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Nociceptor-like rat dorsal root ganglion neurons express the angiotensin-II AT2 receptor throughout development

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ABSTRACT

AT2 receptor (AT2R) plays a functional role in foetal development. Its expression declines in most tissues soon after birth but stays high in sensory areas of the adult nervous system. In the dorsal root ganglia (DRG) the expression pattern of AT2R during development and the identity of the subpopulation expressing it remain unknown. Using a combination of semi-quantitative PCR, western blotting and immunohistochemistry we examined the expression of AT2R at mRNA and protein levels in rat DRGs from embryonic day 15 (E15) until postnatal day 30 (PN30). We found that both AT2R mRNA and protein levels exhibited only minor (statistically non-significant) fluctuations from E15 to PN30. Detailed quantitative analysis of ABC/DAB AT2R staining showed a) that the receptor was present in most neurons at E15 and E18 and b) that postnatally it was predominantly expressed by small DRG neurons. Given that small neurons are putative C-nociceptors and the proposed role of AT2R in neuropathic pain, we next examined whether these AT2R-positive neurons co-localized with Ret and trkA embryonically and with IB4-binding postnatally. Most AT2R-positive neurons expressed trkA embryonically and bound IB4 postnatally. We found strong positive statistically highly significant correlations between AT2R cytoplasmic%intensities and trkA at E15/E18 and with Ret only at E18. Cytoplasmic AT2R also strongly and positively correlated with IB4-binding at PN3, 15 and 30. Our demonstration that a subpopulation of C-nociceptor-like neurons expresses AT2R during development supports a role for this receptor in neuropathic pain.

1. Introduction

Angiotensin-II (Ang-II) receptors are present in the spinal cord and dorsal root ganglia (DRG) (Ahmad et al., 2003; Oldfield et al., 1994). Adult rat and human DRG neurons also express angiotensinogen, the synthesizing enzymes cathepsin-D, ACE and the angiotensin receptors type-1 (AT1R) and type-2 (AT2R) (Anand et al., 2013; Chakrabarty et al., 2008; Patil et al., 2010; Pavel et al., 2008). Thus, a functional renin-angiotensin system is present in DRG neurons and may be involved in the genesis and maintenance of pain (Bali et al., 2014; Chakrabarty et al., 2013; Patil et al., 2010).

Recently, several synthetic drugs with antagonist effect on AT2R were reported to attenuate prostate cancer induced bone pain (Muralidharan et al., 2014), alleviate neuropathic pain (Anand et al., 2015; Rice et al., 2014; Smith et al., 2013) and reverse hypoesthesia induced by *M. ulcerans* in the Buruli ulcer (Anand et al., 2016; Marion et al., 2014). However, the molecular and cellular bases of these actions of AT2R remain poorly understood. To advance our knowledge of the role of AT2R in pathological pain, it is essential to establish what DRG neuronal subpopulations express this receptor.

The DRG comprise subpopulations of neurons with distinct anatomical, electrophysiological and neurochemical characteristics (Krames, 2014; Lawson, 2002; Liu and Ma, 2011). There is a significant correlation between action potential conduction velocity (CV) and cross-sectional area. Thus we recognise three main neuronal subpopulations: neurons with C-fibres (small, slowly conducting, unmyelinated), Aδ (medium, fast conducting, weakly myelinated) and Aα β (large, fastest, heavily myelinated) (Lawson and Waddell, 1991; Lawson, 2002). To play a role in pain, AT2R must be located in nociceptors. This subpopulation includes neurons that bind either the Isolectin-B4 (IB4-positive) or that express trkA (the high-affinity receptor for NGF) or both. Each group contributes ~30% of the C-nociceptors in lumbar DRGs. IB4-positive neurons have C-fibres, are non-peptidergic and predominantly innervate cutaneous and visceral targets (Fang et al., 2006; Zylka et al., 2005). In the adult rat, trkA-positive neurons are peptidergic and variable

Abbreviations: DRG, dorsal root ganglia; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AT2R, angiotensin-II type 2 receptor; IB4, isolectin B4; Ret, receptor for the glial-derived growth factor; GDNF, glial-derived neurotrophic factor; trkA, tropomyosin receptor kinase A; Adr, Adrenal cortex.

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in size; include C, Aδ and Aβ nociceptors and mediate the pro-algesic actions of NGF in inflammatory and neuropathic pain (Fang et al., 2005a; Jankowski and Koerber, 2010; Woolf and Ma, 2007).

Developmentally, C-fibres are generated during a second and third wave of embryonic neurogenesis in the DRG and all initially express trkA (Liu and Ma, 2011). In mice, at embryonic day 15.5 some of these trkA-positive neurons began to switch to small Ret-positive. After birth, these neurons also express the GDNF-family of receptors (GFRα1, 2 and 3) and bind IB4 (Golden et al., 2010; Molliver et al., 1997). Where AT2R fits in this complex picture of nociceptor differentiation is unknown.

The purpose of this work was two-fold: first, to describe the pattern of expression of the AT2R mRNA and its protein in the rat DRG throughout development. Second, we aimed to identify what neuronal subpopulation express AT2R, using classification by size and also the phenotypic markers Ret/trkA and IB4-binding.

2. Materials and methods

We used Wistar rats of 2 embryonic (E15 and E18) and 3 post-natal (PN3, 15 and 30) ages. E0 was defined as the day of mating; PN0 was the day of birth. All procedures had been approved by the Institutional Animal Care and Use Committee (CICUAL #31/2014) of the School of Medical Sciences, UNCuyo.

2.1. Semi-quantitative RT-PCR

mRNA levels were determined by RT-PCR following previously published protocols (Kunda et al., 2014; Marsh et al., 2012). mRNA was extracted using RNeasy (Qiagen) from whole L4/L5 DRGs (this equates to 4 DRG per animal) at each age (5 rats per age). cDNA was synthesized from 200 ng total RNA using the M-MLV kit (Promega). AT2R and GAPDH mRNA were detected using Taq polymerase (Invitrogen) with 35 and 30 cycles, respectively. We performed a negative control containing RNA instead of cDNA to rule out genomic DNA contamination.

Primers sequences were: AT2R forward 5′-CAACTTCAGTTTTGCTGCCAC-3′; reverse 5′-CAGGTCCAAAGAGCCAGTCAT-3′, predicted size 335 bp. For GAPDH, forward 5′-GGTGCTGAGTATGTCGTGGA-3′ and reverse 5′-GGATGCAGGGATGATGTTCT-3′, predicted size 340 bp. All primers were custom-designed and checked using Primer-BLAST. Images of the RT-PCR SYBR Safe (Molecular Probes) stained agarose gels were acquired with an LAS-4000 system (Fujifilm) and quantified with ImageJ software, see Kunda et al. (2014).

2.2. Western blots (WB)

WB were performed as previously described (Acosta et al., 2014). Total protein was extracted from whole L4/L5 DRGs at each age (3 rats per age) using Laemmli buffer supplemented with a protease/phosphatase inhibitor cocktail (HALT, Thermofisher). Samples of ∼20 μg of total protein were run in 8–10% polyacrylamide gels and transferred to a PVDF membrane (Amersham) before blotting. Membranes were probed with 1:1000 goat anti-AT2R (Santa Cruz Biotechnology Cat# sc-48452, K15, RRID:AB_2225720) and 1:2000 mouse anti-α-tubulin (eBioscience, 14–4502-80, RRID:AB_1210457) as loading control. The anti-AT2R antibody has been characterized and it is selective unlike other commercially available antibodies (Anand et al., 2013; Hafko et al., 2013).

Protein bands were developed using ECLPlus (Amersham) and visualized with LAS-4000 system (Fujifilm). WBs were replicated 3 times and quantification was conducted as previously described (Kunda et al., 2014).

2.3. Immunohistochemistry

L4/L5 DRGs from 4 rats per age were fixed in Zamboni's fixative and stored overnight at 4 °C in 30% sucrose. Tissue was then frozen at −80 °C and cut in serial transverse 7 μm cryostat sections.

a. ***Avidin-biotin complex (ABC) immunohistochemistry*** was performed as previously described (Acosta et al., 2012, 2014). The anti-AT2R was used at 1:200. For comparisons between different ages, all sections were incubated simultaneously and treated identically at all stages.
b. ***Double immunofluorescence*** was conducted following previously published protocols (Acosta et al., 2014). Tissue was incubated sequentially in the first primary antibody overnight at 4 °C and in secondary antibody appropriate for that primary antibody for 1hr at room temperature. We used goat anti-AT2R 1:200 (see Section 2.2); rabbit anti-Ret 1:500 (Santa Cruz Biotechnology, sc-167, RRID:AB_631317) and mouse anti-trkA 1:500 (AbD Serotec, 7045-7040, RRID:AB_620277). Secondary antibodies: anti-goat Alexa-594, anti-mouse Alexa-488 and anti-rabbit Alexa-488, all 1:400 (Molecular Probes, Invitrogen). For IB4-binding, tissue was incubated with IB4 conjugated with Alexa-488 (1:500) (Bergman et al., 1999).

2.4. Image analysis

All analysis was performed as previously detailed, see (Fang et al., 2006), on images captured on a Nikon 80i microscope and the measures were performed using HCImage (Hamamatsu). In the co-localization studies, only neurons with visible nuclei were measured. An average of 6–8 fields at ×40 from mid-sections of L4/L5 DRGs were all captured at the same time with identical settings. The cross sectional area and mean cytoplasmic pixel density were determined for each neuron. The mean pixel densities of the 5 most intensely stained and the 5 least intensely stained neurons in the analysed section provided 100% and 0% values respectively.

Neurons were classed by size. For embryonic and early post-natal DRG neurons only 2 groups can be discerned: small and large. At E15, small, mechanical-insensitive neurons are those with areas ≤100 μm^2 while at E18 the limit is ∼120 μm^2 (Lawson and Biscoe, 1979; Lechner et al., 2009). Based on expression of the neurofilament of 200 kDa, which labels large, myelinated neurons (Lawson et al., 1984), the boundary moves to ∼300 μm^2 at PN3 and ∼350 μm^2 at PN15 (Beland and Fitzgerald, 2001). At PN30 we classed them as small (≤400 μm^2, mostly C-fiber neurons), medium-sized (400–800 μm^2, mostly Aδ-neurons) and large (>800 μm^2 mostly Aα β neurons) see (Fang et al., 2005b).

2.4.1. Neurons classed as positive

For each antibody/marker, neurons were classed as positive on the basis of percentage values of neurons that were blindly classed subjectively as clearly stained above background. A subjective score of 1 (visible staining) corresponded to objective AT2R values of ≥20% which were therefore classed as clearly positive.

2.5. Statistics

Because our data failed the DíAgostino-Pearson test for normality, non-parametric tests were used. Results are shown as means ± SEM and comparison between multiple treatment groups was with one way Kruskal-Wallis ANOVA with Dunn's multiple comparisons test. Comparison between 2 treatments was with Mann-Whitney test. All tests were performed with Prism 5 (GraphPad). A level of $p < 0.05$ was considered statistically significant. Correlations were tested with Spearman's non-parametric test.