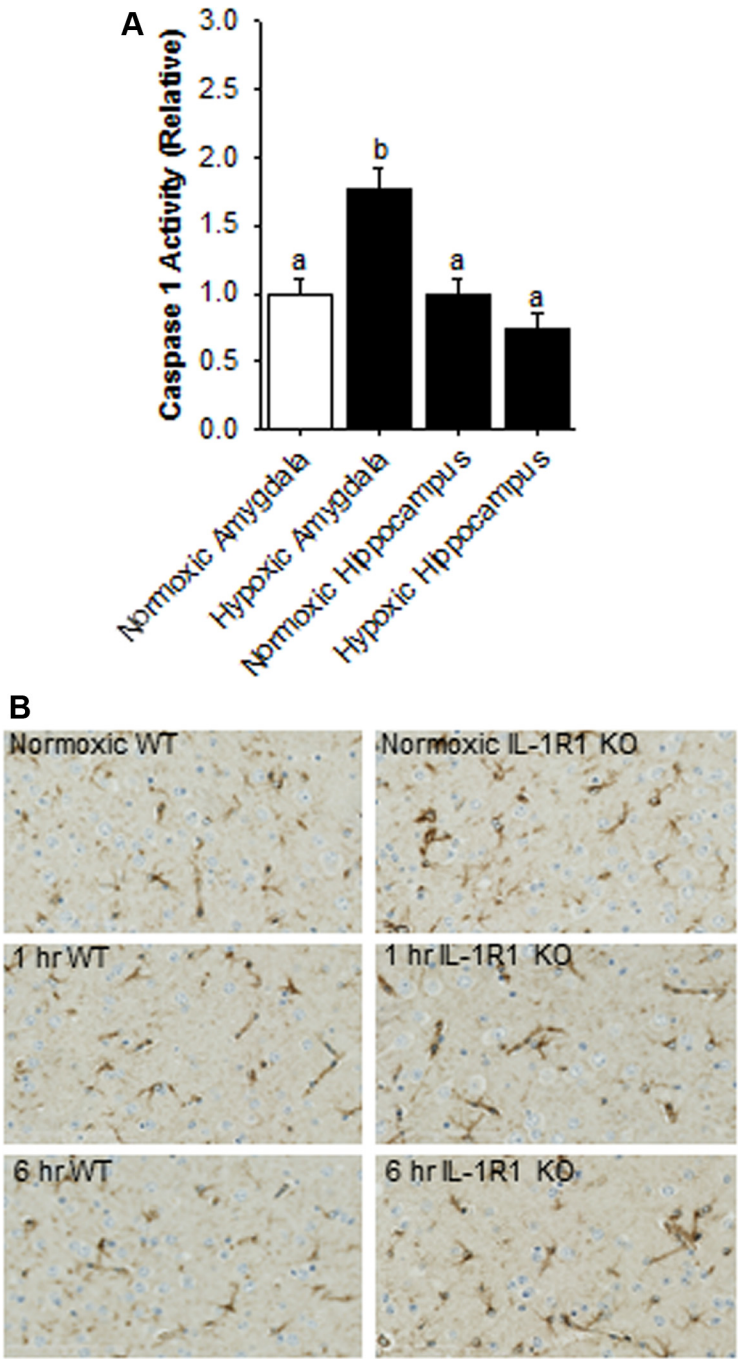

Figure 5. Hypoxia induces brain region-specific activation of caspase 1. ***A***, Wild-type mice were exposed to normoxia or hypoxia for 2 h. Caspase 1 activity was measured in the amygdala and hippocampus 1 h after reoxygenation Results are expressed as means ± SEM; $n = 4$. Bars without a common superscript are different ($p < 0.05$). ***B***, WT mice and IL-1R1 KO mice were treated as in ***A***. Immunohistochemistry was performed for GFAP at 1 and 6 h after reoxygenation. Representative images of the amygdala ($n = 3$).

effects of treatment ($p = 0.055$) and hypoxia ($p = 0.197$) (hypoxia × treatment, $p < 0.05$) were as follows: vehicle normoxic versus vehicle hypoxic, $p < 0.05$ (73.7 ± 2.7% vs 62.4 ± 3.2%), 8-cyclopentyl-1,3-dipropylxanthine plus 3, 7-dimethyl-1-propargylxanthine normoxic versus 8-cyclopentyl-1,3-dipropylxanthine plus 3,7-dimethyl-1-propargylxanthine hypoxic, $p = 0.583$ (73.5 ± 3.4% vs 76.1 ± 3.7%); vehicle hypoxic versus 8-cyclopentyl-1,3-dipropylxanthine plus 3,7-dimethyl-1-propargylxanthine hypoxic, $p < 0.05$ (62.4 ± 3.2% vs 76.1 ± 3.7%).

Discussion

In humans, the causes of a confusional state are numerous and include drugs, toxins, infections, head injuries, and metabolic derangements (Gascon and Barlow, 1970; Mori and Yamadori, 1987). Hypoxemia is a well recognized antecedent to brain injury (Rees et al., 1998) that can precipitate memory loss well beyond hypoxemia-associated confusion or delirium (Berggren et al., 1987). In rodents, hypoxia has long been known to cause retrograde amnesia (Sara and Lefevre, 1972). The inability of rodents to recall a previously learned task or avoid noxious stimuli after hypoxia can be long lived (24 h) as Sara et al. show in rats exposed to 3.5% oxygen (Sara and Lefevre, 1972). In addition, this memory loss can occur without significant brain cell death in mice because, as Kyff et al. (1989) show and we confirm, 2 h of hypoxia above 5% oxygen does not lead to identifiable neuronal death even in the hippocampus (Johnson et al., 2007). Here we show that the acute hypoxia used induced minimal neuroinflammation over the 6 h examined as reflected by no change in the gene expression of GFAP, aquaporin 4, CD11b, F4/80, and PBR and no change in GFAP protein expression. These findings indicate that unlike ischemia where all of the aforementioned astrocyte and microglial markers are rapidly upregulated (Feuerstein et al., 1997, Lu and Sun, 2003, Natale et al., 2003, Taguchi et al., 2007, Taniguchi et al., 2007, Xiong et al., 2009) and significant gliosis occurs, acute hypoxia can be a recoverable event that results in negligible neuropathology.

The mechanism protecting burrowing mammals [especially certain moles that can survive severe oxygen deprivation (3%) for extended times (8 h); Avivi et al., 2006] from acute hypoxia is not clear, but its origin, evolutionarily, appears as a safeguard to burrow collapse. As Fig. 1*A* shows, mice exposed to 6% hypoxia for 2 h developed retrograde amnesia that resolved after 4 h of reoxygenation. This loss of memory was not due solely to a motor deficit that prevented mice from performing the task, be-

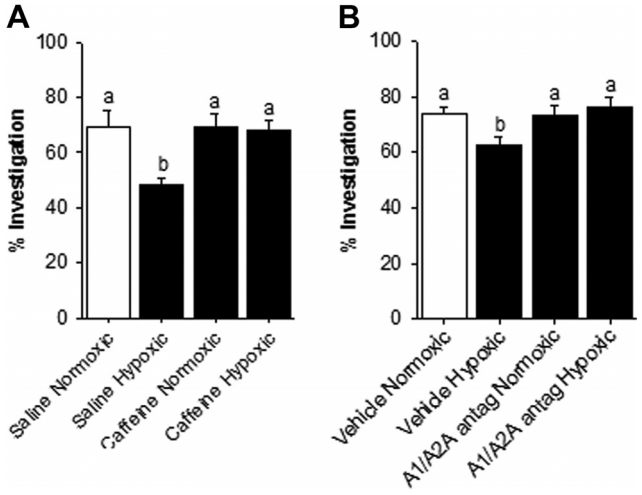

Figure 6. AR blockade speeds recovery of memory formation after hypoxia. ***A***, Wild-type mice treated with/without caffeine were exposed to normoxia or hypoxia for 2 h. Mice were trained in memory formation using novel object recognition after 4 h of reoxygenation. Memory recall (percent investigation) was measured after 5 h of reoxygenation. Results are expressed as means ± SEM; $n = 6$. Bars without a common superscript are different ($p < 0.05$). ***B***, Wild-type mice treated with/without 8-cyclopentyl-1,3-dipropylxanthine plus 3,7-dimethyl-1-propargylxanthine (A1/A2A antag) were exposed to normoxia or hypoxia as in ***A***, and memory was tested as in ***A***. Results are expressed as means ± SEM; $n = 6$. Bars without a common superscript are different ($p < 0.05$).

cause mice had regained normal locomotor activity after 2 h of reoxygenation (Fig. 1*B*).

Post-hypoxia, the ability of mice to form a new memory did not recover until after 6 h of reoxygenation (Fig. 2*A*). Since the training phase for novel object recognition occurred 1 h before the testing phase, memory formation after hypoxia was impaired within the first 5 h of reoxygenation. To determine whether the results obtained were memory type specific, cued fear conditioning and spatially cued Y-maze performance were used as additional tests of memory dysfunction. Fig. 2*B* illustrates that with cued fear conditioning, mice failed to learn when trained after 4 h of reoxygenation and that this was a failure of memory formation and not a deficiency in task performance because, even after 52 h of reoxygenation, mice did not recall the learned cue. Unlike fear conditioning, mouse performance in a spatially cued Y-maze was not impacted by hypoxia (Fig. 2*C*), demonstrating spatial memory, and hence the hippocampus may be less impacted by hypoxia.

Although hypoxia appears tied to anterograde amnesia in humans (Beatty et al., 1987), almost no work has been performed in animal models. A single study by Udayabanu et al. (2008) looked at retrograde and anterograde memory in a mouse model of acute hypobaric hypoxia with a calculated oxygen percentage of 7% (307.4 Torr) for 6 h. They concluded that hypoxia only impacted retrograde memory and not anterograde memory. While they conducted memory formation 2 h post-hypoxia, the tests they used (passive avoidance step-through and elevated plus maze) were strongly dependent on spatial memory and, thus, the hippocampus (Rodgers et al., 1997; Yirmiya et al., 2002). Likewise, in our study spatially cued Y-maze performance was not affected by hypoxia, while our more amygdala-dependent tests (object recognition and cued fear conditioning) were (Moses et al., 2005). Interestingly, Broadbent et al. (2004) showed that in rats spatial memory was impaired when 30–50% of the dorsal hippocampus or 50% of the ventral hippocampus is damaged. Object recognition memory, however, was only impaired after 75–100% of the dorsal hippocampus is injured (Broadbent et al., 2004). Taken together, these findings indicate that acute hypoxia impairs memory that is predominantly decoupled from the hippocampus. Support for this contention is seen in Fig. 5*A*, which illustrates that hypoxia/reoxygenation increases caspase 1 activation in the amygdala but not in the hippocampus.

Brain IL-1β is important to the recovery of memory formation after hypoxia. Fig. 3*C* shows that the caspase 1-specific inhibitor YVAD-CMK (Wu et al., 2010) administered intracerebroventricularly speeds the recovery of memory formation after hypoxia. Since caspase 1 can process other proteins besides pro-IL-1β (Keller et al., 2008), we confirmed these findings by administering IL-1 RA (Fig. 3*B*) and by using IL-1R1 KO mice (Fig. 3*A*). Since YVAD-CMK was administered intracerebroventricularly, our results indicate that brain-generated IL-1β is responsible for impairing memory formation after hypoxia as opposed to IL-1 from the peripheral blood. That a dysregulation in brain IL-1 negatively impacts certain aspects of memory is not surprising. IL-1 is best known for its role in hippocampal-dependent memory (Goshen et al., 2007), and conditions that disrupt IL-1 signaling impair mouse water maze and passive avoidance performance (Yirmiya et al., 2002). As proposed by Goshen and Yirmiya (2002), hippocampal-dependent memory and plasticity are regulated by IL-1 in an inverted U-shaped correlation where low and high brain IL-1 signaling have similar impacts. As for the role of IL-1 in novel object recognition, little is known. Costello et al. (2011) just demonstrated that mice deficient in the single Ig-interleukin-1 related receptor have impaired novel object recognition as well as an upregulation of IL-1α, but not IL-1β, in the brain (Costello et al., 2011). They propose that IL-1α via IL-1R1 may drive certain memory impairments, especially as related to infectious etiologies. Figure 3*E* shows that in hypoxia, activation of ERK1/2 and p38 MAPK may be important to IL-1 regulated memory, because 1 h after reoxygenation these kinases had reduced phosphorylation in IL-1R1 KO mice when compared to wild-type mice. Thus, our results suggest that IL-1β may be more important in disease states involving low oxygen and that MAPK superfamily members downstream of IL-1R1 may be regulatory.

As we and others have shown, brain IL-1β is elicited during activation of the neuroimmune system (Kostulas et al., 1999; Johnson et al., 2007; Dantzer et al., 2008). Basally, mature IL-1β is nearly undetectable in the rodent brain (Layé et al., 2000; Takao et al., 1993; Taupin et al., 1993), but with neuroimmune stimulation, especially ischemic injury, IL-1β is measurable (Saito et al., 1996) and promotes brain injury (Rothwell, 2003). How mature IL-1β is generated in the brain is unknown. Peripherally, the inflammasome is critical to caspase 1 activation, and caspase 1 is responsible for the final enzymatic cleavage of pro-IL-1β to secretable IL-1β (Lamkanfi et al., 2007). Fig. 4*A* demonstrates that there is increased brain caspase 1 activity after hypoxia but that reoxygenation is important to this activity, because non-reoxygenated mice do no show increased caspase 1 activity. Since reoxygenation appears necessary to brain caspase 1 activation, we examined known danger signals potentially relevant to peripheral inflammasome activation and to hypoxia. ROS are considered to play a role in reoxygenation/reperfusion injury, and redox-dependent activation of the NLRP3 inflammasome has recently been shown (Martinon, 2010; Tschopp and Schroder, 2010). Table 1 demonstrates that hypoxia/reoxygenation markedly reduced the GSH/GSSG ratio indicative of ROS generation. As expected, administration of the GSH precursor and antioxidant NAC (Raju et al., 1994) before hypoxia prevented a hypoxia/

reoxygenation-dependent decline in the GSH/GSSG ratio. Additionally, caffeine, which has been shown to have antioxidant properties (Shi and Dalal, 1991; Devasagayam et al., 1996), also prevented a decline in the GSH/GSSG ratio after hypoxia/reoxygenation. Unexpectedly, caffeine (Fig. 4*B*) and more importantly antagonism of the A1+A2A ARs (Fig. 4*C*) prevented reoxygenation-dependent activation of caspase 1 while NAC did not (Fig. 4*D*). Furthermore, caffeine (Fig. 6*A*) and A1/A2A AR antagonism (Fig. 6*B*), but not NAC (data not shown), sped recovery from hypoxia-induced loss of memory formation. These findings indicate that adenosine triggers hypoxia/reperfusion-dependent caspase 1 activation, resulting in a delay in the ability to form new memories. To further confirm that adenosine activates caspase 1 in the brain, mice were perfused with adenosine. Fig. 4*E* shows that adenosine activates brain caspase 1 in a dose-dependent manner and that the EC_{50} of 30 μM is consistent with interstitial adenosine concentrations seen in the microenvironment of hypoxic tissue (10–50 μM) (Sitkovsky and Lukashev, 2005). Figure 4, *F* and *G*, demonstrates that caffeine but not NAC blocked adenosine-dependent activation of caspase 1, additionally illustrating that ARs but not ROS are important to adenosine-dependent caspase 1 activation.

To date, neither adenosine nor its receptors are implicated as direct activators of the inflammasome or of caspase 1. However, AR antagonism, especially via caffeine, is linked to cognitive improvement in certain neurodegenerative diseases (Cunha and Agostinho, 2010) and as a protectant against Alzheimer's disease (Cao et al., 2012). How caffeine achieves this function is unclear, but it has been postulated that since adenosine acts as an inhibitory neurotransmitter, the ability of caffeine to suppress this effect of adenosine is beneficial (Chen et al., 2001). The antioxidant qualities of caffeine have been postulated as a mechanism, but this theory is not consistent with the neuroprotection from ischemia afforded by receptor-specific AR antagonists that lack antioxidant properties (Phillis, 1995) or seen in A2A AR KO mice (Chen et al., 1999). Therefore, caffeine acting as an inhibitor of IL-1β generation, as implicated here, is a potentially new mechanism for its action.

How adenosine activates the inflammasome through ARs needs to be defined. Purines are metabolized to uric acid, which is a well described activator of the inflammasome (Pétrilli and Martinon, 2007). We measured serum uric acid after hypoxia and saw no increase after 1 h of reoxygenation. This finding is consistent with others who have seen that xanthine oxidase, which catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid, is increased after reoxygenation but only at more distant time points (Jones et al., 1968; González-Flecha and Cutrin, 1993; Poulsen et al., 1993; Cherubini et al., 2000). A potential mechanism by which ARs activate caspase 1 is through K^+ flux. When triggered by high extracellular concentrations of K^+, pannexin 1 channels induce activation of caspase 1 (Silverman et al., 2009). Hypoxia causes increased extracellular concentrations of K^+ in brain slice cultures that can be inhibited by AR antagonism (Croning et al., 1995). Importantly, adenosine can stimulate cellular K^+ release through the A1 and A2A ARs (Fredholm et al., 2001, Sanjani et al., 2011).

Finally, the brain is rich in ARs, and they are expressed on a wide range of brain-based cells including microglia (A1, A2A, and A3) (Sperlágh and Illes, 2007), astrocytes (A1, A2A, A2B and A3), neurons (A1 and A2A) (Haskó et al., 2005), and endothelial cells (A2A and A3) (Fredholm et al., 2001; Platts and Duling, 2004). Thus, further work is needed to determine not only how adenosine activates caspase 1 but what are the particular brain cells most responsible for caspase 1 activation and the production of mature IL-1β after hypoxia/reoxygenation. Currently, some specificity to the brain is evident, because hypoxia/reoxygenation did not increase liver caspase 1 activity (data not shown). Taken together, our results indicate that hypoxia/reoxygenation increases caspase 1 activity in the brain, thereby impairing amygdala-based memory formation. These findings are important, because they delineate a new mechanism linking adenosine to activation of the inflammasome.

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