Original article

Screening of differentially expressed genes in the hypothalamus of a rat neuropathic pain model following sciatic nerve injury

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Background Neuropathic pain is induced by injury or disease of the nervous system. Most studies have so far focused only on a few known molecules and signaling pathways among neurons. However, all signal transmissions involved in neuropathic pain appear to be an integral system at different molecular levels. This study was designed to screen the differentially expressed genes of the hypothalamus in chronic constriction injury (CCI) rats and analyze their functions in developing neuropathic pain.

Methods Ten adult female Sprague-Dawley rats ((200±10) g) were used in experimental group and sham group (n=5 in each group). Mechanical allodynia tests were performed to ensure that the CCI rat model was constructed successfully. Total hypothalamus RNAs were isolated from each group. Forward suppression subtractive hybridization (SSH) library of rat hypothalamus was constructed and up-regulated cDNA clones at neuropathic pain states were obtained via suppressed subtractive hybridization technique and the functions of these genes were analyzed bioinformatically.

Results Mechanical allodynia tests showed that the experimental rats had a significantly reduced mechanical allodynia threshold 3 to 13 days after CCI vs sham surgery rats (P <0.01), indicating that the model was successful. Forward SSH library of the rat hypothalamus was constructed successfully and 26 over-expressed expression sequence tags (ESTs) were obtained from these up-regulated cDNA clones.

Conclusion Twenty-six up-regulated genes, involved in the regulation of cell cycle and apoptosis, signal transduction, and neuroprotection, may play key roles in decreasing mechanical withdraw thresholds in CCI rats, which implicates a multidimensional and integrated molecular mechanism at gene level in developing neuropathic pain with the supraspinal contributions.

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Chronic pain is a common and sometimes severely disabling state that affects millions of people worldwide. Such pain can be experienced after nerve injury or as a part of diseases that affects peripheral nerve function.[1] Neuropathic pain is the chronic pain state, which involves certain structural, physiological and pharmacological changes throughout the neuroaxis.[2] Neuropathic pain usually is persistent and can produce sensory deficits and other paradoxical sensations of a qualitative nature, including hyperesthesias, paresthesias and dysesthesias. The qualitative differences in sensation suggest that nerve injury leads to a reorganization of sensory transmission pathways that still persists after healing. Such reorganization in the nervous system implies that the current knowledge of anatomic pain pathways and neurotransmission is insufficient to understand the complexity of chronic neuropathic pain.[3-6]

In this study, we used the rat hypothalamus as a target and constructed a forward suppression subtractive hybridization (SSH) library of chronic pain maintenance phase, through which we intend to characterize the up-regulate of gene expression in the hypothalamus and to identify the key molecular regulation mechanism at pain sensitive states, thereby bringing us closer to a full understanding of the neuropathic pain of molecular mechanisms at supraspinal levels in peripheral nerve injury.

METHODS

Animals

Ten adult female Sprague-Dawley rats ((200±10) g) supplied by the Experimental Animal Center of Beijing, China, were used. The rats were housed in a single room under controlled temperature, humidity and a 12-hour light/dark cycle (08:00–20:00), with ad libitum access to

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normal rat chow and drinking water. All procedures were performed in accordance with the animal care guidelines of the National Institute of Health and the recommendations of the International Association for the Study of Pain; every effort was made to minimize both the animal suffering and the number of animals used.

Chronic constriction injury (CCI) model

All rats were prepared surgically 2–3 days before experimental tests. The rats were subjected to CCI as previously described.[7] Peripheral mononeuropathy (day 0) was produced by loosely ligating the sciatic nerve. Briefly, under sodium pentobarbital anesthesia (40 mg/kg, i.p.), the right sciatic nerve of each rat was exposed at mid-thigh level by blunt dissection through the biceps femoris muscle. Four loosely constrictive ligatures (4-0 chromic gut suture) were tied around the nerve at space of about 1 mm apart. The muscle and skin were closed in layers and a single dose of antibiotic was administered (Ampicillin 8000 U/rat, Sigma Chemicals, USA). Sham operations were performed whereby the nerve was exposed and freed from surrounding tissues without ligation. All operations were done by the same person.

Mechanical allodynia tests

Hind paw withdrawal threshold (PWT) was tested to confirm whether CCI and sham surgery rats (n=5/group) exhibited mechanical hyperalgesia. Sensitivity to mechanical stimuli was assayed using a series of force-calibrated von-Frey filaments (Stoelting Co., Wood Dale, IL, USA). Behavioral studies were carried out in a quiet room between 9:00–11:30 a.m. The rats were placed in a transparent plastic box on a metal mesh floor and were allowed to acclimate to the environment for 30 minutes before testing. The hairless plantar surface of the hindpaw was probed by a series of von Frey filaments. Each filament was tested five times at intervals of 3 seconds. If paw withdrawal was observed at least three times after probing with a filament, the rat was considered responsive to that filament. The response threshold was defined as the lowest filament force required to producing at least three withdrawal responses in the five tests. Withdrawal thresholds to mechanical stimuli in von Frey filament testing were determined before and at the 3rd, 5th, 7th, 13th days after the surgical procedures respectively.

Tissue preparation and RNA analysis

All rats were sacrificed 13 days after surgery. The animals were slightly anesthetized by carbon dioxide in a clear transparent plastic container, and then were immediately decapitated. The brains were dissected and removed as described by Reyes et al.[8] Briefly, a series of six cuts were performed using a razor blade. Viewing the ventral surface of the brain, two coronal cuts were made to isolate a hypothalamic block using the apex of the optic chiasm and the rostral margin of the mammillary bodies as landmarks. This slab was then placed flat and the first two cuts were placed on either side of the chiasm. The third cut was placed just dorsal to the third ventricle. The isolated hypothalamus was immediately frozen in liquid nitrogen, and then stored at −80°C until RNA extraction.

Total RNAs were isolated from hypothalamus tissue using the TRIzol reagent kit (Invitrogen, Carlsbad, CA, USA). The range of A260/280 ratio was 1.7–1.9, and total RNAs were also electrophoresed on a 1% agarose gel to ensure a ratio of 28s:18s rRNA >1.0.

Construction of forward SSH library of rat hypothalamus

In order to get up-regulated cDNA clones at neuropathic pain states, the forward SSH library was done, in which the cDNA from CCI group was used as tester and the cDNA from sham surgery group was used as driver. The total RNAs of hypothalamus tissues of the two groups were used as templates, and double-strand cDNA were synthesized by SMART™ PCR cDNA Synthesis Kit (Clontech, USA). After digestion by Ras I, the double-strand cDNA of the CCI and sham groups served as tester and driver, respectively. Tester cDNA was aliquotted into two separate parts and ligated with adaptor 1 and 2R, respectively, and hybridized by the excessive driver cDNA twice, and then nest PCR amplification was performed. The PCR products were ligated to pGEM-T easy vectors (Promega, USA), which were transformed into *E. coli* DH5α cells (TianGen, China) and screened for blue/white clones. The construction of forward SSH library was accomplished by PCRSelect™ cDNA Subtraction Kit (Clontech). Eighty recombinant clones were selected randomly and sequenced to check the quality of the library.

Bioinformatics analysis of expression sequence tags (ESTs)

The trace files after sequencing were done using the following programs: First, sequences were examined by cross-match software (Phil Green and Brent Ewing, *http://www.phrap.org/consed/consed.html#howToGet*) to screen the vector sequence, then the clean sequences were clustered by BLASTclust (*http://biowulf.nih.gov/apps/blast/doc/blastclust.html*) and aligned by phrap (*http://phrap.org/phredphrapphrap.html*) to reduce redundant sequences and obtain the unigenes. Finally, the unigenes were submitted to NCBI NT databank (*http://www.ncbi.nlm.nih.gov*) to get information regarding functional annotation.

Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Between group data were analyzed using independent-sample t test. A P value less than 0.05 was considered statistically significant.

RESULTS

Nociceptive sensitivity

Non-noxious mechanical stimuli (below a 12 g von Frey filament) did not produce any paw withdrawal response

in animals before CCI surgery. However, the paw withdrawal threshold to innocuous mechanical stimulation was reduced in the left limb in the CCI-treated rats, whereas sham surgery did not affect mechanical sensitivity (Figure). These data show that CCI surgery effectively resulted in mechanical allodynia.

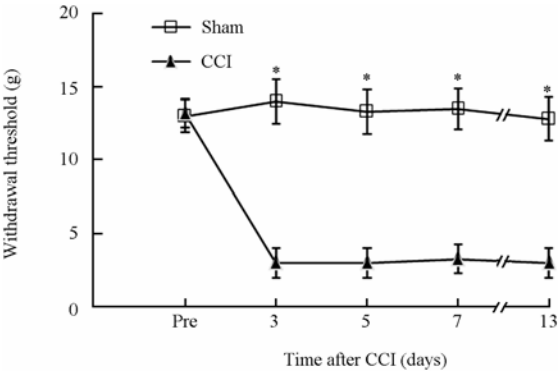

Figure. CCI-induced mechanical allodynia. CCI to the sciatic nerve lowered the hindpaw withdrawal threshold for innocuous mechanical force. CCI rats significantly reduced mechanical allodynia 3 to 13 days post-CCI compared with sham surgery rats ($^{*}P$ <0.01). Data are shown as mean±SEM (n=5/group).

Screening and informatics analysis of the rat hypothalamus SSH library

The forward SSH cDNA library of rat hypothalamus tissues were constructed successfully. In total, 26 positive ESTs were obtained from forward SSH. GeneTools was used for functional annotation of differentially expressed genes. Based on their biological function, those differentially expressed genes were classified into 7 groups: ion transport, signal transduction, inflammatory response, transcription, protein amino acid phosphorylation, G-protein signaling and unknown (Table).

DISCUSSION

Peripheral nerve injury often results in hyperalgesia and allodynia, which are associated with neuropathic pain. The CCI model is a well established one in rats that reliably produces sustained increases in pain sensitivity (i.e., thermal hyperalgesia and mechanical allodynia), and displays features similar to symptoms induced by many chronic pain, such as those with entrapment and diabetic neuropathies, causalgia, and reflex sympathetic dystrophy. Both peripheral and central mechanisms, which involve changes in gene expression, contribute to the pathophysiology of this neuropathic pain models. In our study, one hallmark of the model allows sufficient time to pass which permits complete resolution of the surgical manipulation such that the pain behavior is purely neuropathic, so we decided that the rats were sacrificed 13 day after surgery. Recently, changes in gene expression, protein translation and post-translational protein modification in dorsal root ganglion (DRG) and spinal cord have been reported.[3-6] However, our studies used the hypothalamus of CCI and sham surgery rats to construct a forward SSH library and obtain the up-regulate expressed genes during neuropathic pain states, which may contribute to illuminate the role of gene network in pain sensitive states at supraspinal levels. After the forward SSH cDNA library of rat hypothalamus tissues was successfully constructed, 26 up-regulated expressed genes, which may play key roles in pain sensitive states, were obtained.

Bioinformatical analysis showed that the known over-expressed genes are mainly involved in four processes: the regulation of cell cycle and apoptosis (immunoglobulin superfamily, member 1); signal transduction and neuroprotection (*Rnf14, DDAH, Voltage-gated potassium channel beta subunit 4.1, NADH dehydrogenase subunit 6, tetraspanin 3, lysophospholipase-like 1, prothymosin alpha, transferrin, budding uninhibited by benzimidazoles3 homolog (S. cerevisiae), N-myc downstream regulated gene 2, Coiled-coil-helix-coiled-coil-hel-ix domain containing 5, Rnf25);* gene expression regulation (*Nr4a2, CWC15 homolog (S. cerevisiae), Zinc finger, SWIM domain containing 3, heat shock protein 90 kD alpha (cytosolic), class B member 1*) and intracellular traffic (*Dexras1, mt-Co3, mt-Co2, G-protein coupled receptor 173*).

Indeed, among the mediators activated in CCI model, nitric oxide (NO) and prostaglandins (PGs) have prominent roles in the development of neuropathic pain. Several lines of evidence have shown a role for the NO/cyclic guanosine monophosphate (cGMP) signaling pathway in nociception.[9,10] NO serves as a neurotransmitter both in the brain and the peripheral nervous system, which is synthesized from L-arginine by a family of enzymes termed the NO synthases, including endothelial NOS (eNOS), inducible or macrophage NOS (iNOS), and neuronal NOS (nNOS).[11,12] Lesion-induced NO production has been attributed to have a protect effect on damaged neurons and might be associated with neuronal regeneration, however, induction of NO is also suggested to be involved in neuronal death.[13] Peripheral nerve lesion may cause chronic pain and many studies have demonstrated an important role of NO in the development and maintenance of pathological pain.[14,15] As NO is mainly an excitatory neuromediator, its increased production and release at the intraspinal presynaptic terminal may facilitate afferent synaptic transmission to the dorsal horn neurons, thus contributing to spinal neuronal sensitization and hyperalgesia. In addition, it is conceivable that the enhanced NO release into the spinal cord represents an injury signal by which the pathophysiological processes following nerve injury are carried over from the peripheral to the central nervous system, and NO also has a high potential to induce lasting changes in pre- and postsynaptic excitability.[16] It has been reported that CAPON forms a complex with Dexras1 and NOS, which directs and enhances the delivery and specificity of the NO signal. Furthermore, asymmetric dimethylarginine (ADMA) can regulate NO generation

Table. List of differentially expressed genes of the hypothalamus SSH cNDA library

Gene Bank ID	Genes	Molecular function
BC103648	Ring finger protein 14 (Rnf14)	Zinc ion binding
D86041	N-G,N-G-dimethylarginine Dimethylaminohydrolase (DDAH)	Signal transduction
M27315	Cytochrome c oxidase III, mitochondrial (mt-Co3)	Oxidoreductase activity
J01434	Cytochrome c oxidase II, Mitochondrial (mt-Co2)	Oxidoreductase activity
XM_223828	Ngg1 interacting factor 3 like 1 binding protein 1 isoform 1	Unknown
BC161800	Voltage-gated potassium channel beta subunit 4.1	Ion channel activity
DQ673917	NADH dehydrogenase subunit 6	Signal transducer activity
XM_340809	RAS, dexamethasone -induced 1 (Rasd1)	GTP binding
XM_019328	Nuclear receptor subfamily 4, group A, member 2 (Nr4a2)	Ligand-dependent nuclear receptor activity
NM_206847	Phosphofructokinase, platelet (Pfkp)	6-phosphofructokina-se activity
NM_001005547	Tetraspanin 3	Iron ion transport
BC057636	G-protein coupled receptor 173	G-protein coupled receptor activity
BC087046	Ring finger protein 25 (Rnf25)	Zinc ion binding
NM_175763	Immunoglobulin superfamily, member 1	Coreceptor activity
NM_146106	Lysophospholipase-like 1	Hydrolase activity
AC120736	11 BAC CH230-252B13	Unknown
NM_001024987	CWC15 homolog (*S. cerevisiae*)	RNA binding
BC092569	Prothymosin alpha	Negative regulation of cascade activity
BC091328	Zinc finger, SWIM domain containing 3	DNA binding
AY327504	Transferrin	Iron ion transport
BC099199	Budding uninhibited by benzimidazoles 3 homolog (*S. cerevisiae*)	Signal transduction
NM_001004082	Heat shock protein 90 kDa alpha (cytosolic), class B member 1	ATP binding
BC058446	SMT3 suppressor of mif two 3 homolog2 (*yeast*)	Protein binding
AF334106	N-myc downstream regulated gene 2	Signal transduction
BC158612	Coiled-coil-helix-coiled-coil-hel-ix domain containing 5	Signal transduction
BC064440	Aldolase A, fructose-bisphosphate	Protein binding

through inhibiting of NOS. However, DDAH activity was present to ensure that local concentrations of ADMA did not accumulate to inhibit NO generation.[17] In our study, the up-regulate expressed genes of DDAH and Dexras1 were detected by SSH method. Thus, present data imply that CAPON and/or Dexras1 and DDAH might be involved in the pain process and the development of neuropathic pain through different signaling pathways in nociception.

It has been reported that cytochrome oxidase (COX) and NADH play a vital role in cellular energy generation and the energy production is associated with the expression level of COX.[18-20] Neuronal signal transduction plays an important role in the regulation of neuronal activities. G-proteins and protein kinase cascades are key parts of the signal transduction.[21-23] At the neuropathic pain states, cellular energy generation and activity of neurons were always stayed in the high levels. So many up-regulated genes (*cytochrome c oxidase III, mitochondrial, cytochrome c oxidase II, mitochondrial, NADH dehydrogenase subunit 6, G-protein coupled receptor 173, phosphofructokinase, platelet, lysophospholipase-like 1, aldolase A, fructose-bisphosphate*) were obtained in the forward SSH cDNA library, which may be caused by neuropathic pain.

Emerging evidence for the involvement of ion channels in pain perception such as noxious mechanical stimuli and heat has established ion channels as a potential master regulator of many vital neuronal functions including nociceptive pain modulation.[24] The result of our study revealed that the biological process of ion transport was indeed involved in the pain perception in neuropathic pain, and we found that six genes (*transferrin, ring finger protein 25, tetraspanin 3, voltage-gated potassium channel beta subunit 4.1, ring finger protein 14*) which were related to ion transport were up-regulated in the CCI group.

Among these 26 genes, two differentially expressed unclassfied functional genes were detected in the present study. Although the functions of these genes were not clearly determined, it is assumed that they might play potential roles in the development of neuropathic pain in the hypothalamus.

In summary, we successfully constructed the SSH cDNA library of rat hypothalamus tissues and obtained 26 over-expressed ESTs in the maintenance phase of neuropathic pain, which may play crucial roles in decreasing mechanical withdraw thresholds in CCI rats and may have important functions in developing neuropathic pain. The results imply a multidimensional and integrated molecular mechanism at gene level in developing neuropathic pain with the supraspinal contributions. However, the differentially expressed levels of these ESTs in proteins and their regulatory functions are still unclear and require further investigation.

REFERENCES

1. Makoto T, Kazuhide I, Michael WS. Neuropathic pain and spinal microglia: a big problem from molecules in 'small' glia. Trends Neurosci 2005; 28: 101-107.
2. Myers RR, Campana WM, Shubayev VI. The role of neuroinflammation in neuropathic pain: mechanisms and therapeutic targets. Drug Discov Today 2006; 11: 8-20.
3. Suzuki R, Dickenson AH. Neuropathic pain: nerves bursting

with excitement. Neuroreport 2000; 11: R17-R21.
4. Woolf CJ, Salter MW. Neuronal plasticity: increasing the gain in pain. Science 2000; 288: 1765-1769.
5. Taylor BK. Pathophysiologic mechanisms of neuropathic pain. Curr Pain Headache Rep 2001; 5: 151-161.
6. Zimmermann M. Pathobiology of neuropathic pain. Eur J Pharmacol 2001; 429: 23-37.
7. Bennett GJ, Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 1988; 33: 87-107.
8. Reyes TM, Walker JR, DeCino C, Hogenesch JB, Sawchenko PE. Categorically distinct acute stressors elicit dissimilar transcriptional profiles in the paraventricular nucleus of the hypothalamus. J Neurosci 2003; 23: 5607-5616.
9. Ferreira J, Santos AR, Calixto JB. The role of systemic, spinal and supraspinal L-arginine-nitric oxide-cyclic GMP pathway in thermal hyperalgesia caused by intrathecal injection of glutamate in mice. Neuropharmacology 1999; 38: 835-842.
10. Tao YX, Hassan A, Haddad E, Johns RA. Expression and action of cyclic GMP-dependent protein kinase 1alpha in inflammatory hyperalgesia in rat spinal cord. Neurosci 2000; 95: 525-533.
11. Garthwaite J, Charles SL, Chess-Williams R. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. Nature 1988; 336: 385-388.
12. Bredt DS, Snyder SH. Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. Proc Natl Acad Sci U S A 1989; 86: 9030-9033.
13. Cristino L, Pica A, Della Corte F, Bentivoglio M. Co-induction of nitric oxide synthase, BCL-2 and growth-associated protein-43 in spinal motoneurons during axon regeneration in the lizard tail. Neuroscience 2000; 101: 451-458.
14. Guhring H, Gorig M, Ates M, Coste O, Zeilhofer HU, Pahl A, et al. Suppressed injury-induced rise in spinal prostaglandin E2 production and reduced early thermal hyperalgesia in iNOS-deficient mice. J Neurosci 2000; 20: 6714-6720.
15. MaihÖfner C, Euchenhofer C, Tegeder I, Beck KF, Pfeilschifter J, Geisslinger G. Regulation and immuno-histochemical localization of nitric oxide synthases and soluble guanylyl cyclase in mouse spinal cord following nociceptive stimulation. Neurosci Lett 2000; 290: 71-75.
16. Zimmermann M. Pathobiology of neuropathic pain. Eur J Pharmacol 2001; 429: 23-37.
17. Tran CT, Leiper JM, Vallance P. The DDAH/ADMA/NOS pathway. Atheroscler Suppl 2003; 4: 33-40.
18. Saraste M. Oxidative Phosphorylation at the fin de siècle. Science 1999; 283: 1488-1493.
19. Taanman JW. Human cytochrome coxidase: structure, function, and deficiency. J Bioenerg Biomember 1997; 29: 151-163.
20. Kadenbach B, Huttemann M, Arnold S, Lee I, Bender E. Mitochondrial energy metabolism is regulated via nuclear-coded subunits of cytochrome c oxidase. Free Radical Bio Med 2000; 29: 211-221.
21. Trautwein W, Hescheler J. Regulation of cardiac L-type calcium current by phosphorylation and G proteins. Ann Res Physiol 1990; 52: 257-274.
22. Kholodenko BN. Four-dimensional organization of protein kinase signaling cascades: the roles of diffusion, endocytosis and molecular motors. J Exp Biol 2003; 206: 2073-2082.
23. Heinrich R, Neel BG, Rapoport TA. Mathematical models of protein kinase signal transduction. Mol Cell 2002; 9: 957-970.
24. Lee Y, Lee CH, Oh U. Painful channels in sensory neurons. Mol Cells 2005; 20: 315-324.

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