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Localized inhibition of P2X7R at the spinal cord injury site improves neurogenic bladder dysfunction by decreasing urothelial P2X3R expression in rats

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ABSTRACT

Aims: Reestablishment of bladder function in patients with spinal cord injury (SCI) is a clinical priority. Our objectives were to determine whether SCI-localized inhibition of purinergic P2X7 receptors (P2X7R) improve bladder function by decreasing afferent signals mediated by urothelial P2X3R.

Main methods: Systemic inhibition of P2X7R may improve locomotion in rodent SCI models; however, beneficial effects on bladder function and its physiological mechanisms have not been evaluated. We designed a thermosensitive nanohydrogel (NHG) consisting of the P2X7R antagonist brilliant blue-G (BBG) loaded into silica nanoparticles, embedded with poly(D,L-lactic-*co*-glycolic) acid, and resuspended in 20% pluronic acid. Female Sprague-Dawley rats with a bilateral dorsal lesion at the thoracic T8/T9 region received either 100 μl of an empty NHG, or a NHG containing BBG (BBG-NHG) on top of the spinal tissue. Cystometric properties, spinal immunohistochemistry for P2X7R, and bladder immunohistochemistry for P2X3R were evaluated at four weeks post-SCI.

Key findings: After SCI animals recovered hind-legs use but neurogenic bladder dysfunction remained. SCI rats treated with BBG-NHG for a period of at least two weeks post-SCI experienced fewer non-voiding contractions. The localized inhibition of P2X7R decreased microglia activation. At the lower urinary tract level we observed, unexpectedly, a concomitant reduction of urothelial P2X3 receptors, which are involved in initiation of bladder afferent transmission to start micturition.

Significance: Localized inhibition of P2X7R for two weeks can be associated with reduced number of microglia and attenuated bladder hyperexcitability mediated by downregulation of urothelial P2X3R in rats with neurogenic bladder dysfunction and independently of locomotor improvements.

1. Introduction

Improving bladder function is a major priority for individuals with SCI [1]. The neurogenic bladder in these patients cannot empty efficiently because of abnormal contractions of the detrusor muscle (neurogenic detrusor overactivity, NDO) opposed by a dyssynergic activity of the urethral sphincter during micturition, both of which lead to a host of problems such as incontinence, retention, urinary tract infection, bladder stones, loss of bladder compliance or renal damage [2]. Several pharmacological treatments for NDO are available, but their limited efficacy and bothersome side effects often result in poor patient compliance [3]. Pre-clinical studies investigating spinal cord regeneration after SCI suggest that the amelioration of scar formation at the SCI region can improve locomotor function [4]. However, prospects for restoration of bladder sensory function to regulate micturition remain unclear [5].

At the time of SCI, an excessive release of ATP from damaged neurons and cells in the peri-injury zone contributes to an inflammatory response in which purinergic P2X7-type receptors (P2X7R) play an important role. It has been suggested that P2X7R expressed in microglia may contribute to scar formation over the long term and thus reduce the opportunity for proper tissue regeneration [6,7]. Microglia cells in the brain and spinal cord play a central role in regulating inflammatory

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processes during the early stages following a central nervous system injury [8]. In fact, inhibition of P2X7R attenuates microglia activation and proliferation as well as reduces neuronal injury during brain inflammation [9], and thus makes P2X7R a control switch for triggering major inflammatory response in CNS injuries [10]. These observations suggest that chronic activation of P2X7R may promote a microglia-mediated harmful role and lead to impairments on afferent and efferent bladder pathways.

Initial studies evaluating the protective role of P2X7R inhibition suggested that systemic injections of P2X7R antagonists accelerate recovery of locomotion and reduce cord damage in SCI animals [11]. However, a recent reassessment of these observations failed to fully reproduce the previously observed benefits [12]. Given the substantial literature supporting a neuroprotective role for the central inhibition of P2X7R [6,13], we hypothesized that a region-targeted inhibition of P2X7R at the SCI site would decrease microglia activation and improve bladder sensory function. Using rats with a bilateral dorsal lesion at the T8/T9 thoracic region, we examined the consequences of a localized and sustained release of the P2X7R antagonist brilliant blue-G (BBG), which has an IC_{50} that varies from 10.1 ± 3.7 nM to 12.7 ± 2.2 nM for the rat homomeric P2X7R [14]. Changes in sensory function in the bladder urothelium after BBG treatment were determined by evaluating the expression patterns for urothelial P2X3R, an ionotropic purinergic receptor involved in transmission of bladder afferent activity [15,16]. The urothelial expression patterns for this receptor were evaluated based on our previous observations that hyperexcitability of lumbosacral sensory neurons seems to be importantly mediated by P2X3R in rats with spinal cord injury [17]. The nanohydrogel formulation containing the P2X7R antagonist significantly improved urinary bladder dysfunction.

2. Materials and methods

2.1. Animal model for partial SCI (pSCI)

Female Sprague-Dawley rats weighing 250–300 g were purchased from Envigo (Research Model Services, Houston, TX.) and housed in a pathogen free environment under 12 h light/dark cycles, at a controlled temperature of 25 °C, with ad-libitum access to food and water in plastic cages containing with corn cob bedding. For SCI surgery, rats were anesthetized with 2% isofluorane, and laminectomies were performed at the T8/T9 spinal cord region. For the bilateral dorsal lesion SCI (pSCI) injury model, microscissors were used to transect several dorsal processes, including spinal gray laminae I-V (dorsal horns), dorsal nucleus, dorsal corticospinal tract, dorsolateral/gracile fasciculus, intermediolateral column, lateral spinal nucleus, rubrospinal tract and intercalated nucleus [18,19]. After surgery, all animals received the analgesic drug buprenorphine (0.05 mg/kg) and the anti-inflammatory drug carprofen (50 mg/kg) subcutaneously for either three or four days, respectively. Rats with a pSCI required additional support to empty their bladders (twice a day manual expression) until neurogenic bladder overactivity developed, usually 10–14 days after SCI. In all cases, after sham or SCI surgery rats were singled housed in the described home-cages and conditions for two weeks, when staples were removed. At this time rats were housed in groups of two animals per cage until the end point of the study two weeks later (i.e. a total time of 4 weeks). The methods of injury, as well as the therapeutic measures undertaken, were approved by our institutional IACUC and performed in accordance with the Guide for the Care and Use of Laboratory Animals: Eighth Edition [20] (The National Academies Press, 2011. doi:10.17226/12910). None of the animals utilized for this work became ill or died prior to the experimental endpoint. We made every effort to minimize the suffering and number of animals according to the requirements of our IACUC. Supplementary Fig. 1 shows a summarized description of our experimental approach.

2.2. Nanohydrogel formulation and preparation

Five milligrams of the P2X7R antagonist brilliant blue-G (BBG; Sigma-Aldrich, St Louis, MO) were loaded into 5 mg of silica nanoparticles (SNP; 100 nm diameter) prepared as previously described by our group [21]. Briefly, the BBG-loaded SNP were lyophilized overnight at room temperature, and then coated with D,L-lactide-co-glycolide (PLGA; MW 30 to 60 kDa; Sigma) in an 85:15 ratio. In vitro evaluations confirmed that, depending on the SNP/PLGA ratio the release rate of the P2X7R antagonist could be adjusted in order to facilitate a constant release over a typical period of two weeks as determined by using a spectrophotometer to read supernatant absorbance at 576/280 nm. For application at the SCI region we prepared a 20% solution of pluronic F-127 (Sigma-Aldrich, St Louis, MO) in phosphate-buffered saline solution at 4 °C to maintain the liquid state and mixed 100 μl of this solution with 20 mg of the BBG/SNP/PLGA particles preparation. The in vitro characterization suggested a loading efficiency of about 80%. On the basis of these results, we predicted that the nanohydrogel (NHG) preparation would contain ~8 mg BBG/100 μl (BBG-NHG) for a BBG release of approximately 585 μg BBG/rat/day for a period of least 2 weeks. For control conditions, the described procedure for BBG-NHG was repeated with an empty NHG (E-NHG) preparation that did not include the antagonist. The thermo-sensitive hydrogel becomes semi-solid when reaching temperatures above 34 °C thus remaining in place after application on the SCI area (see Supplementary Fig. 2).

2.3. Experimental groups

Four different groups of animals were evaluated during this study: intact rats [N (group size) = 6], those with spinal cord injury (pSCI; N = 6), animals with pSCI treated with the empty NHG (E-NHG; N = 9), and pSCI animals treated for a period of two weeks with the P2X7R antagonist embedded in the NHG (BBG-NHG; N = 9). Application of the NHG was performed immediately after stopping the bleeding caused by the pSCI procedure (animal to animal variation ranged from 5 to 25 min). Muscle and fascia above the site of the SCI application area were sutured in layers and skin flaps were stapled. Locomotor evaluation by two investigators blinded to the study by reviewing individual videos before pSCI surgery, was performed 3 days after pSCI and weekly for a month. Ability to stand was determined by counting the number of times during a period of 1 min that a SCI rat exhibited exploratory behavior characterized by full weight support on hind legs. From the same video recordings the hindlimb locomotor function was determined with the Basso, Beattie, and Bresnahan scale. After four weeks, rats were anesthetized with urethane (1.1–1.2 g/kg subcutaneous) for cystometric evaluation followed by transcardiac perfusion with ice-cooled Krebs solution and a freshly prepared solution of 4% paraformaldehyde. The spinal cord and the urinary bladder were cryoprotected in 30% sucrose for immunohistological evaluation.

2.4. Cystometric evaluation

Four weeks after surgical procedures, changes in maximal voiding pressure (cm H_2O), duration of intraluminal pressure high frequency oscillations (IPHFO, s), non-voiding contraction (NVC) frequency (NVC/h), and micturition volumes (ml) were evaluated by placing a suprapubic catheter into the urethane anesthetized rats. Intravesical saline infusion was performed using a PE-50 tube at a rate of 0.1 ml/min in intact rats, or 0.2 ml/min in pSCI, pSCI + E-NHG, and pSCI + BBG-NHG. NVC were considered as genuine when observing a change in bladder pressure, without release of perfused saline, of magnitude higher than 5 cm-H2O [22]. Infusion rate was higher in pSCI animals because of the necessary time required to trigger micturition events after bladder hypertrophy. Changes in bladder pressure and voiding events were recorded at a sampling rate of 60 Hz using a pressure and a force transducer, respectively (World Precision Instruments. Sarasota, FL).