Published in final edited form as:

Transl Stroke Res. 2011 June 1; 2(2): 195–201. doi:10.1007/s12975-011-0082-4.

Mild Hypothermia Suppresses Calcium-Sensing Receptor (CaSR) Induction Following Forebrain Ischemia While Increasing GABA-B Receptor 1 (GABA-B-R1) Expression

Jong Youl Kim,

Department of Neurology, University of California, San Francisco and Veterans Affairs Medical Center, 4150 Clement St, San Francisco, CA 94121, USA; Department of Medicine, University of California, San Francisco and Veterans Affairs Medical Center, San Francisco, CA 94121, USA

Nuri Kim,

Department of Neurology, University of California, San Francisco and Veterans Affairs Medical Center, 4150 Clement St, San Francisco, CA 94121, USA

Midori A. Yenari, and

Department of Neurology, University of California, San Francisco and Veterans Affairs Medical Center, 4150 Clement St, San Francisco, CA 94121, USA

Wenhan Chang

Department of Medicine, University of California, San Francisco and Veterans Affairs Medical Center, San Francisco, CA 94121, USA

Abstract

Hypothermia improves neurological outcome from cardiac arrest. The mechanisms of protection are multifold, but identifying some may be useful in exploring potential therapeutic targets. The extracellular calcium-sensing receptor (CaSR) was originally found in parathyroid cells in which the receptor senses minute changes in extracellular $[Ca^{2+}]$ and promotes Ca^{2+} influx and intracellular Ca^{2+} release. The CaSR is broadly expressed in the CNS and colocalized with the inhibitory γ -aminobutyric acid-B receptor 1 (GABA-B-R1). In hippocampal neurons, GABA-B-R1 heterodimerizes with CaSR and suppresses CaSR expression. To study the interplay between these two receptors in the development of ischemic cell death and neuroprotection by hypothermia, we subjected C57/BL6 mice to global cerebral ischemia by performing bilateral carotid artery occlusion (10 min) followed by reperfusion for 1–3 days with or without therapeutic hypothermia (33°C for 3 h at the onset of reperfusion). Terminal deoxynucleotidyl transferase dUTP nick end labeling staining and immunohistochemistry showed that forebrain ischemia increased CaSR expression, decreased GABA-B-R1 expression, and promoted cell death. These changes were particularly evident in hippocampal neurons and could be reversed by mild hypothermia. The induction of CaSR, along with reciprocal decreases in GABA-B-R1 expression, may together potentiate ischemic neuronal death, suggesting a new therapeutic target for treatment of ischemic brain injury.

Keywords

Calcium-sensing receptor; Global cerebral ischemia; Hypothermia; Neuroprotection

Introduction

Neuronal hyperactivity and Ca^{2+} overload are well-established mechanisms of ischemic brain injury [1]. Several pharmaceutical studies have been devised to target established excitatory pathways to prevent neuronal overactivity, but these drugs have failed at the clinical level to date [2, 3]. Histological and electrophysiological studies with animal models of global cerebral ischemia and human biopsies have also suggested that loss of signaling through the inhibitory transmitter γ -aminobutyric acid (GABA) may also contribute to ischemia-induced hyperexcitability. Transient global cerebral ischemia in gerbils markedly reduced both GABA-B-R1 and R2 transcripts in pyramidal cell layers of the hippocampus in conjunction with neuronal death [4]. Preclinical studies showed neuroprotective actions of the GABA-B-R agonist baclofen in animal models and neuronal culture models [5-10], but the effects were modest and only significant at higher drug doses that also caused severe side effects in the animals [8]. This lack of efficacy may be explained by the decreased expression of GABA-B-R protein in affected neurons. However, there are no effective pharmaceutical interventions that are available for the treatment of brain ischemia to date.

Hypothermia is a robust neuroprotectant and has been shown to improve neurological outcome in comatose survivors of cardiac arrest [11, 12] and neonates with hypoxia-ischemia [13-16]. The mechanisms underlying the neuroprotective actions of hypothermia are largely unclear. Because the application of hypothermia is cumbersome and has negative effects on other organ systems, identifying more specific neuroprotective targets, including those modulated by hypothermia, may be useful in the development of more easily implemented treatments.

To that end, we report the extracellular Ca^{2+} -sensing receptor (CaSR), a member of family C G protein-coupled receptors, as a potential pharmaceutical target for treatment of ischemic brain injury. This receptor was originally discovered in parathyroid cells (PTCs) where it senses minute changes in extracellular $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_e$). In PTCs and other cell systems, raising $[\text{Ca}^{2+}]_e$ activates phospholipase C (PLC), promotes Ca^{2+} releases from intracellular stores, and enhances membrane Ca^{2+} influx, leading to intracellular Ca^{2+} accumulation. The CaSR is expressed in many regions of the brain involved in memory, cognition, motor reflexes, thirst, growth, and energy homeostasis [17]. Activation of CaSR by high $[\text{Ca}^{2+}]_e$ promotes axonal and dendritic growth of neurons in the supracervical ganglion [18]. Raising $[\text{Ca}^{2+}]_e$ promotes chemotaxis and pulsatile release of GnRH in neuronal cell lines [19, 20]. Activation of CaSR by high $[\text{Ca}^{2+}]_e$ enhances neuronal excitability in neurons in the subfornical organ [21]. In cultured hippocampal neurons, the CaSR activates nonselective cation channels and Ca^{2+} -dependent K channels and depolarizes their membrane potentials [17, 22-24]. These observations support a role for the CaSR in maintaining $[\text{Ca}^{2+}]_i$, ion permeability, and secretory responses in neurons.

Our recent studies demonstrate that GABA-B-R1 can heterodimerize with the CaSR and suppress its expression and signaling in neurons [25]. Blocking GABA-B-R1 expression in cultured hippocampal neurons increased CaSR expression and promoted cell death [25]. These observations lead us to believe that increased expression of the CaSR may promote cell death due to reduced GABA-B-R expression. Since hypothermia is a robust neuroprotectant, we explored whether this protective effect may be due to blunting the impact of ischemia on CaSR and GABA-B-R1 expression. Here, by using a previously established model of hypothermia protection [26], we show that CaSR expression is increased following ischemic brain insults with reciprocal reduction of GABA-B-R1. These expression changes are prevented by therapeutic hypothermia. Our data suggest that the CaSR could be a potential therapeutic target.

Materials and Methods

Mice were housed and treated according to experimental protocols approved by the San Francisco VA Medical Center's Animal Care Facility and in accordance with NIH guidelines.

Global Cerebral Ischemia and Hypothermia Model

Male C57/B6 males weighing 25–30 g were anesthetized with isoflurane (5% for induction, 2% for maintenance via a facemask) in a mixture of medical air/oxygen (3:1) and were subjected to global cerebral ischemia (GCI) as previously described [26]. Rectal temperature was monitored. Both common carotid arteries (CCAs) were occluded for 10 min, followed by reperfusion. Anesthesia was discontinued approximately 3 h later in all cases, to account for the duration of cooling in the hypothermic groups. Sham-operated mice were subjected to the same surgical procedures and anesthetic exposure, with the exception that the CCAs were not occluded. Mice were recovered for 24 and 72 h.

Mild hypothermia (33°C) was induced immediately after the ischemic procedure by spraying alcohol to the animal's torso, fanning and placement on a cooling blanket. Core body temperature (measured by a rectal probe) reached an average of 33°C within 10 min. This corresponded to brain temperatures of approximately 30°C [27]. Cooling was maintained for 3 h. Body temperature in normothermic mice was maintained at 37°C for the same period of time.

To determine the impact of ischemia and hypothermia on neuronal death, mice were subjected to normothermic ischemia (ische-37°C), mild hypothermic ischemia (ische-33°C), sham surgery under normothermic conditions (sham-37°C), or sham surgery under mild hypothermic conditions (sham-33°C), followed by 72 h reperfusion. To test if changes in CaSR and GABA-B-R1 expression precede neuronal damage, mice were subjected to normothermic ischemia, mild hypothermic ischemia, and sham surgery, and their brain samples were analyzed after 24 h reperfusion ($N=6$ per group).

Tissue Processing

Brains were prepared and analyzed after 24 and 72 h of reperfusion. Briefly, mice were anesthetized and transcardially perfused with normal saline and then with paraformaldehyde (PFA; 4%). Brains were dissected, postfixed in 4% PFA, perfused with 20% sucrose at 48°C for 48 h, frozen, and cryosectioned (10 μ m).

Assessing Ischemic Damage and In Situ Detection of Cells with Fragmented DNA

The extent of ischemic injury in hippocampal CA1, CA3, and dentate gyrus (DG) was determined from hematoxylin and eosin (H&E)-stained sections. DNA fragmentation in neurons was assayed on adjacent sections stained with the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) with the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon). Cryosections were fixed in 1% PFA (10 min) and treated with a mixture of ethanol/acetic acid (2:1) for 5 min at -20°C . After three washes in PBS, sections were incubated in 0.3% hydrogen peroxidase to block endogenous peroxidase activities. Sections were then incubated with terminal deoxynucleotidyl transferase (TdT) enzyme and digoxigenin-labeled dUTP at 37°C for 1 h. The digoxigenin-dUTP incorporation in the fragmented DNA was detected by a peroxidase-conjugated antidigoxigenin antibody and 3,3'-diaminobenzidine (DAB) substrate. Sections stained in the same way, but with TdT eliminated, were used as negative controls, and lactating mammary gland was used as a positive control. The number of TUNEL-positive cells was counted in three separate fields of each region (CA1, CA3, and DG) of hippocampus from

four to six mice in each group by an investigator blinded to the treatment groups. The number of TUNEL-positive cells was normalized and expressed as the percentage of cells counted in the brains of sham animals.

Immunohistochemistry

Brain sections from mice with 24 h reperfusion (six mice per group) were subjected to immunostaining for the expression of CaSR and GABA-B-R1 [25, 28]. Cryosections were incubated sequentially with 0.1% hydrogen peroxidase (3 min), a blocking buffer (0.5% Triton X-100, 0.1% BSA, 1.5% normal horse serum in PBS) for 30 min, and custommade rabbit anti-CaSR (1:100) or guinea pig anti-GABA-B-R1 (1:1,000) antibodies overnight at 4°C [25, 28]. Immunoreactivity was amplified and detected with biotinylated anti-rabbit IgG (1:200; Vector Laboratories) or antiguinea pig IgG (1:500; Sigma), peroxidase-conjugated avidin (ABC Elite, Vector Laboratories), and DAB substrate. Sections were counterstained with hematoxylin.

NeuN, GFAP, and CD11B Immunofluorescence

To determine cell populations that express CaSR in response to ischemia and hypothermia, colabeling for neurons (NeuN), astrocytes (GFAP), or macrophages/microglia (CD11b) with the CaSR was performed by dual-fluorescence immunostaining. After blocking in normal serum for 30 min, brain sections were incubated with anti-NeuN (1:200; Catalog number: MAB377, Chemicon), anti-GFAP (1:200; Catalog number: 3670, Cell Signaling), or anti-CD11b (1:200; Catalog number: ab78457, Abcam) antisera along with anti-CaSR antibody in the blocking solution overnight at 4°C. After three washes with PBS, sections were incubated with corresponding Alexa Fluor 488-conjugated goat anti-mouse IgG (1:200; Invitrogen) and Alexa Fluor 588-conjugated goat anti-rabbit IgG (1:200; Invitrogen) for 2 h at room temperature. After three washes in PBS, sections were mounted with and coverslips were sealed with nail polish.

Statistical Analysis

All data were analyzed with standard statistical methods (*t* test, Systat Software, Inc., San Jose, CA, USA). A *P* value <0.05 was considered significant. All data were expressed as mean±SEM.

Results

To examine the impact of ischemia and hypothermia on neurons *in vivo*, brain sections from mice subjected to GCI and 72 h reperfusion in the presence and absence of hypothermia were stained with H&E for changes in cell morphology and TUNEL for DNA damage. GCI at a normothermia led to extensive cell death in hippocampal CA1, CA3, and DG (Fig. 1a, ische-37°C) when compared with sham controls (sham-37°C). These changes in cell morphology were accompanied by increased numbers of TUNEL-positive injured neurons in the corresponding regions of hippocampus (Fig. 1b, ische-37°C vs sham-37°C). Lowering core body temperature from 37°C to 33°C significantly prevented ischemic neuron damage (Fig. 1a, ische-37°C vs ische-33°C) and reduced numbers of TUNEL-positive cells (Fig. 1b, ische-37°C vs ische-33°C, and Fig. 1c).

In control mice, the CaSR was expressed at modest levels in scattered neurons throughout all regions of hippocampus (Fig. 2a, sham-37°C). By 24 h reperfusion following GCI, CaSR expression was profoundly increased in most if not all neurons in the CA1 and CA3 regions and to a lesser extent in the DG (Fig. 2a, sham-37°C vs ische-37°C). In contrast, GCI caused a reduction in GABA-B-R1 expression in the same regions of hippocampus (Fig. 2b, sham-37°C vs ische-37°C). Hypothermic treatment prevented these ischemia-induced

changes in CaSR and GABA-B-R1 expression (Fig. 2, sham-37°C vs ische-33°C). It should also be pointed out that when these stains were performed, hippocampal neurons remained intact, as seen in the hematoxylin counterstain, suggesting that changes in CaSR and GABA-B-R1 were present before visible cell damage developed (Fig. 2). The impact of ischemia and hypothermia on the CaSR expression was mainly restricted to neurons in the pyramidal layers of hippocampus, shown by extensive colocalization of the CaSR with the neuronal marker NeuN (Fig. 3a, b). There was a little colocalization of CaSR with GFAP and CD11b in brain sections from mice subjected to ischemia, suggesting that astrocytes and macrophages/microglia do not express significant levels of CaSR. We did not observe remarkable expression of CaSR and GABA-B-R1 in other brain regions, such as cortex, and little change was observed under the ischemic condition we applied. This is not surprising, as this GCI model results in remarkable changes in the hippocampus, but less obvious changes are observed in other brain region [26].

Discussion

Understanding of the mechanisms that promote neuronal hyperactivity and Ca^{2+} overload is a critical step in developing regimens to prevent neuron death due to brain ischemia and other brain injuries. Here, we show that GCI induces CaSR expression and promotes neuronal death while hypothermia blocks these effects. Because the CaSR couples to signaling pathways—intracellular Ca release, Ca^{2+} influx, and membrane depolarization—that lead to the accumulation of intracellular Ca^{2+} in many cell systems, including neurons, the overexpression and overactivity of CaSR in hippocampal neurons could readily cause Ca^{2+} overload and neuron death. Furthermore, the reduced expression of GABA-B-R1, which is inversely linked to CaSR expression, may presumably reduce inhibitory neurotransmission under ischemic conditions. The retention of GABA-B-R1 expression by hypothermia may in turn play a role in the observed neuroprotection.

CaSR and GABA-B-R1 receptors belong to the family C GPCR and have strong structural homologies. Studies of the interplay between these two receptors in neurons have just begun. We have previously shown that the CaSR and GABA-B-R1 can form heteromers in neurons and in HEK-293 cells transfected with the receptor cDNAs. Coexpression of the GABA-B-R1 suppressed the CaSR expression and blunted its ability to activate PLC, suggesting that the GABA-B-R1 is a negative regulator of CaSR expression [25]. This notion is further supported by the observations that knocking out the GABA-B-R1 gene in mouse brain *in vivo* and in cultured hippocampal neurons increased the CaSR expression [25]. We, therefore, speculate that initial downregulation of GABA-B-R1 is a main cause for CaSR overexpression in response to ischemia. It, however, remains unclear what causes the downregulation of GABA-B-R1 in ischemic neurons at the first place.

Recent studies show that ischemia also increases CaSR expression in cardiomyocytes and leads to apoptosis [29, 30]. Further investigations indicate that the CaSR activation is important for the cardioprotection induced by ischemic preconditioning of cardiomyocytes [31]. These data together suggest that increased CaSR expression could be an intrinsic cellular response to mild ischemic conditions to sustain vital neuronal and cardiac functions. However, sustained overexpression of CaSR under pathological conditions (cardiac infarction or arrest) can lead to irreversible cell death.

Prior studies in stroke and cerebral ischemia models have shown that increasing GABA-ergic tone through agonists acting at the GABA-A receptor can limit the extent of brain injury [32]. Clomethiazole, a compound thought to act through increasing GABA-ergic tone through the GABA-A receptor, was especially studied at the preclinical level and was shown to be robustly neuroprotective. Clinical studies suggested a beneficial effect in a subgroup of

stroke patients [33], but follow-up studies failed to demonstrate a therapeutic effect [34]. The role of CaSR or GABA-B receptor in stroke is largely unknown, but a few studies in humans suggest that this pathway may be relevant. Brain biopsies from patients with temporal lobe epilepsy, due to trauma, anoxic injury, and other causes, also show reduced GABA-B-R1 expression in sclerotic areas [35].

Our study here suggests CaSR as a potential therapeutic target to limit neuron cell death, since its induction is inhibited by therapeutic hypothermia. To further establish a direct link between CaSR overexpression and neuronal death under ischemic conditions, we have taken a novel genetic approach and tested the effect of GCI on a mouse model with targeted ablation of CaSR gene in hippocampal neurons. Future studies should explore whether these mutant mice or targeting CaSR pharmacologically with antagonists or calcilytics also produces neuroprotective effects in similar models to establish causality in brain ischemia.

Acknowledgments

This work was supported by grants from NIH NIA (R01 AG21353 to WC), NINDS (R01 NS40516, P50 NS014543 to MAY), and the Department of Defense (MAY, WC). This work was also supported by the Department of Veterans Affairs.

References

1. Menon B, Shorvon SD. Ischaemic stroke in adults and epilepsy. *Epilepsy Res.* 2009; 87(1):1–11. [PubMed: 19744830]
2. Ikonomidou C, Stefovskaya V, Turski L. Neuronal death enhanced by N-methyl-D-aspartate antagonists. *Proc Natl Acad Sci USA.* 2000; 97(23):12885–90. [PubMed: 11058158]
3. Ikonomidou C, Turski L. Why did NMDA receptor antagonists fail clinical trials for stroke and traumatic brain injury? *Lancet Neurol.* 2002; 1(6):383–6. [PubMed: 12849400]
4. Vollenweider F, Bendfeldt K, Maetzler W, Otten U, Nitsch C. GABA(B) receptor expression and cellular localization in gerbil hippocampus after transient global ischemia. *Neurosci Lett.* 2006; 395(2):118–23. [PubMed: 16298486]
5. Costa C, Leone G, Saulle E, Pisani F, Bernardi G, Calabresi P. Coactivation of GABA(A) and GABA(B) receptor results in neuroprotection during in vitro ischemia. *Stroke.* 2004; 35(2):596–600. [PubMed: 14726544]
6. Zhou C, Li C, Yu HM, Zhang F, Han D, Zhang GY. Neuroprotection of gamma-aminobutyric acid receptor agonists via enhancing neuronal nitric oxide synthase (Ser847) phosphorylation through increased neuronal nitric oxide synthase and PSD95 interaction and inhibited protein phosphatase activity in cerebral ischemia. *J Neurosci Res.* 2008; 86(13):2973–83. [PubMed: 18512761]
7. Epsztein J, Ben-Ari Y, Represa A, Crepel V. Late-onset epileptogenesis and seizure genesis: lessons from models of cerebral ischemia. *Neuroscientist.* 2008; 14(1):78–90. [PubMed: 17914086]
8. Jackson-Friedman C, Lyden PD, Nunez S, Jin A, Zweifler R. High dose baclofen is neuroprotective but also causes intracerebral hemorrhage: a quantal bioassay study using the intraluminal suture occlusion method. *Exp Neurol.* 1997; 147(2):346–52. [PubMed: 9344559]
9. Canaff L, Zhou X, Hendy GN. The proinflammatory cytokine, interleukin-6, up-regulates calcium-sensing receptor gene transcription via Stat1/3 and Sp1/3. *J Biol Chem.* 2008; 283(20):13586–600. [PubMed: 18348986]
10. Kobuchi S, Shintani T, Sugiura T, Tanaka R, Suzuki R, Tsutsui H, et al. Renoprotective effects of gamma-aminobutyric acid on ischemia/reperfusion-induced renal injury in rats. *Eur J Pharmacol.* 2009; 623(1–3):113–8. [PubMed: 19765583]
11. Hypothermia after Cardiac Arrest Study Group T. Mild therapeutic hypothermia to improve the neurologic outcome after cardiac arrest. *N Engl J Med.* 2002; 346(8):549–56. [PubMed: 11856793]

12. Bernard SA, Gray TW, Buist MD, Jones BM, Silvester W, Gutteridge G, et al. Treatment of comatose survivors of out-of-hospital cardiac arrest with induced hypothermia. *N Engl J Med*. 2002; 346(8):557–63. [PubMed: 11856794]
13. Azzopardi D, Strohm B, Edwards AD, Dyet L, Halliday HL, Juszczak E, et al. Moderate hypothermia to treat perinatal asphyxial encephalopathy. *N Engl J Med*. 2009; 361(14):1349–58. [PubMed: 19797281]
14. Gluckman PD, Wyatt JS, Azzopardi D, Ballard R, Edwards AD, Ferriero DM, et al. Selective head cooling with mild systemic hypothermia after neonatal encephalopathy: multicentre randomised trial. *Lancet*. 2005; 365(9460):663–70. [PubMed: 15721471]
15. Shankaran S, Laptook A, Wright LL, Ehrenkranz RA, Donovan EF, Fanaroff AA, et al. Whole-body hypothermia for neonatal encephalopathy: animal observations as a basis for a randomized, controlled pilot study in term infants. *Pediatrics*. 2002; 110(2 Pt 1):377–85. [PubMed: 12165594]
16. Shankaran S, Laptook AR, Ehrenkranz RA, Tyson JE, McDonald SA, Donovan EF, et al. Whole-body hypothermia for neonates with hypoxic–ischemic encephalopathy. *N Engl J Med*. 2005; 353(15):1574–84. [PubMed: 16221780]
17. Yano S, Brown EM, Chattopadhyay N. Calcium-sensing receptor in the brain. *Cell Calcium*. 2004; 35(3):257–64. [PubMed: 15200149]
18. Vizard TN, O’Keefe GW, Gutierrez H, Kos CH, Riccardi D, Davies AM. Regulation of axonal and dendritic growth by the extracellular calcium-sensing receptor. *Nat Neurosci*. 2008; 11(3):285–91. [PubMed: 18223649]
19. Chattopadhyay N, Jeong KH, Yano S, Huang S, Pang JL, Ren X, et al. Calcium receptor stimulates chemotaxis and secretion of MCP-1 in GnRH neurons in vitro: potential impact on reduced GnRH neuron population in CaR-null mice. *Am J Physiol Endocrinol Metab*. 2007; 292(2):E523–32. [PubMed: 17003237]
20. Bandyopadhyay S, Jeong KH, Hansen JT, Vassilev PM, Brown EM, Chattopadhyay N. Calcium-sensing receptor stimulates secretion of an interferon-gamma-induced monokine (CXCL10) and monocyte chemoattractant protein-3 in immortalized GnRH neurons. *J Neurosci Res*. 2007; 85(4):882–95. [PubMed: 17285630]
21. Washburn DL, Smith PM, Ferguson AV. Control of neuronal excitability by an ion-sensing receptor (correction of anion-sensing). *Eur J Neurosci*. 1999; 11(6):1947–54. [PubMed: 10336663]
22. Ye CP, Yamaguchi T, Chattopadhyay N, Sanders JL, Vassilev PM, Brown EM. Extracellular calcium-sensing-receptor (CaR)-mediated opening of an outward K(+) channel in murine MC3T3-E1 osteoblastic cells: evidence for expression of a functional CaR. *Bone*. 2000; 27(1):21–7. [PubMed: 10865205]
23. Chattopadhyay N, Ye C, Yamaguchi T, Nakai M, Kifor O, Vassilev PM, et al. The extracellular calcium-sensing receptor is expressed in rat microglia and modulates an outward K+ channel. *J Neurochem*. 1999; 72(5):1915–22. [PubMed: 10217268]
24. Chattopadhyay N, Ye CP, Yamaguchi T, Vassilev PM, Brown EM. Evidence for extracellular calcium-sensing receptor mediated opening of an outward K+ channel in a human astrocytoma cell line (U87). *Glia*. 1999; 26(1):64–72. [PubMed: 10088673]
25. Chang W, Tu C, Cheng Z, Rodriguez L, Chen TH, Gassmann M, et al. Complex formation with the type B gamma-aminobutyric acid receptor affects the expression and signal transduction of the extracellular calcium-sensing receptor. Studies with HEK-293 cells and neurons. *J Biol Chem*. 2007; 282(34):25030–40. [PubMed: 17591780]
26. Webster CM, Kelly S, Koike MA, Chock VY, Giffard RG, Yenari MA. Inflammation and NFkappaB activation is decreased by hypothermia following global cerebral ischemia. *Neurobiol Dis*. 2009; 33(2):301–12. [PubMed: 19063968]
27. Yenari MA, Onley D, Hedehus M, de Crespigny A, Sun GH, Moseley ME, et al. Diffusion- and perfusion-weighted magnetic resonance imaging of focal cerebral ischemia and cortical spreading depression under conditions of mild hypothermia. *Brain Res*. 2000; 885(2):208–19. [PubMed: 11102575]
28. Chang W, Pratt S, Chen TH, Nemeth E, Huang Z, Shoback D. Coupling of calcium receptors to inositol phosphate and cyclic AMP generation in mammalian cells and *Xenopus laevis* oocytes and

- immunodetection of receptor protein by region-specific antipeptide antisera. *J Bone Miner Res.* 1998; 13(4):570–80. [PubMed: 9556057]
29. Guo J, Li HZ, Zhang WH, Wang LC, Wang LN, Zhang L, et al. Increased expression of calcium-sensing receptors induced by ox-LDL amplifies apoptosis of cardiomyocytes during simulated ischaemia–reperfusion. *Clin Exp Pharmacol Physiol.* 2010; 37(3):e128–35. [PubMed: 20409080]
30. Jiang CM, Han LP, Li HZ, Qu YB, Zhang ZR, Wang R, et al. Calcium-sensing receptors induce apoptosis in cultured neonatal rat ventricular cardiomyocytes during simulated ischemia/reperfusion. *Cell Biol Int.* 2008; 32(7):792–800. [PubMed: 18455448]
31. Sun J, Murphy E. Calcium-sensing receptor: a sensor and mediator of ischemic preconditioning in the heart. *Am J Physiol Heart Circ Physiol.* 2010; 299(5):H1309–17. [PubMed: 20833954]
32. Gilby KL, Sydserff SG, Robertson HA. Differential neuroprotective effects for three GABA-potentiating compounds in a model of hypoxia-ischemia. *Brain Res.* 2005; 1035(2):196–205. [PubMed: 15722059]
33. Wahlgren NG, Ranasinha KW, Rosolacci T, Franke CL, van Erven PM, Ashwood T, et al. Clomethiazole acute stroke study (CLASS): results of a randomized, controlled trial of clomethiazole versus placebo in 1360 acute stroke patients. *Stroke.* 1999; 30(1):21–8. [PubMed: 9880383]
34. Lyden P, Shuaib A, Ng K, Levin K, Atkinson RP, Rajput A, et al. Clomethiazole Acute Stroke Study in ischemic stroke (CLASS-I): final results. *Stroke.* 2002; 33(1):122–8. [PubMed: 11779900]
35. Munoz A, Arellano JI, DeFelipe J. GABABR1 receptor protein expression in human mesial temporal cortex: changes in temporal lobe epilepsy. *J Comp Neurol.* 2002; 449(2):166–79. [PubMed: 12115687]

Fig. 1.

Hypothermia improves hippocampal neuron survival following GCI. **a** Hematoxylin and eosin and **b** TUNEL staining of hippocampal neurons in C57/B6 mice subjected to GCI and 3 day reperfusion (*Ische*) or sham surgery (*Sham*) at 37°C. Some Sham (*Sham:33°C*) and Ische (*Ische:33°C*) mice were subjected to hypothermic condition at 33°C for 3 h. **c** Cell counts of TUNEL-positive neurons in the hippocampus of ischemic and control mice with or without hypothermia. *Or* oriens layer of hippocampus, *Rad* stratum radiatum of hippocampus, *Hil* hilus of dentate gyrus, *GrDG* granular layer of dentate gyrus; * $P < 0.05$, $N = 6$ /group; scale bar = 50 μ m

Fig. 2.

Cerebral ischemia induces CaSR expression while suppressing GABA-B-R1, a phenomenon reversed by hypothermia. Immunohistological detection of **a** CaSR and **b** GABA-B-R1 in neurons in the CA1, CA3, and DG of the hippocampus from mice subjected to GCI and 1-day reperfusion with (*Ische:33°C*) or without hypothermia (*Ische:37°C*) compared with sham controls (*Sham:37°C*). CaSR induction is largely localized to neuron-like cells within the hippocampus, and this induction is suppressed in hypothermic brains. GABA-B-R1 is present at low levels in uninjured brain (*Sham:37°C*) and is decreased in normothermic animals exposed to GCI (*Ische:37°C*), but expression is markedly increased with hypothermic treatment (*Ische:33°C*). Brain sections were counterstained with hematoxylin. *Rad* stratus radiatum, *GrDG* granular layer of the DG, *Or* oriens, *Hil* hilus; scale bar=50 μ m

Fig. 3.

Ischemia-induced CaSR overexpression was observed mainly in neurons but not astrocytes and microglia. Double immunofluorescent staining of neurons (*NeuN*), astrocytes (*GFAP*), or microglia (*CD11b*) for the expression of CaSR in the hippocampus of mice subjected to GCI and 1 day reperfusion with (*Ische:33°C*) or without hypothermia (*Ische:37°C*) show that CaSR is present in neurons. Neurons still expressed CaSR under conditions of hypothermia (*Ische:33°C*), but at lower levels. Astrocytes and microglia did not colocalize with CaSR signals. Bar=50 μ m