Targeting type-2 metabotropic glutamate receptors to protect vulnerable hippocampal neurons against ischemic damage

Marta Motolese, Federica Mastroiacovo, Milena Cannella, Domenico Bucci, Anderson Gaglione, Barbara Riozzi, Robert Lütjens, Sonia M. Poli, Sylvain Celanire, Valeria Bruno, Giuseppe Battaglia and Ferdinando Nicoletti

Abstract

Background: To examine whether metabotropic glutamate (mGlu) receptors have any role in mechanisms that shape neuronal vulnerability to ischemic damage, we used the 4-vessel occlusion (4-VO) model of transient global ischemia in rats. 4-VO in rats causes a selective death of pyramidal neurons in the hippocampal CA1 region, leaving neurons of the CA3 region relatively spared. We wondered whether changes in the expression of individual mGlu receptor subtypes selectively occur in the vulnerable CA1 region during the development of ischemic damage, and whether post-ischemic treatment with drugs targeting the selected receptor(s) affords neuroprotection.

Results: We found that 4-VO caused significantly reduction in the transcript of mGlu2 receptors in the CA1 region at times that preceded the anatomical evidence of neuronal death. Down-regulation of mGlu2 receptors was associated with reduced H3 histone acetylation at the Grm2 promoter. The transcripts of other mGlu receptor subtypes were unchanged in the CA1 region of 4-VO rats. Ischemia did not cause changes in mGlu2 receptor mRNA levels in the resistant CA3 region, which, interestingly, were lower than in the CA1 region. Targeting the mGlu2 receptors with selective pharmacologic ligands had profound effects on ischemic neuronal damage. Post-ischemic oral treatment with the selective mGlu2 receptor NAM (negative allosteric modulator), ADX92639 (30 mg/kg), was highly protective against ischemic neuronal death. In contrast, s.c. administration of the mGlu2 receptor enhancer, LY487379 (30 mg/kg), amplified neuronal damage in the CA1 region and extended the damage to the CA3 region.

Conclusion: These findings suggest that the mGlu2 receptor is an important player in mechanisms regulating neuronal vulnerability to ischemic damage, and that mGlu2 receptor NAMs are potential candidates in the experimental treatments of disorders characterized by brain hypoperfusion, such as hypovolemic shock and cardiac arrest.

Keywords: ADX92639, Epigenetics, Global ischemia, mGlu2 receptors, Neuronal vulnerability

Introduction

Understanding the mechanisms underlying neuronal vulnerability to ischemic damage may pave the way to novel therapeutic strategies in cerebrovascular disorders. Transient cerebral global ischemia in experimental animal models or humans causes the degeneration of specific populations of vulnerable neurons, such as pyramidal neurons of the CA1 hippocampal subfield [1, 2]. Neurodegeneration develops slowly and becomes manifest only several hours following reperfusion [2]. This relatively long temporal window facilitates the study of the molecular mechanisms underlying the delayed ischemic neuronal death, and allows the translation of these mechanisms into potential therapeutic targets. One of the most widely used animal models of transient global ischemia is the 4-vessel occlusion (4-VO) model in rats [3]. In rats subjected to 4-VO, CA1 pyramidal neurons typically die at 48-72 h after reperfusion, whereas neurons of the CA3 region and the dentate gyrus are relatively spared, at least at this...
timepoint [4, 5]. The use of this model led to the identification of early molecular events occurring in neurons destined to die, such as derepression of the gene silencer, REST [6], down-regulation of the Ca\textsuperscript{2+}-impermeable AMPA receptor subunit, GluA2 [2, 4, 7], activation of nuclear factor-κB (NFκB) and cyclooxygenase-2 [8, 9], and induction of the Wnt inhibitor, Dickkopf-1 [5]. These findings laid the groundwork for pharmacological studies showing that AMPA receptor antagonists are able to protect hippocampal CA1 neurons against ischemic damage [10–12].

Subtype-selective ligands of metabotropic glutamate (mGlu) receptors have shown efficacy as neuroprotective drugs in models of transient global ischemia [13]. Pellegrini-Giampietro and colleagues have consistently shown that mGlu1, but not mGlu5, receptor antagonists are protective against ischemic damage of CA1 pyramidal neurons in gerbils exposed to global ischemia and in organotypic hippocampal slices exposed to oxygen/glucose deprivation [14, 15, 16]. Expression and function of mGlu5 receptors are decreased in the hippocampal CA1 region at early times after transient global ischemia [17–19], whereas mGlu1 mRNA levels are either decreased [17] or unchanged [18].

Mixed orthosteric agonists of mGlu2 and mGlu3 receptors (compounds LY354740 and LY379268) showed also neuroprotective activity in the gerbil model of transient global ischemia [20, 21], raising the important question of which of the two subtypes should be specifically targeted by therapeutic interventions. Interestingly, studies carried out in cultured neurons suggest that activation of mGlu3 receptors is neuroprotective, whereas activation of mGlu2 receptors is not harmful under control conditions, but amplifies neuronal damage if combined with neurotoxic insults, such as N-methyl-D-aspartate (NMDA) [22] or β-amyloid peptide [23]. In situ hybridization analysis in the hippocampus of rats subjected to transient global ischemia showed either increases [18] or reductions [17] in mGlu2 receptor mRNA at 24 h after reperfusion, with no changes in the transcript of mGlu3 receptors. Whether expression of mGlu2 and mGlu3 receptors differs in vulnerable and resistant hippocampal subregions in response to global ischemia is unknown.

We now report that transient global ischemia causes an epigenetic down-regulation of mGlu2 receptors, which selectively occurs in the vulnerable CA1 region. Post-ischemic treatment with a selective negative allosteric modulator (NAM) of mGlu2 receptors protected CA1 neurons against ischemic damage, whereas treatment with a positive allosteric modulator (PAM) of mGlu2 receptors extended ischemic damage to CA3 neurons.

**Results**

**Temporal profile of neuronal damage in CA1 and CA3 regions in rats subjected to transient global brain ischemia**

Transient global ischemia induced by 4-VO in rats caused the expected loss of CA1 pyramidal neurons at 72 h following reperfusion, with no detectable neuronal death at 6, 12, or 24 h (images at 24 and 72 h are shown in Fig. 1). Neurons of the CA3 region and the dentate gyrus were largely resistant to ischemic damage, at least at 72 h after reperfusion (Fig. 1).

**Global ischemia caused an early down-regulation of mGlu2 receptors in the CA1 region**

We measured the transcripts of mGlu1, mGlu2, mGlu3, and mGlu5 receptors by qPCR in microdissected CA1 and CA3 regions of sham-operated and 4-VO rats at times that preceded neuronal death (12 and 24 h after reperfusion). We were surprised to find that sham-operated rats showed much lower mGlu2 mRNA levels in CA3 than in CA1 region at both times (Fig. 2a). This distribution pattern was unique to mGlu2 receptors, at least with respect to mGlu1, mGlu3, and mGlu5 receptors (Fig. 2b–d). 4-VO Rats showed a substantial decrease in mGlu2 mRNA levels in the CA1 region, with no changes in the CA3 region. This reduction was more prominent at 24 h than at 12 h after reperfusion (-57 % and -46 % vs. the respective values of sham-operated rats, respectively) (Fig. 2a). Ischemia did not cause significant changes in mGlu1, mGlu3, and mGlu5 receptor mRNA levels in the two hippocampal subregions (Fig. 2b–d). At 24 h, mGlu1 mRNA levels were higher in CA3 than in CA1 region in both sham-operated and ischemic rats (Fig. 2c).

**Global ischemia up-regulated HDAC2 and reduced histone acetylation in the mGlu2 receptor gene promoter in the CA1 region at times that precede neuronal death**

In order to dissect the epigenetic programming leading to changes in the expression of mGlu2 receptors in response to global ischemia, we measured the transcript encoding for type-1 and type-3a DNA-methyltransferases (Dnmt1 and -3a) and type-1, -2, and -3 histone deacetylases (Hdac1, -2 and -3) in the CA1 and CA3 regions in 4-VO rats at 6, 12, and 24 h after reperfusion and at corresponding times in sham-operated rats. We also measured the transcript of Gadd45β, which is the product of a DNA-damage responsive gene and is involved in mechanisms of DNA demethylation [24–26], and the transcript of the glucocorticoid receptor (GR, encoded by the Nr3c1 gene), which is known to regulate the expression of Hdac2 [27]. Transient global ischemia caused a selective increase in the transcripts of Hdac2 and GRs in the vulnerable CA1 region at 12 h after reperfusion (Fig. 3b, c). The transcript...
encoding for Gadd45β was strongly up-regulated at early times after reperfusion (6 and 12 h) in both CA1 and CA3 (Fig. 3d; see also ref. [28]), suggesting that the resistant CA3 region was also reactive to the ischemic insult at early times after reperfusion. The transcripts of Dnmt1 and Dnmt3a did not change in CA1 in response to global ischemia. Increases in Dnmt3a mRNA levels were only observed in the CA3 region at 6 h after ischemia (Fig. 3a). Hdad1 and Hdad3 mRNA levels did no change in CA1 of 4-VO rats. We only found a reduction of Hdad1 mRNA levels in CA3 at 24 h after ischemia with respect to the corresponding group of sham-operated rats (Fig. 3b).

Knowing that the mGlu2 receptor gene (Grm2) gene promoter is targeted by HDAC2 [29], we extended the analysis to the HDAC2 protein levels and to histone H3 acetylation at the mGlu2 receptor gene promoter in the CA1 region at 12 and 24 h after reperfusion. Immunoblot analysis showed a significant increase in HDAC2 protein levels at 12 h, and a reduction at 24 h after reperfusion (Fig. 4a). ChIP analysis of acetylated-H3 histone bound to the Grm2 promoter showed a trend to a reduction (-50%) at 12 h, and a significant reduction (-53%) at 24 h after reperfusion (Fig. 4b).

Effect of post-ischemic treatment with selective mGlu2 receptor ligands on neuronal damage in 4-VO rats

Because orthosteric ligands do not differentiate between mGlu2 and mGlu3 receptors, we used two brain permeant allosteric modulators to specifically examine the influence of mGlu2 receptors on neurodegeneration/neuroprotection in the 4-VO model of transient global ischemia. As a selective mGlu2 PAM, we used a commercially available compound (LY487379), which has no intrinsic agonist activity at mGlu2 receptors, but markedly potentiates glutamate-evoked responses at mGlu2 receptors [30, 31]. To selectively inhibit mGlu2 receptors, we used compound ADX92639, the characterization of which is reported below.

In vitro pharmacological profile of ADX92639

Compound ADX92639 was tested in an agonist or antagonist mode with a FLIPR-based intracellular Ca²⁺ mobilization assay using HEK293 cells stably expressing rat or human recombinant mGlu receptor subtypes. ADX92639 exhibited a high potency as an mGlu2 receptor antagonist, with apparent IC₅₀ values of 175 nM and 145 nM at human and rat mGlu2 receptor clones, respectively (Fig. 5a). The compound had no activity as an agonist, antagonist, PAM or NAM at all other mGlu receptor subtypes (data not shown). In addition, ADX92639 was inactive up to 10 μM in competition binding assay on membranes expressing 71 G-protein coupled receptors, transporters, enzymes, and ion channels (binding profile at Cerep, data not shown).
Schild-plot experiments carried out in human mGlu2 receptor clones showed that ADX92639 induced a rightward shift of the glutamate concentration-response curve (in the FLIPR assay) together with a decrease in glutamate efficacy (Fig. 5b). This profile is consistent with a NAM activity of ADX92639 at mGlu2 receptors. Reversibility experiments (Fig. 5c) showed that the effect of ADX92639 observed in the Schild-plot analysis was not due to a non-reversible competitive effect of the compound.

The non competitive nature of the antagonist activity of ADX92639 was confirmed by competition experiments using [3H]LY341495, in which ADX92639 was unable to displace specifically bound [3H]LY341495 to membranes from human mGlu2 receptor clones, as opposed to LY341495 and glutamate (Fig. 5d).

Repeated treatment with ADX92639 did not cause changes in body temperature in rats

Knowing that mGlu2 receptor ligands may cause changes in body temperature [32–35] we measured body temperature in rats after oral administration of ADX92639 or its vehicle. ADX92639 or vehicle was administered three times with intervals of 24 h. Values of body temperature, collected at different times from 1 to 58 h after the first administration, did not differ between the groups of rats treated with ADX92639 and vehicle. In none of the timepoints values were lower than 35.8 °C in both groups of rats (Table 1).

Treatment with the mGlu2 receptor negative allosteric modulator, ADX92639, protected CA1 neurons against ischemic damage, whereas treatment with the mGlu2 receptor enhancer, LY487379, extended ischemic damage to CA3 neurons

In a first set of experiments, 4-VO rats were treated orally with 30 mg/kg of ADX92639 or vehicle at 12, 36, and 60 h after reperfusion. Sham-operated rats were only treated with vehicle. CSF exposure of ADX92639 after an oral dose of 30 mg/kg is largely (4 fold) above the in vitro IC₅₀ value at the mGlu2 receptor; in addition, 30 mg/kg is the dose of ADX92639 that has previously shown to produce maximal effects in several behavioural models of
cognition [36]. Treatment with ADX929639 in 4-VO rats was highly protective against ischemic neuronal death in the CA1 region at 72 h after reperfusion. As expected, global ischemia did not cause detectable changes in the number of viable neurons in the CA3 region, regardless of treatment with ADX92639 (Fig. 6a, b).

Additional groups of 4-VO rats were treated s.c. with the mGlu2 PAM, LY487379 (30 mg/kg) or vehicle at 12, 24, 36, 48, and 60 h after reperfusion. Sham-operated rats were also treated with LY487379 or vehicle at the corresponding times. Treatment with LY487379 in 4-VO rats caused a non-significant trend to the amplification of neuronal death in the CA1 region, and, interestingly,
extended ischemic damage to the CA3 region (Fig. 6c, d). The two sham-operated rats treated with LY487379 showed no signs of neuronal death in the CA1 region (number of viable neurons = 10,601 and 9599/mm²) with respect to sham-operated rats treated with vehicle (see Fig. 6c), suggesting that the compound was not neurotoxic on its own (i.e., in the absence of ischemia).

**Discussion**

The Grm2 gene encoding the mGlu2 receptor is highly plastic and undergoes epigenetic modifications in response to environmental stressors and drug treatments. For example, exposure to prenatal stress, activation of mineralocorticoid receptors by corticosterone, and treatment with atypical antipsychotic drugs all cause an epigenetic down-regulation of mGlu2 receptors in forebrain regions, such as the hippocampus and frontal cortex [29, 37, 38]. Expression of mGlu2 receptors is heavily regulated by acetylation mechanisms [38–41], and HDAC2 in particular was found to bind to the Grm2 promoter and inhibit mGlu2 receptor expression [29]. A high sensitivity to epigenetic regulation is a distinct feature of the mGlu2 receptor. Of note, the mGlu3 receptor, which shares structural and functional similarities with the mGlu2 receptor, is less susceptible to epigenetic regulation [29, 37, 40].

Present data offer the first demonstration that a specific insult (transient global ischemia) causes epigenetic changes in mGlu2 receptors that might be related to mechanisms of neurodegeneration/neuroprotection. Expression of mGlu2 receptors in the CA1 region was down-regulated in response to ischemia at times that preceded the anatomical evidence of neuronal death. Histone acetylation at the Grm2 promoter was also reduced in CA1 at early times after reperfusion (12 and 24 h), whereas HDAC2 and its transcriptional
activator, GR [27], showed an early up-regulation (12 h) following reperfusion. HDAC2 levels decreased in the CA1 region at 24 h after reperfusion perhaps as a result of a negative feedback regulation. These data suggest the following scenario: transient global ischemia causes the GR-mediated induction of HDAC2, which, in turn, associates with the Grm2 gene promoter causing a long-lasting histone deacetylation and inhibition of gene expression. After global ischemia, the mGlu2 receptor was down-regulated in the vulnerable CA1 region, but not in the resistant CA3 region. This raised two questions that may be relevant to the study of selective neuronal vulnerability to ischemic damage: (i) is the down-regulation of mGlu2 receptors neurotoxic or neuroprotective?; (ii) why is the mGlu2 receptor specifically down-regulated in the CA1 region after ischemia? Studies carried out in neuronal cultures challenged with excitotoxins or β-amyloid peptide suggest that activation of mGlu2 receptors is detrimental to neurons at least within the context of a neurodegenerative process [22, 23]. Present data are fully consistent with this hypothesis. We used a NAM (compound ADX92639), which showed a high degree of selectivity for mGlu2 receptors and did not cause changes in body temperature following systemic administration. ADX92639, given orally several hours after reperfusion, was highly protective against CA1 damage. In contrast, pharmacological enhancement of mGlu2 receptors with the selective PAM, LY487379, increased neuronal damage. This suggests that the epigenetic down-regulation of mGlu2 receptors occurring in the CA1 region at 12-24 h after reperfusion represents a potential defensive mechanism. However, the residual activity of mGlu2 receptors still contributes to the progression of neuronal damage unless the receptor is pharmacologically inhibited in the post-ischemic period.

The specificity of mGlu2 receptor changes for the CA1 region could be simply explained by assuming that it is only this region that is sensitive to ischemic insult. However, global ischemia caused an early up-regulation of the death-related protein, GADD45β, in both CA1 and CA3 (see also ref. [28]). This suggests that a death gene program is induced in both vulnerable and resistant neurons after global ischemia, and that resistant neurons harbor a molecular repertoire that restrains the development of the program. We were surprised to find that mGlu2 receptor mRNA levels were much lower in CA3 than in CA1 both under basal conditions and in...
Table 1 Effects of ADX92639 or its vehicle on body temperature at different times (1-58 h) after first administration

| Hours | Vehicle | ADX92639 |
|-------|---------|----------|
| 0     | 36.9 ± 0.3 | 37.0 ± 0.1 |
| 1     | 36.4 ± 0.3 | 36.5 ± 0.3 |
| 3     | 35.9 ± 0.1 | 36.1 ± 0.1 |
| 6     | 35.8 ± 0.1 | 35.8 ± 0.1 |
| 9     | 37.0 ± 0.1 | 36.9 ± 0.2 |
| 24    | 36.2 ± 0.1 | 36.2 ± 0.4 |
| 25    | 35.9 ± 0.0 | 35.8 ± 0.1 |
| 27    | 35.9 ± 0.0 | 35.9 ± 0.1 |
| 30    | 36.2 ± 0.2 | 36.3 ± 0.2 |
| 33    | 36.8 ± 0.3 | 36.1 ± 0.2 |
| 48    | 36.0 ± 0.1 | 35.9 ± 0.0 |
| 49    | 36.1 ± 0.0 | 36.1 ± 0.0 |
| 52    | 36.1 ± 0.1 | 36.1 ± 0.1 |
| 55    | 36.2 ± 0.0 | 36.2 ± 0.1 |
| 58    | 36.7 ± 0.0 | 36.5 ± 0.3 |

Effect of oral treatment with ADX92639 (30 mg/kg) or vehicle on body temperature in normal rats. ADX92639 or vehicle were administered three times with 24 h of interval. Rectal temperature was measured at different times (1-58 h) after the first administration of ADX92639 or vehicle. Value are means ± S.E.M. (n = 3)

response to ischemia. The low expression of mGlu2 receptors might contribute to the intrinsic resistance of CA3 neurons to ischemic damage unless the function of these receptors is pharmacologically amplified at critical time windows after reperfusion. This explains why post-ischemic treatment with the mGlu2 receptor PAM, LY487379, extended neuronal death to the CA3 region, which was otherwise resistant. We wish to highlight that LY487379 did not cause any apparent sign of neuronal damage in non-ischemic rats, suggesting that pharmacological activation of mGlu2 receptor is not toxic per se but may play a permissive role on neuronal damage in the presence of an ischemic insult.

The molecular mechanism by which activation of mGlu2 receptors becomes detrimental for neurons committed to die is unknown. mGlu2 receptors are coupled to G_\alpha proteins and their activation leads to inhibition of adenylate cyclase activity and inhibition of voltage-sensitive Ca^{2+} channels with ensuing reduction of neurotransmitter release [42]. It is possible that either inhibition of GABA release from CA1 interneurons [43, 44] or activation of intracellular death-related mechanisms in pyramidal neurons are involved in the permissive role of mGlu2 receptors on neuronal damage. However, our analysis of Grm2 expression was not carried out at cellular level, and we cannot exclude that mGlu2 receptors present in cells other than pyramidal neurons are involved in the pathophysiology of ischemic neuronal death and can be targeted by neuroprotective drugs. For example, inflammatory mechanisms and microglial activation contribute to neuronal degeneration in models of transient global ischemia, including the 4-VO model [45–47]. mGlu2 receptors are present in microglia [48, 49], and activation of mGlu2 receptors induces a pro-inflammatory and neurotoxic phenotype in microglia [48, 50, 51]. Microglial cells respond to mGlu2 receptor activation with an enhanced production of tumor-necrosis factor-α and other mechanisms that are detrimental to neighbor neurons [50, 51]. On the basis of data obtained in an in vitro model of oxygen-glucose deprivation, it has been suggested that activation of microglial mGlu2 receptors by the glutamate released from ischemic neurons may contribute to the overall process of neuronal death [51]. Thus, it cannot be excluded that mGlu2 receptor blockade in microglia might have contributed to the protective effect of ADX92639 we have seen in the 4-VO model.

Our findings raise the possibility that mGlu2 receptor NAMs might be helpful in the treatment of cardiac arrest, hypovolemic shock, severe hypotension or other pathologies that may reduce brain perfusion below a critical threshold and cause damage of vulnerable hippocampal neurons. To support this possibility it will be important to extend the study to other mGlu2 receptor NAMs and also to induce transient global ischemia in rats lacking mGlu2 receptors [52, 53]. Of note, preclinical studies show that mGlu2 receptor NAMs have a potential therapeutic value in the treatment of cognitive dysfunction associated with major depression, which is often resistant to conventional antidepressant medication [54, 55]. The neuroprotective effect of mGlu2 receptor NAMs may be an added value if these drugs are used for the treatment of depression associated with vascular dementia or chronic cardiovascular disorders causing brain hypoperfusion and neuronal damage.

Conclusions

We found that the mGlu2 receptor was epigenetically down-regulated in the vulnerable CA1 region during the development of ischemic damage. This change might represent a protective mechanism aimed at restraining ischemic neuronal damage, because post-ischemic treatment with an mGlu2 receptor NAM reduced the extent of ischemic damage. These findings may be relevant in the mGlu receptor field for three reasons. First, they suggest that expression of mGlu2 receptors undergoes plastic changes under pathological conditions and this mechanism might be related to the intrinsic vulnerability of particular neuronal populations to ischemic damage. Second, they are in line with previous findings from our group demonstrating that pharmacological activation of mGlu2 receptors, which is safe under normal conditions,
may be detrimental to neurons challenged by a toxic insult. Third, and more important, they suggest that selective mGlu2 receptor NAMs may be beneficial in the treatment of brain ischemia or hypoperfusion.

Materials and methods
Induction of transient global ischemia in rats
Adult male Sprague Dawley rats (Charles River, Calco, Italy), weighing 200-250 g, were housed under controlled conditions (ambient temperature, 22 °C; humidity, 40 %) on a 12 h light-dark cycle with food and water ad libitum. Animal experiments were performed in full compliance with the ARRIVE guidelines. All efforts were made to minimize the number of animals and animal suffering. The experimental protocol was approved by the Ethical Committee of Neuromed Institute (Pozzilli, Italy) and by The Italian Ministery of Health (D.M. 227/2011-B). Transient global ischemia was induced by the 4-VO method [3], as described previously [5] with minor modifications. In brief, animals were anesthetized with

Figure 6
Neuronal damage analysis of post-ischemia treatment with an mGlu2 receptor NAM, ADX92639, or PAM, LY487379. Nissl staining and neuronal density in the CA1 and CA3 regions of sham-operated and 4-VO rats treated with selective mGlu2 receptor ligands. In (a) and (b), vehicle or the mGlu2 receptor NAM, ADX92639 (30 mg/kg), were orally administered three times at 12, 36, and 60 h after reperfusion; sham-operated rats were only treated with vehicle. Rats were killed at 72 h after reperfusion. Values are means ± S.E.M. of 3-8 rats per group. *p < 0.05 vs. sham-operated rats (*) or vs. 4-VO rats treated with vehicle (#) (One-way ANOVA + Fisher LSD; F(2,18) = 10). In (c) and (d), vehicle or the mGlu2 receptor PAM, LY487379 (30 mg/kg), were s.c. injected five times at 12, 24, 36, 48 and 60 h after reperfusion. Sham-operated rats were only treated with vehicle (data of two sham-operated rats treated with LY487379 are reported in the Results section). Rats were killed at 72 h after reperfusion. Values are means ± S.E.M. of 3-5 rats per group. *p < 0.05 vs. sham-operated rats (*) or vs. 4-VO rats treated with vehicle (#) (One-way ANOVA + Fisher LSD; CA1: F(2,12) = 29.7, p < 0.05; CA3: F(2,12) = 5.9, p < 0.05)
chloral hydrate (350 mg/kg, i.p.), and positioned in a stereotaxic apparatus with the head tilted down at approximately 30° from the horizontal plane; an incision was made behind the occipital bone directly overlying the first two cervical vertebrae. The paraspinal muscles were separated from the midline, and the right and the left alar foramina of the first cervical vertebrae were exposed. A 0.5 mm electrocautery needle was inserted through each alar foramen, and both vertebral arteries were electrocauterized and permanently occluded. Afterwards, both common carotid arteries were exposed and isolated with a 3-0 silk thread, and the incision was sutured. Twenty-four hours later, animals were anesthetized with isoflurane (induction, 3 %; maintenance, 2 %), the wound was reopened, and both common carotid arteries were occluded with aneurismic clips for 10 min. Anesthesia was temporarily discontinued during carotid artery occlusion. Only rats showing complete loss of consciousness with loss of righting reflex and bilateral midriasis were included in the study. After 10 min of occlusion, isoflurane anesthesia was restored, both carotid arteries were re-opened and visually inspected to ensure adequate reflow, and the wound was sutured. Body temperature was monitored and maintained at 37 °C with rectal thermometer coupled to a heating blanket. After reperfusion, animals were kept for 2 h at 37 °C before being transferred to their home cages. For sham operation, animals were subjected to the same anesthesia and surgical procedures, except that carotid arteries were not occluded.

Biochemical analysis
Animals were killed by decapitation at 6, 12, 24, and 72 h after reperfusion. The brain was quickly removed and placed on an ice-cold rat brain matrix. A 2-mm thick slice containing the dorsal hippocampus was rapidly removed, and the CA1 and CA3 subfields were microdissected on ice under stereomicroscope, immediately frozen on liquid nitrogen, and stored at -80 °C.

RNA isolation, reverse transcription and qPCR
Total RNA from CA1 and CA3 hippocampal regions was extracted using Trizol reagent (Invitrogen) according to manufacturer’s protocol. The RNA was then treated with DNase (Qiagen) and single strand cDNA was synthesized from 2 μg of total RNA using superscript III (Invitrogen) and random hexamers. Real-time PCR was performed on 20 ng of cDNA by using specific primers and Power SYBR Green Master Mix (Applied Biosystem) on an Applied Biosystems Step-One instrument. Thermal cycler conditions were as follows: 10 min at 95 °C, 40 cycles of denaturation (15 sec at 95 °C), and combined annealing/extension (1 min at 60 °C). Primers used were as follows: Hdac1 Forw CTGTCCTCCGATTTGAGTC and Rev TCAGACTTCTTTCGATGGGTG; Hdac2 Forw AGTGTCGTAGCTGC and Rev GATTTGGCTCCTTTAT; Gadd45α Forw TGGAAGGTGGACAGTGAG and Rev CTTCAGAGTTCTCTGC; Dnmt1 Forw CCATCGTCAAAAGGGAAGGG and Rev GCCATCAGCTGATGATA; mGlu1 receptor Forw CTGTCACCAATGCTCAGCTC and Rev TCCAAAGTATAC and Rev ATAGCTGTCTGTGGGATCT; mGlu3 receptor Forw CAAGTGACTACAGGTGCAG and Rev CTGTCACAAATGCTCAGCTC; Dnmt2 Rev TCGTCCTGTCTCAGCACTC and Rev GCCATCCTTTTATTCTCCTCCT; Nr3c1 Forw CCATCGTGAAAAAGGGAAGG and Rev CAGCTAACATCTCTGGGAAT; mGlu4 receptor Forw CTGGAAGATAGCAGATG and Rev AGGTCGTGAGATCAGAGATTCCTGC; mGlu5 receptor Forw GCTGC and Rev CTCCAGAGTTCTCTGC; and Rev ACTCACCTTC and Rev GCTTTCCTCGACACCT and Rev GCCATCCTTTTATTCTCCTCCTCCT; mGlu2 receptor Forw GCTGC and Rev ACTCACCTTC and Rev GCTTTCCTCGACACCT and Rev GCCATCCTTTTATTCTCCTCCTCCT; and mGlu3 receptor Forw TGAAGATGTCGCTG and Rev GGTGTC; Hdac3 Forw TGGAAGGTGGACAGTGAG and Rev CTTCAGAGTTCTCTGC; Dnmt3a Forw CCATCGTGAAAAAGGGAAGG and Rev CAGCTAACATCTCTGGGAAT; Gadd45β Forw GC TGGCCATAGACAGAGAAGG and Rev GCCATCCTTC and Rev GTGACAGATGTTGACGAGCT and Rev TGGAGGCTGGAGAG; and β-actin Forw GTTGACATCCGTAAAGACC and Rev TGGAGGCTGGAGAGAG. mRNA copy number of each gene analyzed was calculated from serially diluted standard curves simultaneously amplified with the samples and normalized against β-actin copy number.

Chromatin immunoprecipitation
Chromatin immunoprecipitation assay was performed by standard procedures. Briefly, tissue was cross-linked using 1 % formaldehyde at room temperature for 10 min. The crosslinking reaction was stopped by adding glycine to a final concentration of 0.125 M. Tissue was then washed three times with ice-cold PBS supplemented with protease inhibitors (Sigma-Aldrich), homogenized in lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5 % NP40, protease inhibitors), and nuclei lysis buffer (1 % SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.0, protease inhibitors), and then sonicated on ice using a Covaris S220 ultrasonicator. Following sonication, chromatin fragments of 0.2-0.6 kb in length were obtained. Ten percent of the sonicated lysate was saved and successively used to quantify the total amount of DNA present in different samples before immunoprecipitation (Inputs). The chromatin solution was precleared with salmon sperm DNA/protein A-agarose 50 % gel slurry for 1 h at 4 °C and immunoprecipitated overnight at 4 °C with antibodies against acetyl-histone H3 (Upstate) or the respective isotype matched control Ig. After precipitation, the chromatin-antibody complexes were collected using Protein A Sepharose beads and washed. Samples were then eluted with 1 % SDS, 100 mM NaHCO₃ at room temperature for 15 min, reverse cross-linked with NaCl 100 mM at 65 °C overnight, and treated with proteinase-K. Protein-free DNA was extracted by phenol/chloroform/isoamyl alcohol, precipitated with 100 % ethanol, and analyzed by real-time PCR.
using the following specific primers for Grm2 promoter: 
Forw GATCTGCTGGAAGCTGCTG and Rev CCTCC TCTGTTCCTCTGGACT. Levels of histone acetylation at the mGlu2 receptor gene promoter were expressed as percentage of the input DNA that was immunoprecipitated by the anti acetyl-histone H3 antibody using the following equation: % (acetDNA/IP/total input) = 2^[Ct(10 % input) - 3.32] · Ct(acetDNA · IP)] × 100 %.

Western blot analysis
CA1 regions were dissected and homogenized at 4 °C in a buffered solution composed of Tris-HCl pH 7.5, 10 mM; NaCl, 150 mM; SDS 10 %, EDTA, 5 mM; PMSE, 10 mM; IGEPAL, 1 %; leupeptin, 1 µg/ml; and aprotinin, 1 µg/ml. Equal amounts of proteins (30 µg) from supernatants were separated by 10 % SDS polyacrilamide. After separation, proteins were transferred on immuno-blots PVDF membranes. Membranes were incubated with a mouse monoclonal anti-HDAC2 antibody (1:5,000, overnight at 4 °C, Upstate), and then incubated for 1 h with an anti-mouse secondary antibody (1:7,000, peroxidase-coupled). Immunostaining was revealed by the enhanced substrate (Bio-Rad).

Histological analysis
Animals were killed at 24 or 72 h after reperfusion. Brains were fixed in Carney’s solution, embedded in paraffin, and sectioned at 10 µm. Sections were deparaffinized and processed for staining with thionin (Nissl staining) for histologic assessment of neuronal degeneration. Surviving neurons were counted in the CA1 and CA3 hippocampal subfield under a light microscope at 20× magnification. Neurons with a rounded shape similar to that commonly observed in sections from control animals were considered to be viable. Cells were counted from three sections of the dorsal hippocampus within a dissector area of 1,225 µm² (35 × 35 µm) randomly positioned by the software (Image pro plus 6.0) over the region of interest. The results are expressed as cell density per mm².

Drugs
2,2,2-trifluoro-N-[4-(2-methoxyphenoxy)phenyl]-N-(3-pyridinylmethyl)ethanesulfonamide hydrochloride (LY487379) was purchased from Tocris. ADX92639, was provided from Addex Therapeutics. The molecular structure of ADX92639 could not be disclosed by Addex. General features of compound ADX92639 are the following: molecular weight = 332; partition coefficient (ClogP) = 1.8; topographical polar surface area (tPSA) = 79 Å²; H-bond donor/H-bond acceptor (HBD/HBA) = 0/6; kinetic solubility at pH 7.4 = 0.137 mg/ml; intrinsic clearance (Clint) in rats = 12 µl/min/ mg prot.

Characterization of the receptor profile of ADX92639
FLIPR-based intracellular Ca²⁺ mobilization assay
A fluorescent cell-based Ca²⁺ mobilization assay was performed on HEK293 cells stably expressing rat or human recombinant mGlu receptor subtypes, as described previously [56]. ADX92639 was tested at different concentrations, up to 30 µM, as an agonist, positive allosteric modulator (PAM), or negative allosteric modulator (NAM). ADX92639 was also tested in competition binding assay on membranes expressing 71 targets including G-protein coupled receptors, membrane transporters, enzymes, and ion channels (Cerep, Poitiers, France). In a Schild-plot analysis in mGlu2 receptor-expressing cells, the concentration-response curve to glutamate was tested in the presence of increasing concentrations of ADX92639. To assess the reversibility of the effect of ADX92639, cells expressing the mGlu2 receptor were treated with the compound and then either washed three times with PBS or not. Cells were then stimulated with glutamate at the EC₅₀ value.

[³H]LY341495 binding on membranes from cells expressing human mGlu2 receptor
For the study of [³H]LY341495 binding, we used the same buffer and conditions described by Wright et al. (2001). Cell membrane homogenates were washed three times with ice-cold assay buffer (10 mM potassium phosphate buffer containing 100 mM potassium bromide, pH 7.6). Potassium bromide was added because bromide ions are known to enhance [³H]LY341495 binding [57]. Membranes were incubated in the presence of 1 nM [³H]LY341495 at 5 °C for 30 min in the presence of increasing concentrations of non-radioactive LY341495, glutamate, or ADX92639. Bound and free [³H]LY341495 were separated by filtration.

Measurements of body temperature in rats treated with ADX92639
The effect of ADX92639 on body temperature was measured in male Sprague-Dawley rats of the same age and weight of those used for the induction of global ischemia. Rats were treated orally with either ADX92639 (30 mg/kg, suspended in 1 % carboxymethylcellulose) or vehicle. ADX92639 and vehicle were administered three times at time 0, and then after 24 and 48 h. The rectal temperature was measured at different times after the first administration of either ADX92639 or vehicle (see Table 1) by a
thermistor inserted about 2 cm into the rectum. The probe was left in place for about 10 sec.

Drug treatments in 4-VO rats

Twenty-six 4-VO rats satisfying the inclusion criteria (loss or righting reflex, no response to painful stimuli, and midriasis during carotid artery occlusion), and 8 sham operated rats were used for pharmacological experiments. Two groups of 4-VO rats (n = 8 per group) received oral administration of ADX92639 (30 mg/kg, suspended in 1% carboxymethylcellulose) or vehicle. ADX92639 or vehicle were administered three times in the post-ischemic period at 12, 36 and 60 h after reperfusion. Three sham-operated rats receiving three oral administration of 1% carboxymethylcellulose at the same time-points were used as controls. No sham operated rats received ADX92639. Two additional groups of 4-VO rats (n = 5 per group) were injected s.c. with either LY487379 (30 mg/kg, dissolved in arachid oil) or vehicle at 12, 24, 36, 48 and 60 h after reperfusion. Sham-operated rats were treated s.c. with either LY487379 (n = 2) or vehicle (n = 3) at the same time-points. All animals were killed at 72 h after reperfusion for histological assessment of neuronal death in the CA1 and CA3 regions.

Abbreviations

4-VO: 4-vessel occlusion; PAM: positive allosteric modulator; NAM: negative allosteric modulator.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MM and MC designed and performed biochemical experiments, and wrote the manuscript; FM, AG and DB performed in vivo experiments; BR performed biochemical experiments; RL, SMP and SC performed the experiments and wrote the manuscript; FN designed the experiments and wrote the manuscript; All authors read and approved the final manuscript.

Author details

1 Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Neuromed, 86077 Pozzilli, Italy. 2 Addex Therapeutics, Plan-les-Ouates, Geneva, Switzerland. 3 Department of Physiology and Pharmacology, Sapienza University of Rome, Piazzale Aldo Moro, 5, 00185 Rome, Italy. 4 Present address: Pragma Therapeutics, 74166 Saint-jean-en-Genevois, France.

Received: 29 September 2015 Accepted: 15 October 2015

Published online: 24 October 2015

References

1. Schmidt-Kastner R, Freund TF. Selective vulnerability of the hippocampus in brain ischemia. Neuroscience. 1991;40(3):599–636.
2. Pellegrini-Giampietro DE, Gorter JA, Bennett MV, Zukin RS. The GluR2 (GluR-B) hypothesis. Ca2+-permeable AMPA receptors in neurological disorders. Trends Neurosci. 1997;20(10):464–70.
3. Puliselli WA, Brierley JB. A new model of bilateral hemispheric ischemia in the unanesthetized rat. Stroke. 1979;10:267–72.
4. Calderone A, Jover T, Mashiok T, Noh KM, Tanaka H, Bennett MV, et al. Late calcium ETDA rescues hippocampal CA1 neurons from global ischemia-induced death. J Neurosci. 2004;24(44):9903–13.
5. Cappuccio I, Calderone A, Busceti CL, Biagioni F, Pontarelli F, Bruno V, et al. Induction of Dickkopf-1, a negative modulator of the Wnt pathway, is required for the development of ischemic neuronal death. J Neurosci. 2005;25(10):2647–57.
6. Calderone A, Jover T, Noh KM, Tanaka H, Yokota H, Lin Y, et al. Ischemic insults derepress the gene silencer REST in neurons destined to die. J Neurosci. 2003;23(9):1211–22.
7. Pellegrini-Giampietro DE, Zukin RS, Bennett MV, Cho S, Puliselli WA. Switch in glutamate receptor subunit gene expression in CA1 subfield of hippocampus following global ischemia in rats. Proc Natl Acad Sci U S A. 1992;89(21):10499–503.
8. Clemens JA, Stephenson DT, Smales MB, Dixon EP, Little SP. Global ischemia activates nuclear factor-kappa B in forebrain neurons of rats. Stroke. 1997;28(5):1073–80.
9. Kostinhalo J, Koponen S, Chan PH. Expression of cyclooxygenase-2 mRNA after global ischemia is regulated by AMPA receptors and glucocorticoids. Stroke. 1999;30(9):1900–5.
10. Buchan AM, Li H, Cho S, Puliselli WA. Blockade of the AMPA receptor prevents CA1 hippocampal injury following severe but transient forebrain ischemia in adult rats. Neurosci Lett. 1991;132(2):255–58.
11. Kawasaki-Yatsugi S, Yatsugi S, Koshiya K, Shimizu-Sasamata M. Neuroprotective effect of YM090, an AMPA-receptor antagonist, against delayed neuronal death induced by transient global cerebral ischemia in gerbils and rats. Jpn J Pharmacol. 1997;74(5):253–60.
12. Colbourne F, Li H, Buchan AM, Clemens JA. Continuing post-ischemic neuronal death in CA1: influence of ischemia duration and cytoprotective doses of NOX and SNX-111 in rats. Stroke. 1999;30(3):662–8.
13. Bruno V, Battaglia G, Copani A, D’Onofrio M, Di Iorio P, De Blasi A, et al. Metabotropic glutamate receptor subtypes as targets for neuroprotective drugs. J Cereb Blood Flow Metab. 2001;21(9):1013–33.
14. Pellegrini-Giampietro DE, Cozzi A, Peruginelli F, Leonardo P, Meli E, Pelliccari R, et al. 1-Aminooctanoic-1,5-dicarboryl-xly acid and (S)-(+)-2-(3-carboxy-bicyclo[1.1.1]pentyl-glycine, two mGlu1 receptor-prefering antagonists, reduce neuronal death in vitro and in vivo models of cerebral ischemia. Eur J Neurosci. 1999;11(10):3637–47.
15. Pellegrini-Giampietro DE, Peruginelli F, Meli E, Cozzi A, Albanari-Torregrossa S, Pelliccari R, et al. Protection with metabotropic glutamate 1 receptor antagonists in models of ischemic neuronal death: time-course and mechanisms. Neuropharmacology. 1999;38(10):1593–600.
16. Meli E, Ricca R, Attucci S, Cozzi A, Peruginelli F, Moroni F, et al. Activation of mGlu1 but not mGlu5 metabotropic glutamate receptor contributes to post-ischemic neuronal injury in vitro and in vivo. Pharmacol Biochem Behav. 2002;73(2):439–46.
17. Ivensen L, Mulvihill E, Hallemann B, Diemer NH, Kaiser F, Sheardown M, et al. Changes in metabotropic glutamate receptor mRNA levels following global ischemia increase of a putative presynaptic subtype (mGluR4) in highly vulnerable rat brain areas. J Neurochem. 1994;63(2):625–33.
18. Rosdahl D, Seitzberg DA, Christensen T, Balchen T, Diemer NH. Changes in mRNA for metabotropic glutamate receptors after transient cerebral ischemia. Neuroreport. 1994;5(5):593–6.
19. Yeh TH, Wang HL. Global ischemia downregulates the function of metabotropic glutamate receptor 5 in hippocampal CA1 pyramidal neurons. Mol Cell Neurosci. 2005;29(3):484–92.
20. Bond A, O’Neill MJ, Hicks CA, Monn JA, Lodge D. Neuroprotective effects of a systemically active group II metabotropic glutamate receptor agonist. LY354740 in a gerbil model of global ischemia. Neuroreport. 1998;9(6):1191–3.
21. Bond A, Ragunooothy N, Monn JA, Hicks CA, Ward MA, Lodge D, et al. LY372068, a potent and selective Group II metabotropic glutamate receptor agonist, is neuroprotective in gerbil global, but not focal, cerebral ischemia. Neurosci Lett. 1999;273(3):191–4.
22. Corti C, Battaglia G, Molinaro G, Rizzo B, Pittaluga A, Corsi M, et al. The use of knock-out mice unveils distinct regions for mGlu2 and mGlu3 metabotropic glutamate receptors in mechanisms of neurodegeneration/ neuroprotection. J Neurosci. 2007;27(31):8297–307.
24. Barreto G, Schäfer A, Marhold J, Stach D, Swaminathan SK, Handa V, et al. Gadd45a promotes epigenetic gene activation by repair-mediated DNA demethylation. Nature. 2007;445(7128):671–5.

25. Rai K, Huggins UJ, James SR, Karfp AR, Jones DA, Cairns BR. DNA demethylation in zebrafish involves the coupling of a deaminase, a glycosylase, and Gadd45. Cell. 2008;135(7):1201–12.

26. Cortellino S, Xu J, Sannai M, Moore R, Caretti E, Cigalino A, et al. Thymine DNA glycosylase is essential for active DNA demethylation by linked deamination-base excision repair. Cell. 2011;146(1):67–79.

27. Gräff J, Rei D, Guan JS, Wang YY, Seo J, Hennig KM, et al. An epigenetic blockade of cognitive functions in the neurodegenerating brain. Nature. 2012;483(7388):222–6.

28. Chen J, Uchimura K, Steetler RA, Zhu RL, Nakamanya M, Jin K, et al. Transient global ischemia triggers expression of the DNA damage-inducible gene GADD45 in the rat brain. J Cereb Blood Flow Metab. 1998;18(6):646–57.

29. Kurita M, Holloway T, García-Bea A, Kozlenkov A, Friedman AK, Moreno JL, et al. HDAC2 regulates atypical antipsychotic responses through the modulation of mGlu2 promoter activity. Nat Neurosci. 2012;15(9):1245–54.

30. Johnson NP, Baez M, Jagdmann Jr GE, Britton TC, Large TH, Callagaro DO, et al. Discovery of allosteric potentiators for the metabotropic glutamate 2 receptor: synthesis and subtype selectivity of N-(4-(2-methoxyphenoxy)phenyl)-(2,2,2-trifluoroethylsulfonyl)pyrid-3-ylmethyamine. J Med Chem. 2006;49(15):3189–92.

31. Schaffhauser H, Rowe BA, Morales S, Chavez-Noriega LE, Yin R, Jachev C, et al. Pharmacological characterization and identification of amino acids involved in the positive modulation of metabotropic glutamate receptor subtype 2. Mol Pharmacol. 2003;64(4):798–810.

32. Iijima M, Shimazaki T, Ito A, Chaki S. Effects of metabotropic glutamate 2/3 receptor antagonists in the stress-induced hyperthermia test in singly housed mice. Psychopharmacology (Berl). 2007;190(2):233–9.

33. Wieronska JM, Stachowicz K, Biański P, Palucha-Poniewiera A, Pilk A. On the mechanism of anti-hyperthermic effects of LY379268 and LY487379, group II mGlur receptor antagonists, in the stress-induced hyperthermia in singly housed mice. Neuropsychopharmacology. 2012;36(1):322–31.

34. Gleason SD, Li X, Smith IA, Ephen JD, Wang XS, Heinz BA, et al. mGlu2/3 agonist-induced hyperthermia: an in vivo assay for detection of mGlu2/3 receptor antagonist and its relation to antidepressant-like efficacy in mice. CNS Neurol Drug Targets. 2013;12(5):554–66.

35. Alnawo A, Ver Donck L, Drinkenburg WH. Blockade of the metabotropic glutamate (mGlur2) modulates arousal through vigilance states transitions: evidence from sleep-wake EEG in rodents. Behav Brain Res. 2014;270:56–67.

36. Lambeng N, Kálnichovský M, Perry B, Schneider M, Roerig-US, I Girard F, et al. ADX92369, a potent and selective negative allosteric modulator of metabotropic glutamate receptor 2 (mGlur2) improves recognition memory in rodent models relevant to Alzheimer’s disease. Soc Neurosci Annu Meet, New Orleans, USA. 2012;620(14).

37. Matricciono F, Tuerling P, Maccari S, Nicoletti F, Guidotti A. Pharmacological activation of group II metabotropic glutamate receptors corrects a schizophrenia-like phenotype induced by prenatal stress in mice. Neuropsychopharmacology. 2012;37(14):2929–38.

38. Nasca C, Bigio B, Zelk D, Nicoletti F, McEwen BS. Mind the gap: glucoconotcids modulate hippocampal glutamate tone underling individual differences in stress susceptibility. Mol Psychiatry. 2015;20(7):755–63.

39. Chiechio S, Zammatorto M, Morales ME, Busceti CL, Drago F, Gereau 4th RW, et al. Epigenetic modulation of mGlu2 receptors by histone deacteylase inhibitors in the treatment of inflammatory pain. Mol Pharmacol. 2009;75(5):1014–20.

40. Nasca C, Xenod X, Barone Y, Canuso A, Scaccianoce S, Matricciono F, et al. L-acetylcarnitine causes rapid antidepressant effects through the epigenetic induction of mGlu2 receptors. Proc Natl Acad Sci U S A. 2013;110(12):4804–9.

41. Zammatorto M, Sortino MA, Parenti C, Gereau 4th RW, Chiechio S, HDAC and HAT inhibitors differently affect anaesthesia mediated by group II metabotropic glutamate receptors. Mol Pain. 2014;10:68.

42. Nicoletti F, Bockjaet J, Collingridge GL, Conn PJ, Ferraguti F, Schoepf DD, et al. Metabotropic glutamate receptors: from the workbench to the bedside. Neuropharmacology. 2011;60(7–8):1017–41.

43. Penner JC, Shinozaki H, Miles R. Dual modulation of synaptic inhibition by distinct metabotropic glutamate receptors in the rat hippocampus. J Physiol. 1995;485(1):121–34.

44. Porc JC, McKinney RA, Gahwiler BH, Thompson SM. Differential control of GABA release at synapses from distinct interneurons in rat hippocampus. J Physiol. 2000;528:123–30.

45. Spencer SJ, Mouhate A, Pittman QJ. Peripheral inflammation exacerbates damage after global ischemia independently of temperature and acute brain inflammation. Stroke. 2007;38(5):1570–7.

46. Hua F, Ma J, Ha T, Xie Y, Kelley J, Williams DL, et al. Activation of Toll-like receptor 4 signaling contributes to hippocampal neuronal death following global cerebral ischemia/reperfusion. J Neuroimmunol. 2007;190(1-2):101–11.

47. Chu K, Yin B, Wang J, Peng G, Liang H, Xu Z, et al. Inhibition of P2X7 receptor ameliorates transient global cerebral ischemia/reperfusion injury via modulating inflammatory responses in the rat hippocampus. J Neuroinflammation. 2012;9:69.

48. Taylor DL, Diemelt ET, Czuzner ML, Pocock JM. Activation of group II metabotropic glutamate receptors underlies microbial reactivity and neurotoxicity following stimulation with chromogranin A, a peptide up-regulated in Alzheimer’s disease. J Neurochem. 2002;82(5):1179–91.

49. Geurts JJ, Wolsvik G, Bi B, van der Valk P, Polman CH, Troost D, et al. Altered expression patterns of group I and II metabotropic glutamate receptors in multiple sclerosis. Brain. 2003;126(pt 8):1755–66.

50. Taylor DL, Jones F, Kukota ES, Pocock JM. Stimulation of microbial metabotropic glutamate receptor mGlu2 triggers tumor necrosis factor alpha-induced neurotoxicity in concert with microbial-derived Fas ligand. J Neurosci. 2005;25(11):2922–6.

51. Kassial V, Schlichter LC. Mechanisms of microglia-mediated neurotoxicity in a new model of the stroke penumbra. J Neurosci. 2008;28(9):2221–30.

52. Cendin L, Kantomene S, Barker GR, Hanna L, Murray L, Warburton EC, et al. Study of novel selective mGlu2 agonist in the tempo-rimo-ammonic input to CA1 neurons reveals reduced mGlu2 receptor expression in a Wistar strain with an anxiety-like phenotype. J Neurosci. 2011;31(18):6721–31.

53. Zhou Z, Karlsson C, Liang T, Xiong W, Kimura M, Tapocik JD, et al. Loss of metabotropic glutamate receptor 2 escalates alcohol consumption. Proc Natl Acad Sci U S A. 2013;110(42):16963–8.

54. Goeidler C, Ballard TM, Knoflach F, Wichmann J, Gatti S, Umbricht D. Cognitive impairment in major depression and the mGlu2 receptor as a therapeutic target. Neuropharmacology. 2013;64:337–46.

55. Celerine S, Sebhat J, Wichmann J, Mayer S, Schaffner C, Gatti S. Novel metabotropic glutamate receptor 2/3 antagonists and their therapeutic applications: a patent review (2005 - present). Expert Opin Ther Pat. 2015;25:690–9.

56. Kalinichev M, Rouiller M, Girard F, Royer-Urion L, Bouniqua B, Finn T, et al. ADX271743, a potent and selective negative allosteric modulator of metabotropic glutamate receptor 2, in vitro and in vivo characterization. J Pharmacol Exp Ther. 2013;344(3):624–7.

57. Wright RA, Arnold MB, Wheeler WJ, Ormstein PL, Schoepf DD. [3H]LY341495 binding to group II metabotropic glutamate receptors in rat brain. J Pharmacol Exp Ther. 2001;298(2):453–60.