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Expression of spinal cord microRNAs in a rat model of chronic neuropathic pain

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ABSTRACT

Neuropathic pain is accompanied by significant alterations of gene expression patterns in the somatosensory nervous system. The spinal cord is particularly prone to neuroplastic changes. Since the expression of microRNAs (miRNAs) has been linked to numerous pathophysiological processes, a contribution of miRNAs to the maladaptive plasticity of the spinal cord in neuropathic pain is possible. Aim of the present study therefore was to characterize the specific expression pattern of miRNAs in the rat spinal cord. Furthermore, we evaluated the time-dependent changes in expression patterns of spinal miRNAs in the chronic constriction injury (CCI) model of neuropathic pain in rats. Results from miRNA microarrays revealed a distinct expression pattern of miRNAs in the rat spinal cord. MiRNAs-494, -720, -690 and -668 showed the highest signal intensities. Members of the let-7 family as well as miR-124 belong to the group of the most highly expressed miRNAs. Induction of neuropathic pain by CCI did not lead to relevant differences in spinal miRNA expression levels compared to sham-operated animals at any studied time point. Therefore, modulation of miRNAs does not seem to contribute significantly to the changes in gene expression that cause neural plasticity in the spinal cord in this model of chronic neuropathic pain.

1. Introduction

Neuropathic pain is caused by a lesion or dysfunction of the somatosensory nervous system [22]. These damages lead to thorough changes in the structure and function of the somatosensory system. Treatment of neuropathic pain remains a great challenge and in a significant number of patients, its treatment is inadequate [12]. Improving the understanding of the mechanisms underlying neuropathic pain might eventually lead to more specific, mechanism-oriented and thus more efficient treatment strategies [6]. Evidence suggests that the differential expression of multiple pain-associated genes in the spinal cord plays a key role in the development and maintenance of neuropathic pain [19].

MiRNAs are known to be important mediators of gene regulation in response to cell-to-cell signaling [2]. Functional (mature) miRNAs recognize and bind to partially homologous sequences in the 3′-untranslated region (3′-UTR) of target messenger RNAs (mRNAs) [7]. Several miRNAs are involved in the regulation of genes responsible for nervous system development and neural plasticity [16,17]. The changes in pain sensitivity induced by peripheral nerve injury are characterized by altered gene regulation and protein expression [11]. This may be accompanied by changes in the expression of miRNAs involved in nociceptive pathways.

In the present study, the basal expression level of miRNAs in rat spinal cord was investigated. Furthermore, we addressed the question of whether the expression of miRNAs in the spinal cord was affected during the development of the painful peripheral neuropathy using the chronic constriction injury model of neuropathic pain.

2. Experimental procedures

2.1. Animals

This study was performed with permission of the local animal use and care committee in accordance with the German animal protection law and the guidelines of the International Association for the Study of Pain. Chronic constriction injury (CCI) was used as a model of neuropathic pain [8]. Male Wistar rats weighing 350–400 g were anesthetized with an intraperitoneal injection of pentobarbital (60 mg/kg), and the left sciatic nerve was ligated with four loose ligatures proximal to the trifurcation. Sham procedure comprised equal treatment but without ligation of the sciatic nerve. Each group consisted of 6 animals. Animals were investigated 4 h, 24 h, 6 days and 12 days after surgery.

Abbreviations: CCI, chronic constriction injury; DRG, dorsal root ganglion; MeV, multi experiment viewer; miRNA, microRNA; SNI, spinal nerve injury.

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The development of mechanical allodynia was examined as described previously [14]. Behavioral testing was performed before CCI surgery and before tissue extraction to assess the development of neuropathic pain. Afterwards, animals were deeply anesthetized with intraperitoneal pentobarbital and the spinal cord (L4–L6) was quickly removed and immediately frozen in liquid nitrogen.

2.2. RNA isolation

Total RNA of rat spinal cord was isolated using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. RNA quantity was determined by UV spectrophotometry (Nanodrop, Thermo Scientific, USA) and RNA integrity was verified by gel electrophoresis using 2.5 μg of total RNA per lane.

2.3. MicroRNA-microarray

MiRNA expression profiling was performed using the miCHIP microarray platform as described previously [9,10]. In brief, 500 ng of total RNA was labeled with a Cy3-conjugated RNA linker (Biospring, Germany) and hybridized on the microarray. miCHIP is based on a locked nucleic acid (LNA) technology, whereby LNA-modified, Tm-normalized miRNACURY capture probes (Exiqon, Denmark), designed to target 590 and 349 unique mouse and rat miRNAs, respectively (miRNAbase v11), were printed on Codelink slides (GE Healthcare, USA). Microarray images were generated using the Genepix 4200AL laser scanner (Molecular Devices, USA) in batches using the Genepix auto PMT (Photo Multiplayer).

2.4. Computational analysis

The Genepix software saves signal into TAB-delimited files using the '.GPR' file extension. GPR files are median normalized by using the miCHIP R-library running on Bioconductor (www.bioconductor.org). Specifically, to perform the median normalization, the miCHIP library carries out the following manipulation of the data: (i) it removes all the signals from absent features and from features that have been flagged, (ii) it calculates the median intensity of the replicated spots for each capture probe (note that the correct signal is contained in the column 'F532 Median—B532'), (iii) it computes the total median intensity of the signal within each experiment and (iv) it uses the total median intensity to median normalize the individual samples.

As output, the miCHIP-library generates a TAB delimited file which contains the signal intensities from each sample within the experiment organized in columns. Median normalized values are then imported into the MeV microarray analysis software (Multi Experiment Viewer, www.tm4.org). Within MeV, data are first filtered for low intensity signals with cut off set to 0.5. As second step we perform the Hierarchical clustering (with Pearson correlation), which allows us to evaluate the relationship across the samples. Finally, we perform SAM (serial analysis of microarray), which allows to identify miRNAs which are differentially co-regulated across different treatment.

2.5. MicroRNA-qPCR assay

1 μg of total RNA was reverse transcribed using the high capacity RNA-to-cDNA master mix according to the manufacturer's protocol (Applied Biosystems, Carlsbad, USA). qPCR assays for miR-30b (Assay ID: 000602, Applied Biosystems), miR-100 (Assay ID: 000437, Applied Biosystems), miR-10a (Assay ID: 000387, Applied Biosystems), miR-99a (Assay ID: 000435, Applied Biosystems) and miR-720 (Assay ID: 001629, Applied Biosystems) were used according to the manufacturer's instructions. U6 (Assay ID: 001973, Applied Biosystems) was used for normalization. qPCR conditions: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 60 s on an Applied Biosystems 7300HT thermocycler (Applied Biosystems). All samples were run in duplicates and PCR was repeated at least twice. Relative expression was estimated using the ΔΔCq-method [25] and the relative expression software tool [26].

2.6. Statistical analysis of in vivo experiments

Paw withdrawal thresholds assessed with the Plantar Aesthesiometer as a sign of mechanical allodynia were compared using paired student's *t* test (SPSS 16, SPSS Inc., Chicago, IL, USA) $p < 0.05$.

3. Results

The withdrawal thresholds as response to mechanical stimulation with von Frey-type filaments were not significantly altered on either side as early as 4 or 24 h after nerve ligation (Fig. 1). Animals that underwent CCI procedure showed significantly decreased paw withdrawal thresholds of the left injured paw 6 and 12 days after surgery as a sign of mechanical allodynia. No changes were observed after sham procedure or in the right paw.

To functionally investigate a possible link between miRNA expression and the development of a painful peripheral neuropathy we first analyzed the expression of miRNAs in the rat spinal cord using miRNA microarrays (miCHIP [9]). A large number of miRNAs was identified as expressed in rat spinal cord (Fig. 2, Table S1, supplementary material). While ranking the normalized signal intensities for expression levels we identified miR-494, -720, -690, -668, -875-3p, -24-1*, -709, -207, -433* and -138* as the most highly expressed miRNAs in rat spinal cord. In accordance with previous reports, we identified members of the let-7 family (i.e. let-7e and 7c) as well as miR-124 and miR-34b-3p among the group of highly expressed miRNAs in the rat spinal cord.

We next analyzed whether induction of chronic neuropathic pain led to temporal changes of miRNA expression patterns in the rat spinal cord. To study this, the relative expression of spinal cord miRNAs in CCI vs. sham operated animals was initially assessed by

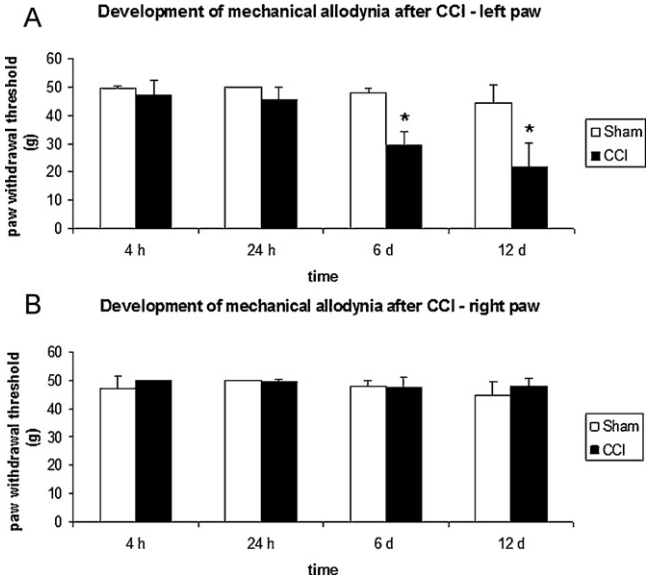

Fig. 1. Results from assessment of mechanical allodynia in the left, injured (A) and the right, uninjured paw (B). Paw withdrawal threshold (g) as response to mechanical stimuli of the plantar surface of the respective paw. Significant reduction of the withdrawal threshold of the left paw 6 and 12 days after CCI in comparison to baseline values and threshold of the sham group represents development of mechanical allodynia as sign of neuropathic pain. Data are presented as mean + SD, $p < 0.05$.