Riluzole Normalizes Early-Life Stress-Induced Visceral Hypersensitivity in Rats: Role of Spinal Glutamate Reuptake Mechanisms

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BACKGROUND & AIMS: The molecular basis underlying visceral hypersensitivity in functional irritable bowel syndrome remains elusive, resulting in poor treatment effectiveness. Because alterations in spinal non-neuronal (astrocytic) glutamate reuptake are suspected to participate in chronic pain, we asked whether such processes occur in visceral hypersensitivity. **METHODS:** Visceral hypersensitivity was induced in Sprague–Dawley rats by maternal separation. Separated adults were given a systemic administration of riluzole (5 mg/kg), an approved neuroprotective agent activating glutamate reuptake. Visceral hypersensitivity was assessed using colorectal distension (40 mm Hg). Somatic nociception was quantified using Hot Plate, Randall–Sellito, and Hargreaves tests. Spinal proteins were quantified using immunofluorescence and Western blot. The dependence of visceral sensory function upon spinal glutamate transport was evaluated by intrathecal injection of glutamate transport antagonist DL-*threo*-β-benzyloxyaspartate (TBOA). For in vitro testing of riluzole and TBOA, primary cultures of astrocytes were used. **RESULTS:** We show that riluzole counteracts stress-induced visceral hypersensitivity without affecting visceral response in nonseparated rats or altering nociceptive responses to somatic pain stimulation. In addition, maternal separation produces a reduction in glial excitatory amino acid transporter (EAAT)-1 with no change in EAAT-2 or γ-amino butyric acid transporters. Stress was not associated with changes in glial fibrillary acidic protein or astrocytic morphology per se. Furthermore, visceral normosensitivity relies on spinal EAAT, as intrathecal TBOA is sufficient to induce hypersensitivity in normal rats. **CONCLUSIONS: We identify spinal EAAT as a therapeutic target, and establish riluzole as a candidate to counteract gastrointestinal hypersensitivity in disorders such as irritable bowel syndrome.**

Keywords: Irritable Bowel Syndrome; Maternal Separation; Astrocytes; Spinal Cord.

BASIC–ALIMENTARY TRACT

Visceral hypersensitivity is a core symptom of functional gastrointestinal disorders such as irritable bowel syndrome (IBS) and is a pressing clinical issue that remains an ongoing challenge for the pharmaceutical industry.[1,2] The ineffectiveness of current medications against visceral hypersensitivity is attributable at least in part to the lack of a unifying hypothesis regarding the chain of events generating pain. Despite this absence of consensus, stress occurring in early life is known to have a key detrimental upstream influence on nociceptive pathways and in the manifestation of IBS symptoms.[3–5] Hence, the enhanced visceromotor response following pup isolation from dams (maternal separation) is viewed as an IBS-relevant model in animals.[6] In addition, central sensitization (ie, an increased activation of spinal nociceptive neurons to peripheral stimuli) may be an end mechanism accounting for visceral hyperalgesia in IBS,[7] presumably due to a sustained glutamatergic sensory neurotransmission. However, glutamate receptor antagonists show a constellation of side effects strongly restricting their utilization in patients. Interestingly, beyond glutamate receptor targeting, the efficacy of glutamatergic neurotransmission may be modulated by acting on glutamate reuptake systems. Indeed, the proper and regulated action of glutamate relies on its fast elimination from extracellular synaptic milieu as a result of the scavenging machinery, namely the excitatory amino-acid transporters (EAAT)-1 and EAAT-2.[8] These transporters are expressed by non-neuronal glial cells, primarily astrocytes, whose endfeet cover virtually all synapses in the central nervous system. Remarkably, the importance of spinal EAAT in nociception has been emphasized by recent evidence showing that selective intrathecal inhibition of glial glutamate transport in naïve rats results in pain behaviors.[9] Moreover, a reduction in EAAT expression occurs in chronic pain states[10–12] as a component of the profound phenotypic alterations taking place in spinal astrocytes, which encompasses an increased synthesis of glial fibrillary acidic protein (GFAP) and a release in sensitizing soluble factors.[13] These alterations are consistently observed in a variety of pain models, giving astrocytes an emerging key role in the maintenance of chronic pain. However, there is a paucity of data regarding the

Abbreviations used in this paper: EAAT, excitatory amino-acid transporter; GAT, γ-amino butyric acid transporter; GFAP, glial fibrillary acidic protein; IBS, irritable bowel syndrome; PBS, phosphate-buffered saline; TBOA, DL-*threo*-β-benzyloxyaspartate.

0016-5085/$36.00
doi:10.1053/j.gastro.2010.03.003

involvement of astrocytes in visceral pain, especially with regard to the possible role of glutamate uptake system.

Riluzole (2-amino-6-trifluoromethoxybenzothiazole) is a neuroprotective drug already approved for amyotrophic lateral sclerosis.[14] Riluzole is a potent activator of glutamate reuptake both in vitro and in vivo. In addition, pain-modulatory properties of riluzole have been reported,[11,15] but its influence on visceral pain remains unknown. Therefore, in the present study, we assessed whether riluzole could reduce visceral hypersensitivity induced by maternal separation and how this could shed some insight on underlying mechanisms in stress-induced visceral pain. We found that riluzole given intraperitoneally normalizes visceral hypersensitivity in maternally separated rats. Moreover, it failed to affect visceral responses in nonseparated animals and had no effects on somatic nociception; maternal separation produces a reduction in EAAT-1 in the spinal cord; stress did not result in changes in EAAT-2, γ-amino butyric acid transporter (GAT), and γ-amino butyric acid expression or in astrocytic morphological alterations; direct intrathecal inhibition of EAAT using DL-*threo*-β-benzyloxyaspartate (TBOA) is sufficient to produce a visceral hypersensitivity in naïve rats. Taken together, the data presented here add another dimension to the use of riluzole in therapeutics, establishing its relevance to counteract visceral hypersensitivity, and point to spinal glial glutamate reuptake as a pharmacological target for pain in functional bowel disorders.

Materials and Methods

Animals

Male Sprague–Dawley rats weighing 280–300 g were housed and bred in the local animal facility with food and water ad libidum, on a 12:12-hour reversed dark–light cycle with temperature at 20°C ± 1°C. Animals were group-housed by 4 to 5 per cage in usual plastic cages with sawdust bedding in an enriched environment with shredded paper and a cardboard roll. All experiments were in full accordance with the European Community Council Directive (86/609/EEC). Behavioral assessments were always carried out randomly by an experimenter blind to treatment groups.

Drug Administration

Riluzole and TBOA were purchased from Tocris (Bristol, UK). For intrathecal injection, rats were sedated by light isofurane anesthesia and vehicle (phosphate-buffered saline [PBS], 10 μL) or TBOA (1.2 μg or 12 μg in 10 μL vehicle) was administrated. Correct positioning was assessed by a tail flick upon needle insertion. Balloons were immediately inserted in preparation for colorectal distension. The absence of spinal damage was assessed prior to distension, and animals presenting any sign of paralysis or spontaneous pain behaviors were immediately culled. Colorectal distensions were carried out 8–10 minutes after intrathecal injections. This timing was selected from previous studies that showed that it was at the longer range used to see maximal effects of TBOA delivered intrathecally.[9] For riluzole studies, rats were weighed and a slight volume correction was applied to the stock solution (1.5 mg/mL, in PBS) for the intraperitoneal injection to achieve a final administration of 5 mg/kg riluzole. Control rats were injected with vehicle (1 mL PBS). Colorectal distension was carried out 30 minutes later. For nociceptive testing (Hot Plate, Randall–Sellito, and Hargreaves tests), riluzole was injected 30 minutes prior to testing.

Colorectal Distension

Animals were sedated using isofurane, and 7-cm–long deflated balloons attached to polyethylene tubing were inserted intrarectally through the anus. Rats were left to recover for 10 minutes and the tubing was connected to a barostat machine. The protocol consisted in a tonic distension at a constant pressure of 40 mm Hg during 10 minutes. Visceromotor response to distension was quantified as the number of abdominal contractions resulting in animal arching during the procedure. At the end of the distension, balloons were deflated and the animals were culled.

Hot-Plate Test

Rats were placed with all 4 paws on the hot plate (Incremental Hot Plate; Stoelting, Dublin, Ireland) heated at 55°C and the latency to hindpaw lick or shake was measured. The cutoff time was set at 20 seconds to avoid tissue damage. Baseline latencies were recorded 30 minutes prior to the experiment.

Paw Pressure Test, Randall–Sellito

Responses to noxious mechanical stimuli were measured with a Digital Randall–Sellito test (Stoelting). Rats were handled and partly restrained. Following forepaw positioning, an increasing pressure was applied to the dorsal surface. Nociceptive threshold was defined as the pressure in grams eliciting the paw withdrawal. Measurements were performed in triplicate. A cutoff weight was set at 400 g. Baseline responses were recorded 1 day before drug administration.

Paw Withdrawal Test, Hargreaves

Paw withdrawal response to noxious radiant heat stimuli was assessed using Plantar Test (Plantar Test Analgesia Meter; Stoelting). Rats were placed in individual Plexiglas chambers on a glass platform heated at 37°C and habituated for 5 minutes. A mobile radiant heat source was located under the platform and focused onto the mid-plantar surface of each hindpaw sequentially. The paw withdrawal latency was defined as the time from radiant heat onset to paw withdrawal. The apparatus was calibrated to give a paw withdrawal latency of 7–9 seconds prior to drug injection. A total of 6 paw withdrawal latencies (in triplicate, each separated by at