Genes Associated with Individual Variation of Electroacupuncture Anti-allodynic Effects in Rat

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The present study aims to identify and characterize genes that cause differences between non-responders and responders to electroacupuncture (EA) on mechanical allodynia following peripheral nerve injury. Under sodium pentobarbital anesthesia, animals were subjected to unilateral transection of the superior caudal trunk at the level between S1 and S2 spinal nerves. EA stimulation (2Hz, 0.3 ms, 0.2-0.3 mA) was delivered to Zusanli (ST36) for 30 min 2 weeks after the surgery. The degree of mechanical allodynia was assessed quantitatively by touching the tail with von Frey hair (2.0 g) at 10 min intervals. The rats, which showed an EA-induced decrease of response frequencies under 10 %, were classified as non-responders and those displaying an EA-induced decrease of response frequencies 20 % or more were classified as responders. Results from oligonucleotide microarray, to which cDNAs from the spinal dorsal horn (DH) were applied, showed that hemoglobin beta chain complex and chondroitin sulfate proteoglycan-5 decreased and limbic system-associated membrane protein increased in the non-responder group, whereas calcium-independent alpha-latrotoxin receptor homolog-3 increased in the responder group. These results suggest that The functional abnormality of molecules regulating cell adhesion, intracellular signal transduction and cell differentiation in the spinal DH may be involved in the anti-allodynic effect of EA.

Key words : Electroacupuncture, Allodynia, Non-responder, Responder, Microarray

Introduction

Acupuncture has long been used in Asian countries for the treatment of various diseases including pain, with few side effects, and recently considered a new alternative method of medicine in Western countries[1]. Electroacupuncture (EA) is a modified technique of acupuncture using electrical stimulation and its analgesic effects have been shown in various animal species and humans[2]. However, the analgesic effects responding to EA show marked individual variations, which also provoke both clinical and experimental difficulties.

Individual difference in acupuncture analgesia in normal rats[3-5], has been reported. The neuropeptide cholecystokinin octapeptide (CCK-8) and its receptor subfamilies have been suggested to be associated with non-responder for EA analgesia[3,5,6]. Recently, cDNA microarray study for normal rats exhibited that Brain factor-1, dopamine β-hydroxylase and acetylcholinesterase T subunit were expressed abundantly in the responder group, whereas neurodegeneration markers, such as tau and cathepsin B were abundant in the non-responder group[7].

Peripheral nerve injury often results in chronic neuropathic pain, which is characterized byspontaneous burning pain, hyperalgesia and allodynia[8]. Previous findings of our group[9,10] and others[11] have indicated that low-frequency EA stimulation produce an anti-allodynic effect via the endogenous opioid system, although Dai et al.[12] reported that high-frequency EA failed to suppress mechanical allodynia. Additionally, potential genes and proteins associated with EA analgesic effects in an animal model of neuropathic pain have also been identified[13,14]. Individual variation in EA analgesia on neuropathic pain, however, is poorly understood. Therefore, the present study, using a rat model of neuropathic pain[15], was performed to identify the expression of extensive genes from the spinal dorsal horn (DH), which is affected by the injury of the associated peripheral nerve, in the non-responder and responder group to EA.

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Materials and Methods

Young adult male Sprague-Dawley rats (Sam:TacN(SD)BR, 200-220 g) were housed in groups of four, with water and food available ad libitum. The room was maintained with a 12-hour light/dark cycle (08:00-20:00 light, 20:00-08:00 dark) and kept at 23±2℃. The rats were subjected to the neuropathic surgery and behavioral test for mechanical allodynia as previously described by Na et al.[15]. Briefly, under sodium pentobarbital anesthesia (40 mg/kg, i.p.), the right superior caudal trunk was exposed and transected at the level between the S1 and S2 spinal nerves innervating the rat's tail. In sham surgery, the surgery procedure was the same as above conditions, except for the actual cutting of the spinal nerves. The rat was lightly immobilized in a plastic holder (5.3×15, 5.6×17, 6.0×24 cm in diameter × length) with the hind legs and the tail protruding on a plate. The mechanical allodynia was assessed by normal innocuous stimulation of the tail with the von Frey hair (bending force: 2.0 g). The mechanically sensitive area was first determined by rubbing various areas of the tail with the von Frey hair. Testing was performed by gently poking the most sensitive spot with the von Frey hair. An abrupt tail movement in response to the von Frey hair stimulation was considered to be an abnormal response attributed to mechanical allodynia. The stimulation was repeated 10 times at 10 s intervals for each animal. In each session, the test stimuli were delivered to the most painful spot under the same conditions. The degree of response was expressed as a percentage of response frequency and was determined as follows;

Response frequency (%)
= (Number of abnormal responses/stimulation) × 100

Two weeks after the neuropathic surgery, the behavioral tests were performed with the von Frey hair, and rats displaying well-developed behavioral signs of mechanical allodynia were selected. Two stainless steel acupuncture needles 0.25 mm in diameter were inserted to a depth of 5 mm in each hind leg, one into Leg Samni (ST36) which is located at the anterior tibial muscle approximately 10 mm below the knee joint, and the second into the anterior tibial muscle 5 mm distal to the first one. The two acupuncture needles were connected with the output terminals of an electric stimulator. Train-pulses (2 Hz, 0.3 ms pulse width, 0.2-0.3 mA) were delivered to the inserted needles for 30 minutes. The behavioral tests were performed again during and after EA stimulation.

Since the peak analgesia usually appeared at 30 min after the beginning of EA and the anti-allodynic effect was rapidly recovered following EA stimulation[9,10], the decreased percentage of tail response frequencies of the time point and 10 minutes following EA stimulation was taken as the index for the effectiveness of EA analgesia. The preliminary analysis also showed statistically significant analgesia in the two time points when the decreased average percentage of response frequencies was more than 10-20% compared with that before EA stimulation. In addition, when rats were only restrained in holders without EA treatment or with acupuncture needles inserted at the same loci without electrical stimulation, the percent changes of response frequencies appeared less than 10 %. Thus, the rats, which showed an EA-induced decrease of response frequencies under 10%, were classified as non-responders and those displaying an EA-induced decrease of response frequencies 20% or more were classified as responders. The rats whose response frequencies were in the range from 10% to 19% were discarded.

Rats were sacrificed by CO_2 asphyxiation followed by decapitation. For spinal cord isolation, laminectomy was performed. The spinal cord at the level S1 was dissected and the dorsal part of the spinal cord was carefully separated from the ventral part by cutting through the central canal. The dorsal spinal cord sample was frozen immediately in liquid nitrogen and kept at -70℃. Frozen specimens were placed in TRIzol reagent (Invitrogen Life technologies, Gaithersburg, MD, USA) and homogenized with a polytron homogenizer before isolation of total RNA. An aliquot of total RNA was used to confirm the absence of significant degradation by electrophoresis in a 1% agarose gel.

The microarray analysis was performed using Affymetrix Rat Genome U34A arrays according to the manufacturer's protocol. Each chip contains 8799 probe pair sets known transcripts and expressed sequence tags (ESTs) clusters (see http//www.Affymetrix.com). Two sets of oligonucleotide microarray membranes for each group were used. Briefly, total RNA (10 μg) from the spinal dordal horn (DH) was reverse transcribed using an oligo-dT primer encoding the T7 RNA polymerase promoter. First strand cDNA synthesis reactions were carried out at 37℃ using Superscript II reverse transcriptase. Second strand cDNA synthesis was finished using E. coli DNA ligase and T4 DNA polymerase. Each double-stranded cDNA sample was purified by phenol:chloroform:isoamyl alcohol (25:24:1) (Invitrogen Life Technologies). The biotinylated cRNA was synthesized using ENZO BioArray high yield RNA transcript labeling kit (Affymetrix, Inc., Santa Clara, U.S.A.) and cRNAs were purified using RNaeasy mini kit (Qiagen, Hilden, Germany). Biotin-labeled cRNA (20 μg) were fragmented at 94℃ for 35

min in 200 mM Tris-acetate, pH 8.1, 500 mM potassium acetate (Sigma-Aldrich), and 150 mM magnesium acetate (Sigma-Aldrich) to a mean size of 35-200 nucleotides.

GeneChip arrays were prehybridized with 150 ㎕ hybridization buffer [100 mM MES (2-[N-Morpholino] ethanesulfonic acid, Sigma-Aldrich), 1 M [Na^+], 20 mM EDTA, 0.01% tween 20 (Sigma-Aldrich)] for 10 min at 45℃ in an Affymetrix Genechip Hybridization Oven at 60 rpm. Hybridization was performed in a final volume of 300 ㎕, containing 15 μg fragmented biotinylated cRNA, 50 pM control oligonucleotide B2 (Affymetrix), eukaryotic hybridization controls (bioB, bioC, bioD, creX, 30 pM each) (Affymetrix), 0.1 ㎎/㎖ herring sperm DNA (Promega, Madison, U.S.A.), 0.5 ㎎/㎖ acetylated bovine serum albumin (BSA) (Invitrogen Life Technologies) in 1 × hybridization buffer. The samples were heated to 95℃ for 5 min and 45℃ for an additional 5 min and then spun down. Two hundred microliters of the hybridization buffer were added to the Genechips and hybridizations were performed at 45℃ for 16 hr. After hybridization, Microarrays were washed and stained according to EukGE-WS2 protocol of Genechip Fluidics Station (Affymetrix). The microarrays were scanned with a Genechip System Confocal Scanner (Hewlett-Packard Co., Palo Alto, CA). The analysis of scan data was performed using GeneChip Software MAS-5.0 (Affymetrix). For additional informations about the statistical analysis, see the Affymetrix Statistical Algorithms Reference Guide at: http://www.affymetrix.com/support/technical/technotes/statistical_reference_guide.pdf.

Results

As results, among the whole rats (n=52), twenty-six rats (50%) showing an EA-induced decrease of response frequencies less than 10% were classified as non-responders. Fifteen rats (29%) showing an EA-induced decrease of response frequencies more than 20% were classified as responders. The remaining 11 rats (21%) showing an EA-induced decrease of response frequencies between 10% and 19% were excluded.

Gene expression difference between non-responders and responders when compared with the sham group were screened. A total of twenty-four genes are differently expressed in the two experimental groups. In non-responder group, 8 genes are differently expressed in comparison with genes of the control group(Table 1). Among these 8 genes, 3 genes decreased and 5 genes increased. In the responder group, 16 genes are differently expressed in comparison with genes of control group(Table 2). Among these 16 genes, 10 genes decreased and 6 genes increased.

Table 1. The genes that were expressed significantly different in non-responders vs. control.

ID*	Gene Name	Fold**
M94919	hemoglobin beta chain complex (Hbb)	-1.68
AI639315	Transcribed sequences	-1.42
U33553	chondroitin sulfate proteoglycan 5 (Cspg5)	-1.57
AF060173	SV2 related protein (Svop)	1.59
M83680	GTPase Rab14 (Rab14)	1.58
AA892921	EST	1.52
U31554	limbic system-associated membrane protein (Lsamp)	1.65
X96437	immediate early response 3 (Ier3)	1.41

Table 2. The genes which were expressed significantly different in responders vs. control.

ID*	Gene Name	Fold**
AA799576	Similar to nucleolar protein GU2 (LOC361848), mRNA	-1.18
AA818843	postsynaptic protein Cript (Cript)	-1.15
AA894084	Transcribed sequences	-1.42
AA894304	Ultraviolet B radiation-activated UV118 mRNA, partial sequence	-1.12
AA944422	calponin 3, acidic (Cnn3)	-1.27
AI104399	triosephosphate isomerase 1 (Tpi1)	-1.33
AI172064	lectin, galactose binding, soluble 1 (Lgals1)	-1.36
U44948	cysteine and glycine-rich protein 2 (Csrp2)	-1.29
X54640	basigin (Bsg)	-1.26
X75253	phosphatidylethanolamine binding protein (Pbp)	-1.32
AF063103	calcium-independent alpha-latrotoxin receptor homolog 3 (Cirl3)	2.12
M10094	RT1 class Ib, locus Aw2 (RT1-Aw2)	1.19
AA858621	CaM-kinase II inhibitor alpha (LOC287005)	1.09
AI178971	Similar to alpha globin (LOC287167), mRNA	1.05
U75923	isoleucine-tRNA synthetase (Iars)	1.09
U78977	ATPase, class II, type 9A (Atp9a)	1.15

*ID indicates the accession number of GenBank (NCBI). **Fold means ratio of hybridization intensity. Down regulated genes are expressed as negative fold changes.

Discussion

In normal rats, previous studies reported individual differences for responsiveness to EA or acupuncture analgesia on nociceptive pain[3-6], suggesting that from 20% up to 40% of Wistar rats[4] and about 40% Sprague-Dawley rats[3] were not respondent to EA analgesia assessed by a tail-flick test. These results displayed similarity in the ratio of non-responders, although the strains of rats used were different. In the present study, neuropathic rats produced by transecting some spinal nerves innervating the tail also showed individual variation in responsiveness to EA stimulation. However, up to 50% of Sprague-Dawley rats were not respondent to EA analgesia on mechanical allodynia. The ratio of non-responder rats was higher in the present study than that in the previous studies. In a neuropathic pain model, spinal nerve injury can induce various changes in the spinal DH, which may alter the responsiveness for EA stimulation or EA pathway in the CNS. Thus, this study intended to focus on differently expressed genes in the DH of spinal cord S1 affected by the spinal nerve transection, comparing non-responders and responders.

In our microarray analysis, the genes, being differently expressed between non-responders and responders, were few. Furthermore, only one gene was detected under the criteria of 2-fold difference(Table 2), and CCK or its receptors, which have been considered as potent factors causing individual variation on EA in normal rats[3,5,6], were not differently expressed between non-responders and responders in neuropathic rats. Thus, it can be supposed that the difference of gene expression of the spinal DH between non-responders and responders in neuropathic rats was very small. This finding is in corroboration with Lee's results[7] that the genes identified from microarray, being differently expressed between responders and non-responders in normal rats, were relatively few when compared with other cDNA microarray studies.

As shown in Table 1 and 2, twenty-four genes were differently expressed in non-responder and responder neuropathic rats. These genes are involved in a number of biological processes, including signal transduction, cell adhesion and cell differentiation. Especially notable among them are hemoglobin beta chain complex (Hbb), chondroitin sulfate proteoglycan 5 (Cspg5), limbic system-associated membrane protein (Lsamp) and calcium-independent alpha-latrotoxin receptor homolog 3 (Cirl3). Hbb is expressed 1.68 times less in non-responders than in responders. Cspg5, neuroglycanC, is expressed 1.57 times less in non-responders. Lsamp, cell adhesion molecule regulating neuronal outgrowth, is expressed 1.65 times more in non-responders. Cirl3, which is involved in orphan G protein-coupled receptors, is expressed 2.12 times more in responders(Table 1 and 2). However, the functions of these genes as associated with acupuncture analgesic mechanism are unknown.

Hemoglobin beta chain complex (Hbb) was less expressed in non-responders than in responders. The presence of hemoglobin containing alpha-globin and beta-globin in the CNS[16] and macrophages[17] has been reported. Hemorphins, hemoglobin-derived oligopeptides with opioid activity, were first isolated from enzymatically treated bovine blood and were found to inhibit enzymes degrading enkephalins[18,19]. Hemorphins are largely distributed in various areas such as the cerebrospinal fluids, the pituitary gland and hypothalamic tissue[20]. Spinorphin, the sequence of which matches a conserved region of beta-globin, was isolated from bovine spinal cord. Like hemorphins, spinorphin is an endogenous inhibitor of enkephalin-degrading enzymes such as neutral endopeptidase, aminopeptidase and dipeptidyl aminopeptidase[21], and has analgesic activity on morphine-resistant pain pathways[22] and anti-allodynic effect[23]. Enkephalins are well known to be involved in the pain-modulating mechanism of EA in the spinal cord[2]. However, Enkephalins are short-lived, being rapidly degraded by enkephalin-degrading enzymes. Thus, these fragments of beta-globin can be supposed to protect enkephalins from degradation, which result in potentiating EA-mediated analgesia in the spinal DH. This view is supported by Takeshige's result[4] that D-phenylalanine, an enkephalinase inhibitor, converted non-responders into responders to EA.

The chondroitin sulfate proteoglycan 5 gene (Cspg 5; neuroglycan C) was less expressed in non-responders than in responders. Neuroglycan C (NGC) is a transmembrane chondroitin sulfate proteoglycan exclusively expressed in the CNS[24]. NGC has been known to be involved in maintenance of brain functions and neuronal circuit formation in the developmental change of the CNS, and to participate in the repair processes of injured neuronal circuits of the CNS[25].

The limbic system-associated membrane protein gene (Lsamp) was more highly expressed in non-responders. The Lsamp gene encodes the limbic system-associated membrane protein (LAMP) that is an immunoglobulin superfamily member (IgSF) with 3 Ig domains and a glycosyl-phosphatidyl inositol anchor and that exhibit high sequence homologies with other members of the IgSF (Opioid-binding cell adhesion molecule and neurotrimin)[26]. LAMP is expressed by cortical and subcortical neurons comprising the limbic system and is present on dendrites and somata of neurons in lamina II and the intermediolateral nucleus in the adult spinal cord[27]. Although, so far, the functions of LAMP are insufficiently understood, this protein has been reported to be essential for proper targeting of limbic connections[26] and to be involved in axon targeting in the spinal cord[28].

Taken together, the functional abnormality of cell adhesion molecules regulating neuronal outgrowth may be able to affect functional and structural reorganization of the spinal DH following spinal nerve injury, which in turn may alter the responsiveness to EA stimulation or impair EA pathway resulting in non-responders to EA anti-allodynic effect.

The calcium-independent alpha-latrotoxin receptor homolog 3 (Cirl3) gene was highly expressed more than 2-fold in responder neuropathic rats when compared with non- responders. Alpha-latrotoxin is a potent neurotoxin from black widow spider venom that triggers neurotransmitter release[29]. The calcium-independent alpha-latrotoxin receptor (Cirl), also called latrophilin, belongs to a family of closely related orphan G protein-coupled receptors (GPCRs) homologous to the secretin receptor family[30,31]. In this family of three closely homologous proteins, Cirl-3 is expressed predominantly in brain similarly to Cirl-1[32]. However, the functions of Cirl-3 are poorly understood.

Conclusion

In conclusion, it was suggested that individual variation of response to EA might be caused by differential expression of genes associated with signal transduction, cell adhesion, and cell differentiation. Gene expression profiles substantiating individual variation on EA in neuropathic rats will help other investigators to research intrinsic molecular mechanism on the anti-allodynic effect of EA.

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