Assessment of Pain and Spinal Cord levels of Calcitonin Gene Related Peptide and Interleukin-6 in a Model of Neuropathic Pain in Female Rats

Gihan Sharara[1], Hala M. Abou Heif [2] and Abeer El-Emam Deif [2]

Departments of [1]Biochemistry and [2]Physiology Faculty of Medicine, Alexandria University
gihansharara@hotmail.com

Abstract: Background: Neuropathic pain is a form of chronic pain that arises from functional changes in the pain sensory system after peripheral nerve injury. It is manifested by spontaneous pain, hyperalgesia and allodynia. Calcitonin gene related peptide (CGRP) is 37 amino acid neuropeptide that is widely distributed in the central and peripheral nervous system and is involved in processing of nociceptive information. Evidences from human and animal studies whether estrogen is pro or antinociceptive is inconsistent. Transcutaneous electric nerve stimulation (TENS) is a non pharmacologic therapy for pain used to produce analgesia in inflammatory conditions. **Aim** of the present work was assessment of pain and spinal cord levels of CGRP and interleukin-6 (IL-6) in a model of neuropathic pain induced in female rats by chronic constriction injury (CCI) of sciatic nerve. The possible modulation of neuropathic pain by estradiol and TENS was also assessed in ovariectomized rats with CCI of sciatic nerve. **Material & Methods**: The study was carried out on 48 adult female rats divided into six groups (8 rats/each). Group 1: sham operated rats, group II: CCI of sciatic nerve in gonadally intact female rats, group III: ovariectomized (OVX) rats, group IV: OVX+CCI, group V: OVX+CCI/17 β estradiol, group VI: OVX+ CCI/TENS. All ovariectomized rats were included in the study 3 weeks after ovariectomy for depletion of circulating estrogen. Exogenous estrogen was given to ovarietomized rats of group V as 6μg/kg body weight (b.wt.) per day subcutaneous for 3 weeks starting on the same day of CCI-surgery (day 0). TENS was delivered to OVX+CCI rats (group VI) starting from day 0 and continued for 3 weeks on alternate days under light ether anaesthesia. Pain behavior was assessed by pin prick test. Mechanical hyperalgesia was defined as increased hindpaw withdrawal frequencies percent (PWF%) to mechanical stimuli. The assessment began on the day of CCI surgery (day 0) before the operation (pre-operative, baseline data) and followed up weekly on post-CCI days 8, 14 and 21 (post operative assessment). Ovariectomized rats that were not subjected to CCI (group III) followed the same schedule for assessment of PWF%. At the end of the experiment, rats were sacrificed, lumbar spinal cord was dissected and used for later assessment of mRNA expression of calcitonin gene-related peptide (CGRP) using an Reverse transcriptase –Polymerase chain reaction (RT-PCR) method. Also levels of CGRP and IL-6 in the spinal cord were assessed by Enzyme Immunoassay Kits. **Results**: The expression of CGRP in the spinal cord tissue, levels of CGRP and IL-6 were significantly higher in lumbar spinal cord after CCI, OVX or OVX+CCI compared with sham operated rats. After estrogen or TENS therapy, a significant decrease of expression of CGRP, levels of CGRP and IL-6 were found compared with untreated (OVX+CCI) group. However, the values of CGRP and IL-6 after treatment were still above control level. Rats in group II (CCT of sciatic nerve in gonadally intact female rats) had significant increase of PWF% on post-CCI days 8, 14 and 21. Group III: OVX rats had significant increase of PWF% on day 0 and post-CCI days 8,14 and 21 versus sham operated rats. Group IV (OVX+CCI) showed significant increase of PWF% on days 8, 14 and 21 post-CCI compared to baseline. These values were significantly higher than those of sham operated rats. PWF% in group IV (OVX+CCI) tended to be higher at all the time points of post-CCI follow up than corresponding values in group II (CCI in gonadally intact females) and the difference was statistically significant on day 14. Group V (OVX+CCI/17 β estradiol) showed significant decrease of PWF% starting from day 8 and continued on days 14 and 21 post-CCI versus baseline, but remained significantly higher than sham operated rats. Group VI: OVX+CCI/TENS showed significant decrease of PWF% on post-CCI days 8, 14 and 21 compared with baseline, but remained significantly higher than sham operated controls. No statistically significant differences in PWF% were found between estradiol and TENS treated groups on post CCI days 8, 14 and 21. **Conclusion**: increased spinal cord levels of CGRP and IL-6 could be involved in the mechanism of mechanical hyperalgesia after CCI surgery of sciatic nerve and ovariectomy. Administration of estradiol or TENS significantly attenuated the development of hyperalgesia in (OVX+CCI) group, possibly by decreasing spinal cord levels of CGRP and IL-6. The findings suggested that TENS can be considered as alternative to hormonal therapy for neuropathic pain in ovariectomized rats.

[Gihan Sharara, Hala M. Abou Heif and Abeer El-Emam Deif. **Assessment of Pain and Spinal Cord levels of Calcitonin Gene Related Peptide and Interleukin-6 in a Model of Neuropathic Pain in Female Rats.** J Am Sci 2012;8(6):145-157]. (ISSN: 1545-1003). http://www.sciencepub.net/american. 18

Key words: Neuropathic pain, CGRP expression, IL-6, estrogen, TENS.

Abbreviations: calcitonin gene related peptide (CGRP), interleukin-6 (IL-6), 17 β estradiol (E_2), transcutaneous electric nerve stimulation (TENS), CCI: chronic constriction injury, ovariectomy (OVX), paw withdrawal frequency percent (PWF%), Reverse transcriptase-Polymerase chain reaction (RT-PCR)

1. Introduction

Neuropathic pain is a form of chronic pain that arises from functional changes in the pain sensory system after peripheral nerve injury.[1] It is manifested by spontaneous pain, hyperalgesia (exaggerated responses to noxious stimuli) and allodynia (painful stimulation to innocuous stimuli).[1] Evidence suggests that nerve injury or disease-elicited inflammatory and immune responses in injured nerves contribute to the initiation and maintenance of neuropathic pain.[2] The production of proinflammatory cytokines, neurotrophic factors and other pain mediators by invading macrophages and other inflammatory cells possibly plays important roles in peripheral mechanisms of neuropathic pain.[3] Additionally, pain related neuropeptides including substance P (SP) and calcitonin gene related peptide (CGRP), normally contained in intact axons are released by degenerating ones. Both peptides play important roles in inflammation and immunity.[4] Thus, injured nerves are an ideal milieu for neuroimmune interactions, that contribute to the initiation and maintenance of neuropathic pain.[4] The pathophysiology of peripheral nerve injury-induced neuropathic pain disorders can be investigated using the chronic constriction injury (CCI) model of **Bennett and Xie**.[5]

CGRP is a 37 amino acid neuropeptide that is distributed widely throughout the central and peripheral nervous system, including the peripheral and central circuits of primary sensory neurons, as well as motor neurons in the spinal cord.[6] CGRP has been implicated in the processing of nociceptive information in the spinal cord, where it is found in the terminals of unmyelinated (C) and thinly myelinated (Aδ) afferent fibers.[7] CGRP can enhance the release of glutamate and aspartate in the spinal cord, CGRP may produce its effect on pain transmission and modulation through interactions with SP and excitatory amino acids. Alternatively, CGRP may be involved in pain transmission independently of SP or excitatory amino acids. It has been reported that CGRP itself produces a depolarization.[8]

The neuropathic pain is managed by pharmacological and non pharmacological intervention, however, this pain syndrome can not be controlled with conventional analgesic.[1]

Estrogen is known to influence multiple functions in brain tissue, including neuronal development, plasticity and survival, neurotransmitter and neuropeptide synthesis and neurotransmitter receptors.[9] There are recent studies on the effects of estradiol on the spinal cord and on the peripheral nervous system.[10] Animal experiments[11] as well as observations in humans[12] have shown that somatosensory perception and pain sensitivity are influenced by estrogen, but little is known about the underlying mechanisms. In the rat and mouse, both estrogen receptors α (ERα) and ERβ have been shown to be expressed in dorsal horn of adult spinal cord, in laminae I and II, an area involved in receiving and processing of nociceptive information.[13] However, the evidence from both human and animal studies whether estrogen is pro-or anti-nociceptive is inconsistent.[14]

Transcutaneous electrical nerve stimulation (TENS) is a commonly utilized non pharmacological treatment for pain [15]. TENS was initially reported for pain relief by **Wall & Sweet** in 1967[16] and was based on the mechanisms described in the gate control theory for pain. Data show that TENS activates opioid receptors spinally and supraspinally to reduce hyperalgesia associated with inflammation.[17] In the spinal cord, a complicated neurochemistry involves activation of acetylcholinergic and serotonergic receptors by TENS to reduce hyperalgesia.[18]

Therefore the aim of the present work was assessment of pain behavior and spinal cord levels of CGRP and interleukin-6 (IL-6) in animal model of neuropathic pain in female rats and also to assess the possible effects of estrogen and TENS therapy on neuropathic pain in ovariectomized rats with CCI.

2. Material and Methods

The study was carried out on 48 adult female albino rats weighing between 200-250 grams. They were housed in cages under standard laboratory conditions. Food and water were provided *ad libitum*. All protocols followed in this study were approved by the Ethics Committee of Experimental Animals, Faculty of Medicine, Alexandria University. Six groups were studied (8 rats/ group). They were:

- **Group (I):** Sham operated.
- **Group (II):** CCI of sciatic nerve in gonadally intact cycling female rats.
- **Group (III):** Ovariectomized (OVX) group.
  Ovariectomized females were included in the study 3 weeks after ovariectomy operation. This time lag after ovariectomy is required to deplete circulating estrogen.[19]
- **Group (IV):** OVX+CCI group
  Ovariectomized females were operated upon for CCI of sciatic nerve after 3 weeks from ovariectomy operation.
- **Group (V):** OVX + CCI/17- β estradiol (E_2).
  This group received 17-β estradiol replacement therapy (Sigma, st. Louis Mo) in a dose of 6μg/kg b.wt./day[20] subcutaneously dissolved in corn oil starting on the same day of CCI surgery till the end of the experiment (3 weeks). The sham operated rats received the corn oil vehicle.
- **Group (VI):** OVX+CCI/TENS
  TENS therapy was started on the same day of CCI surgery while the rats were still under anaesthesia.

I-Surgical procedures

1-Chronic constriction injury (CCI) surgery of sciatic nerve

Rats were deeply anaesthetized with sodium pentobarbital (50 mg/kg b.wt.) via intraperitoneal injection. The surgery procedure was first described by **Bennett & Xie**.[5] The left sciatic nerve was exposed high on the thigh, near the trochanter and proximal to its trifurcation, approximately 7mm of the common sciatic nerve was freed from adhering tissue. Four chromic gut sutures (4/0) were loosely tied around the nerve at intervals of approximately 1mm, and ligatures were tied loosely enough, so that on visual inspection, blood flow was not obstructed. The surgical incision was sutured in layers. Prophylactic antibiotics were used to prevent infection.

2-Ovariectomy

Ovariectomies were performed under sodium pentobarbital anaesthesia via bilateral incision in the dorsal flank. The fallopian tubes were ligated with 3/0 silk sutures below the ovaries which were then removed.[21]

Sham operated rats were subjected to anaesthesia, the ovaries were exposed but not removed. Also, the sciatic nerve was dissected exposed but not ligated.

II-Assessment of pain behavior

Pin prick test

Nerve injured rats were routinely tested for the presence of pain-like behaviours prior to, and from 1 week after surgery according to the previously described methods.[22] During testing, the rats were placed in specialized cages with a metal mesh floor allowing access to the planter surface of the injured hindpaw. The animals were allowed to habituate for 15 minutes prior to initiation of behavioral testing. The pin prick test was done by pressing the planter surface of the hindpaw with the point of safety pin, at an intensity sufficient to produce a reflex withdrawal response in normal unoperated animal, but without skin penetration.[23]

The schedule of assessment was: baseline (preoperative), taken before CCI-sciatic surgery on the same day of the operation (day 0), followed by weekly assessment of PWF% on days 8, 14 and 21 post-CCI surgery. Ovariectomized rats without CCI surgery were included in the study 3 weeks after ovariectomy and examined for PWF% using the same schedule used for other rats with CCI surgery.

Mechanical stimuli were given ten times applied to the injured hindpaw through the wire mesh floor. The stimuli were administered at intervals of three to four seconds. The occurrence of paw withdrawal in each of these ten trials was expressed as percent withdrawal frequency [(number of paw withdrawals/number of trials) X 100]. Avoidance responses such as lifting, shaking or licking the paw, and running away were regarded as positive responses.[24,25] The procedure was repeated, 3 times during each examination and the average was recorded. Mechanical hyperalgesia was defined as exaggerated or increased paw withdrawal frequencies (PWF%) of the injured hindpaw relative to the control.[23]

Rats that exhibited motor deficiency after CCI surgery (Such as paw dragging or limping) or those which failed to develop mechanical hyperalgesia were excluded from further testing.

III-Transcutaneous electric nerve stimulation (TENS)

TENS was applied to rats after CCI surgery of sciatic nerve through self adhesive surface electrodes using the every way E 704 SD timer TENS (the manufacturer is Everyway, Medical Instruments Co., Ltd Taipei, Taiwan). The device delivers symmetrical biphasic square pulses. It was adjusted on burst mode. Bursts occur twice every second. Pulse width was 60 μs and amplitude 30 mA. The frequency was fixed at 100 HZ which is intermediate between low and high frequencies. The timer was adjusted to 15 minutes per setting. TENS was delivered under light ether anaethesia starting from the day of CCI surgery and continued on alternate days (3 times/week) for the total duration of the experiment (3 weeks). Electrodes were placed ipsilateral on the same side of sciatic injury. One electrode was placed on the paraspinal musculature of lumbar area on the skin innervated by dorsal rami of spinal nerves L1 through L6. The L1 through L6 segments of the spinal cord include segments that innervate the affected rat hindpaw. The second electrode was on the hindpaw.[25]

At the end of the experiment, rats were sacrificed and lumbar spinal cord was dissected and prepared for further analysis of CGRP and IL-6 levels.

IV-Biochemical tests

The spinal cord tissue was divided into 3 portions: one for detection of CGRP expression mRNA, the second part was for determination of CGRP level and lastly for determination of IL-6 levels.

1- Detection of CGRP expression mRNA by reverse transcriptase polymerase chain reaction (RT-PCR)[26]

About 30 mg of spinal cord tissues were stored at –80°C in lysis buffer containing guanidium thiocyanate and β-mercaptoethanol for RNA extraction.

RNA extraction

Total RNA was extracted and purified from homogenized spinal cord tissue using the ‘‘RNeasy Fibrious tissue mini kit’’ (Qiagen, Hilden, Germany) including the DNA digestion step. The concentration of

extracted RNA was measured spectrophotometrically at 260 nm.

RT-PCR

For amplification of the targets, reverse transcription and PCR were run in two separate steps. Briefly, equal amounts of total RNA (6 µg) were heat denatured and reverse transcribed by incubation at 42°C for 90 min with 12.5 U avian myeloblastosis virus reverse transcriptase (AMV) (Promega Corp., Madison, WI,

USA), 20 U ribonuclease inhibitor RNAsin (Promega Corp.), 200 nM deoxy nucleoside 5'-triphosphate mixture, and 1 nM oligo-dT primer in a final volume of 30 µL of 1× avian myeloblastosis virus reverse transcriptase buffer. The reactions were terminated by heating at 97°C for 5 min and cooling on ice. The cDNA samples were amplified in 50 µL of 1× PCR buffer in the presence of 2.5 U Taq DNA polymerase (Promega Corp.), 200 nM deoxy-nucleoside 5'-triphosphate mixture, and the appropriate primer pairs (1 nM of each primer. These sets of primers of CGRP were designed from GenBank (accession No. G35510 and 691379, respectively):

Forward primer 5'-TCCAAACTGGTCACACCTCACT-3',

Reverse primer 5'-CAGCTTTGGTGACAGCATCTCT-3'.

PCR consisted of a first denaturing cycle at 97°C for 5 min, followed by a variable number of cycles of amplification defined by denaturation at 96°C for 1.5 min, annealing for 1.5 min, and extension at 72°C for 3 min. A final extension cycle of 72°C for 15 min was included. Annealing temperature was adjusted to 55°C [(26)].

Agarose gel electrophoresis

All PCR products were electrophoresed on 2% agarose stained with ethidium bromide and visualized with a UV transilluminator.

Semi-quantitative determination of PCR products

Semi-quantitation was performed using a gel documentation system (BioDO, Analyser) supplied by Biometra. According to the following amplification procedure, relative expression of each gene was calculated following the formula [(26)]

R = Densitometrical units of each gene/Densitometrical units of β-actin

β-actin primers were designed from GenBank (accession No. J00691).

2-Determination of CGRP level in lumbar spinal cord:

The rest of the tissue was weighed and was then homogenized with a glass-glass homogenizer in 10 mM phosphate buffer saline (PBS) (pH 7). The homogenates were centrifuged for 10 minutes at 10,000 g and the supernatants were mixed with bovine serum albumin (BSA) 0.1% immediately.[(27)] The supernatant was used to assess the level of CGRP by Enzyme Immunoassay kit[(28)] (Phoenix Pharmaceuticals, INC.)

3-Determination of interleukin-6 (IL-6)

The spinal cord tissue was well-rinsed in PBS and then homogenized in a 200-µl lysis/extraction reagent (Sigma-Aldrich). The samples were centrifuged at 14,000 rpm for 10 min at 4°C, and then the supernatants were assayed for IL-6, using rat IL-6 ELISA provided by Bender Med System, INC (BMS 625 rat IL-6). The kit is an enzyme linked immunosorbent assay for quantitative detection of rat IL-6 in tissues[(29)]. The total protein concentration in all samples was measured using the Lowry method [(30)] . Levels of spinal cord tissue IL-6 were expressed in pg/mg protein.

Statistical Methods

After data entry and careful meticulous revision the file was transferred to SPSS format (Statistical Padcage for Social Sciences) version 10 and the following tests were performed: descriptive analysis (mean±SD), analysis of the variance (ANOVA) and least significant difference for post-hoc comparison among different studied groups at the same time. The Mann-Whitney U test was used to compare between pre-operative (baseline) and post-operative data in each of the studied groups. The F test was used to compare the biochemical parameters among the studied groups. A level of $P<0.05$ was defined as statistically significant.

3. Results

I. Pain behavior test

Paw withdrawal frequency percent (PWF%) (Tables I & II and Figures 1&2).

Group II (CCI surgery of sciatic nerve in gonadally intact females) had significant increase of PWF% on post-CCI days 8, 14 and 21 compared with baseline as well as compared with sham operated rats. Group III (OVX) rats had significantly higher values of PWF% compared with sham operated rats on day 0 and on post-CCI days. Values of PWF% became significantly increased in group III (OVX) rats compared with baseline on post-CCI day 14. Group IV (OVX + CCI) showed significant increase of PWF% values on days 8, 14 and 21 compared with (day 0) baseline data. All the values of PWF% assessed in OVX + CCI group were significantly higher than those of sham operated rats. Values of PWF% tended to be higher on post CCI days 8, 14 and 21 than corresponding values of PWF% in group II and the difference was statistically significant on day 14. Group V: OVX + CCI/17 β estradiol showed significant decrease of PWF% starting from day 8 post-CCI versus baseline data and continued on days 14 and 21 to be significantly decreased compared with

baseline. Values of PWF% in group V: OVX+CCI/17 β estradiol became significantly lower than those of untreated group IV (OVX + CCI) on days 14 and 21 post-CCI, but remained significantly higher than sham operated controls. In group VI: TENS therapy resulted in significant decrease of PWF% on post-CCI days 8, 14 and 21 versus baseline. Values of PWF% were significantly lower in group VI than values in untreated group IV (OVX + CCI) on post-CCI days 14 and 21, but remained significantly higher than controls. No statistically significant differences in PWF% were found between TENS and estradiol treated groups on post-CCI days 8, 14 and 21.

II. Result of biochemical tests

CGRP gene expression in lumbar spinal cord (Figure 3, Table III)

Gene expressions of CGRP were evaluated using RT-PCR and agarose gel electrophoresis (Figure 3 a, b, c,d). As shown in Table III, the expression of CGRP increased significantly in the group II CCI , group III OVX and group IV OVX + CCI than the sham operated control group. After estradiol therapy (group V) and TENS (group VI), CGRP expression was shown to be significantly reduced in the spinal cord compared to the groups II and III, however they still showed higher expression than the controls, Table III (F= 16.52, *P*=0.001*) .

CGRP and IL-6 Levels in lumbar spinal cord (Table IV)

Mean values of CGRP were significantly higher in lumbar spinal cord in group II (CCI) and in group III (OVX) rats as compared with sham operated rats. In group VI (OVX+CCI), the mean value of CGRP was significantly higher compared with sham operated rats and as compared with either (CCI) or (OVX) groups alone. After estradiol or TENS therapy, values of CGRP were significantly lower in groups V, V1 respectively as compared to untreated group IV. However, the values of CGRP after treatment in groups V and V1 were still significantly higher than sham operated control rats group I, Table IV (F = 195.8, *P* = 0.0001*).

Mean values of IL-6 were significantly higher in lumbar spinal cord in groups II (CCI) and III (OVX) rats as compared to group I (sham operated). In group VI (OVX + CCI), the mean value of IL-6 was significantly higher compared with sham operated rats and as compared with either (CCI) group II or (OVX) group III alone. After estradiol or TENS therapy values of IL-6 were significantly lower in groups V and V1 respectively as compared to untreated group IV. However the value of IL-6 after treatment were still significantly higher in groups V and VI compared with sham operated control rats, Table IV (F = 85.62, *P* = 0.0001*).

Table I: Comparison of paw withdrawal frequency percent (PWF%) between baseline pre-operative and post-operative values on days 8, 14 and 21 in each of the studied groups.

Studied groups	Pre-operative	Post-operative (post-CCI sciatic surgery)		
		Day 8	Day 14	Day 21
Group 1(sham operated) $\overline{X}$ ±SD U *p*	25.0±5.3	26.3±5.2 0.50 0.321	25.0±5.3 0.01 0.500	23.8±5.2 0.46 0.321
Group II (CCI) $\overline{X}$ ±SD U *p*	25.0±5.3	57.5±4.6 13.1 0.000	68.8±3.5 19.51 0.000	72.5±4.6 19.14 0.000
Group III (OVX) $\overline{X}$ ±SD U *p*	61.3±6.4	63.8±5.2 0.80 0.203	67.5±7.1 1.83 0.043	71.3±6.4 3.13 0.004
Group IV (OVX + CCI) $\overline{X}$ ±SD U *p*	65.0±5.3	76.3±5.2 4.3 0.000	81.3±6.4 5.55 0.000	90.0±5.3 9.43 0.000
Group V (OVX + CCI/E_2) $\overline{X}$ ±SD U *p*	63.8±5.2	53.8±5.2 3.85 0.001	51.3±6.4 4.29 0.000	42.5±4.6 8.68 0.000
Group VI (OVX +CCI/TENS) $\overline{X}$ ±SD U *p*	70±5.3	56.3±5.2 5.22 0.000	47.5±4.6 9.07 0.000	40.0±7.6 9.16 0.000

p <0.05 was defined as statistically significant

Table II: Comparison of paw withdrawal frequency percent (PWF%) in the six studied groups in the same time interval either pre-operative or post-operative on days 8, 14 and 21.

Studied groups	Pre-operative	Post-operative (post-CCI sciatic surgery)		
		Day 8	Day 14	Day 21
Group 1(sham operated) $\overline{X}$ ±SD	25.0±5.3[a]	26.3±5.2[a]	25.0±5.3[a]	23.8±5.2[a]
Group II (CCI) $\overline{X}$ ±SD	25.0±5.3[a]	57.5±4.6[b]	68.8±3.5[b]	72.5±4.6[b]
Group III (OVX) $\overline{X}$ ±SD	61.3±6.4[b]	63.8±5.2[b]	67.5±7.1[b]	71.3±6.4[b]
Group IV (OVX + CCI) $\overline{X}$ ±SD	65.0±5.3[b]	76.3±5.2[b]	81.3±6.4[c]	90.0±5.3[b]
Group V (OVX + CCI/E_2) $\overline{X}$ ±SD	63.8±5.2[b]	53.8±5.2[b]	51.3±6.4[b]	42.5±4.6[c]
Group VI (OVX +CCI/TENS) $\overline{X}$ ±SD	70±5.3[b]	56.3±5.2[b]	47.5±4.6[b]	40.0±7.6[c]
F *p*	215.3 0.00001*	209.88 0.00001*	154.8 0.0001*	165.25 0.0001*

Same letters has no significant difference.

Table III: Calcitonin gene related peptide (CGRP) gene expression in the spinal cord among the studied experimental groups

Parameter CGRP EXPRESSION	Group I Sham operated	Group II CCI	Group III OVX	Group IV OVX + CCI	Group V OVX + CCI/E_2	Group VI OVX + CCI/TENS
Number	8	8	8	8	8	8
Mean	1.46[a]	6.78[b]	6.16[b]	6.78[b]	2.38[c]	3.91[c]
SD	0.19	0.26	0.15	0.26	0.16	0.21
F *p*	16.52 0.001*					

The same small letters indicate that there was no significant difference, while the different letters indicate that there was a significant difference.

Table IV: Comparison of spinal cord levels of calcitonin gene related peptide (CGRP) and interlekin-6 (IL-6) among the studied groups.

Parameter studied	Group I Sham operated	Group II CCI	Group III OVX	Group IV OVX + CCI	Group V OVX + CCI/E_2	Group VI OVX + CCI/TENS	F (p)
CGRP (ng/gm) Mean±SD	29.1±2.9[a]	78.6±3.6[b]	53.9±4.7[c]	109.6±10.0[d]	59.6±4.0[c]	49.3±5.0[c]	195.8 0.00001*
IL-6 (pg/mg protein) Mean±SD	54.7±2.6[a]	140.1±6.3[b]	106.4±10.8[c]	197.7±7.5[d]	93.1±6.4[c]	90.7±7.1[c]	85.62 0.0001*

Same letters has no significant difference.

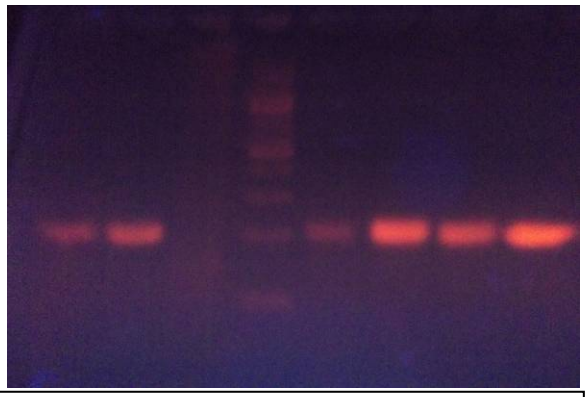

Figure 3a. Agarose gel electrophoresis profile showing PCR products of CGRP gene (210 bp).
Lane 1,2,4,5,6: Group III OVX**; Lane 3**: Group I control; **M**: DNA size marker 50 – 1000 bp

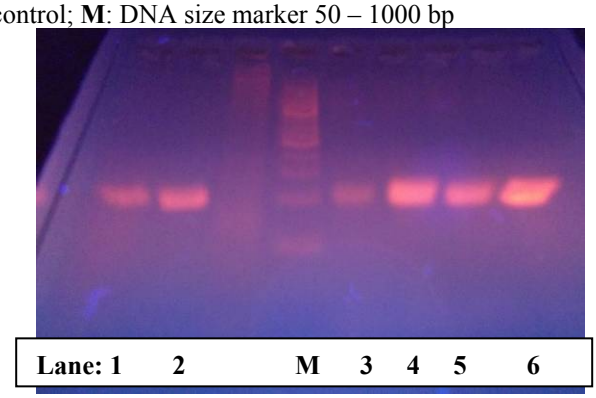

Figure 3b. Agarose gel electrophoresis profiles showing PCR products of CGRP gene (210 bp). Lane 1,2,4,5,6: Group IV OVX + CCI**;** Lane 3: Group I control; M: DNA size marker 50 – 1000 bp

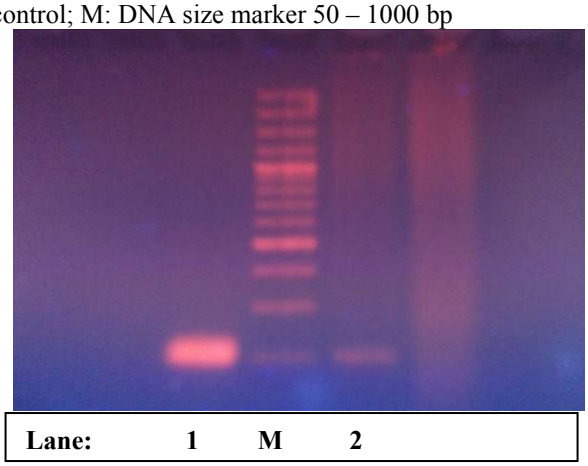

Figure 3c. Agarose gel electrophoresis profiles showing PCR products of CGRP gene (210 bp). **Lane 1:**Group IV OVX + CCI; **Lane 2:** Group VI: OVX + CCI/TENS; M: DNA size marker 50 – 1000 bp

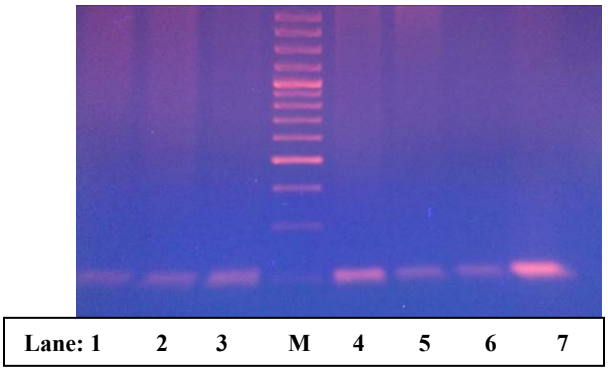

Figure 3d. Agarose gel electrophoresis profiles showing PCR products of CGRP gene (210 bp). **Lane 1-7** : Group V OVX + CCI/E2; M: DNA size marker 50 – 1000 bp

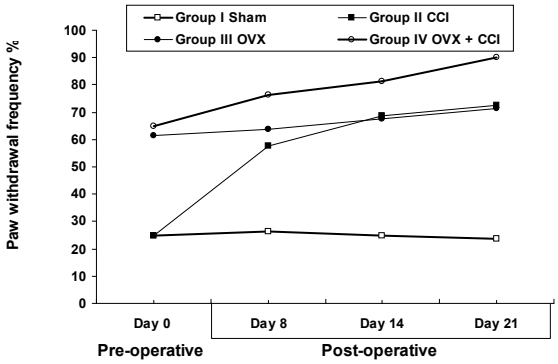

Fig. (1): Mean values of paw withdrawal frequency percent (PWF%) on pre-operative (day 0) and post-operative days 8,14 and 21 for group I: sham, group II: chronic constriction injury of sciatic nerve (CCI surgery), group III: ovariectomy OVX) and group IV: OVX+CCI.

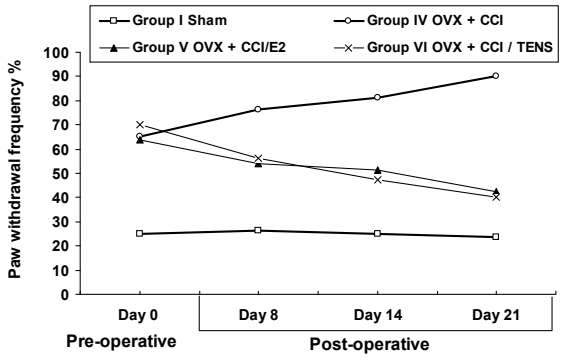

Fig. (2): Mean values of paw withdrawal frequency percent (PWF%) on pre-operative (day 0) and post-operative days 8,14 and 21 for group I: sham, group IV: ovariectomy and chronic constriction injury of sciatic nerve (OVX+CCI), group V: OVX+CCI/17 β estradiol (E_2), group VI: OVX+CCI/TENS.

4. Discussion

Neuropathic pain is defined as pain initiated or caused by a primary lesion or dysfunction in the nervous system.[1] A thorough understanding of the mechanisms and modulatory factors underlying neuropathic pain associated with peripheral nerve injury is imperative for the development of effective treatment strategies.[1] The effects of gender on nociceptive processing has received attention recently.[14]

The present work was conducted to determine whether CGRP and IL-6 levels in lumbar spinal cord are involved in CCI-induced neuropathic pain of sciatic nerve in ovariectomized female rats compared to gonadally intact female rats and the possible modulation of this pain behavior by exogenous estrogen or TENS therapy.

In the present work CCI of sciatic nerve produced a significant increase of PWF% as a sign of mechanical hyperalgesia on post CCI follow up days 8,14 and 21 in ovariectomized rats (OVX+CCI) group IV and in gonadally intact rats group II compared with sham operated rats.

CCI of rat sciatic nerve is a widely used model in inducing chronic neuropathic pain similar to the clinical features of human causalgia and reflex sympathetic dystrophy.[1,31] CCI surgery was shown to induce significant mechanical and thermal hyperalgesia.[1,31] The behavioral signs of pain observed in rats with CCI of sciatic nerve in the present work were similar to those reported in other studies.[1,31]

It was hypothesized that mechanical hyperalgesia develop as a result of two events: the first is associated with an injury discharge that is evoked early in the injured afferents.[32,33] The second is associated with spontaneous ectopic discharge that is developed late in the nearby uninjured nociceptive afferents as a result of degeneration of injured afferents that may take several days to develop.[31,32] This ectopic discharge result in sensitization of central neurons in the spinal cord as well as peripheral sensitization of uninjured receptor terminals, leading to later part or delayed phase of hyperalgesia.[32,33]

The synaptic and cellular mechanisms of pain-related central sensitization in the spinal cord are not fully understood yet [34]. CGRP has been identified as an important molecule in spinal nociceptive processing and the ensuring behavioral responses.[35] It plays a pro-nociceptive role after peripheral nerve injury upon its release from primary afferent neurons in preclinical models of neuropathic pain[36]. CGRP sensitizes the responses of dorsal horn neurons to innocuous and noxious peripheral stimulation[37] and to intraspinally administered excitatory amino acids.[38]

Current results showed that the expression of CGRP increased significantly in the group II CCI, group III OVX and group IV OVX + CCI than the control group. **Bird *et al.*** [34] found that CGRP increased neuronal excitability and facilitated synaptic transmission at the level of substania gelatinosa (SG) neurons of dorsal horn of spinal cord of arthritic rat pain model compared to control rats. The facilitation of synaptic transmission involved endogenously activated CGRP receptors expressed in dorsal horn of spinal cord including SG neurons [34]. The enhanced CGRP receptor function may involve a change in the coupling to downstream effector systems such as kinases and ion channels as well as increased receptor expression or affinity [39]. **Jang *et al.*** [40] found that intraplanter injection of CGRP1 receptor antagonist (CGRP8-37) in the affected hindpaw immediately before L5 spinal nerve ligation (L5 SNL), delayed the onset of lesion induced mechanical hyperalgesia. Moreover, the administration of CGRP receptor antagonist after L5 SNL significantly attenuated mechanical hyperalgesia. These findings suggest a role for CGRP in the induction ad maintenance phases of pain induced by L5 SNL. Similarly, intrathecal administration of CGRP receptor antagonist alleviated mechanical and thermal allodynia induced in rats by spinal cord hemisection suggesting that CGRP may play a role in chronic central neuropathic pain.[41] Another study showed that spinal nerve ligation (SNL) surgery induced an increase in expression of CGRP and neuropeptide Y (NPY) of the spinal cord injured neurons. The maladaptive changes in CGRP and NPY expression in the spinal cord may be mediated by the bioactivity of 5-hydroxytryptamine (5-HT)/5-HT2A receptors in neuropathic pain and the blockade of 5-HT2A receptors could reverse the evoked upregulation of both CGRP and NPY in the spinal cord contributing to the inhibition of neuropathic pain [42]

In a previous study, **Parkitna *et al.*** [43] found significant increase of CGRP in lumbar spinal cord, 3 and 14 days after CCI surgery of sciatic nerve and suggested that increased CGRP levels contributed to induction and maintenance phases of neuropathic pain after sciatic injury. Similarly, in the present study CGRP was increased in lumbar spinal cord 21 days after CCI in gonadally intact female rats versus sham operated rats. These findings are consistent with other studies[44,45] .

Hirose *et al.* [44] reported that after peripheral nerve injury, the expression of CGRP in dorsal root ganglion (DRG) neurons is induced by mechanical allodynia. These results indicate that a subpopulation of DRG neurons express CGRP in response to peripheral nerve injury in the spinal dorsal horn. The increase in CGRP in the deep laminae may affect the excitability of postsynaptic neurons, and may have a role in neuronal plasticity following peripheral nerve injury. Considering the previous reports and current study, the up regulation of CGRP in the spinal cord in

this current model seems to be correlated with pain-related behavior. These finding demonstrated the importance of centrally released neurotransmitters such as CGRP from intact nerves in the pathogenesis of neuropathic pain.[44,45]

Other results [46] also confirm the increase of CGRP expression after peripheral nerve injury, which subsequently decreased in a transgenic mouse model where nuclear factor kappa B (NF-κB) was selectively inhibited. This reduction of CGRP expression correlated with pain behavior and inflammation after peripheral nerve injury. This suggests that CGRP expression in neuropathic pain might be mediated via NF-kB. [46]

IL-6 belongs to the hematopoietic cytokine/growth factor family, which plays important roles in mediating immune and inflammatory responses.[47] In the present work, level of IL-6 in lumbar spinal cord was significantly increased after CCI surgery in gonadally intact (group II) and in ovariectomized rats (OVX+CCI) group IV compared with sham operated rats. The present findings suggest that IL-6 could be involved together with CGRP in the pathogenesis of neuropathic pain after CCI of sciatic nerve. Our findings are consistent with those of other investigators that IL-6 was upregulated in lumbar spinal cord and increased incrementally overtime in nerve injured rats and was implicated in the development of neuropathic pain in these rats.[48-50] It was reported that partial sciatic nerve ligation increased IL-6 expression and shifted it from small to medium and large size damaged DRG neurons. Increased Prostaglandin E_2 (PGE_2) in injured DRG neurons is a novel mechanism that might probably facilitate the de novo synthesis of pain-related cytokines such as IL-6 which contributes to the neuropathic pain [49]. It was found that intrathecal administration of IL-6 antibodies attenuated the development of mechanical hyperalgesia induced by L5 spinal nerve transection in rats.[51]

Considerable evidence indicates sex-related differences in pain responses and in the effectiveness of various analgesic agents. Though the mechanisms underlying these effects remain unclear, the influence of gonadal hormones on nociceptive processing represents one plausible pathway thereby such sex differences could emerge.[14]

In the present work, group III OVX rats had significantly higher PWF% which is a sign of mechanical hyperalgesia compared with sham operated rats on post CCI days 8,14 and 21. In group IV (OVX+CCI) values of PWF% tended to be higher at all time points examined than corresponding values in group II (CCI in gonadally intact female rats) but the difference was statistically significant on day 14 post CCI. These findings suggest that lack of estrogen potentiated mechanical hyperalgesia induced by CCI surgery of sciatic nerve. Estrogen treated rats (group V: OVX+ CCI/17 β estradiol) had significantly lower PWF% on days 14 and 21 post CCI compared with untreated rats (group IV: OVX+CCI), thus estrogen therapy significantly attenuated the developing mechanical hyperalgesia after sciatic injury.

Our findings are consistent with **Sanoja & Cervero**[52]. They found that mechanical hyperalgesia developed 3-4 weeks after ovariectomy. When estrogen therapy was started by the 5th week after ovariectomy, significant attenuation of mechanical hyperalgesia occurred by the 6th week, however, thermal hyperalgesia was less responsive. **Shir *et al.*** [53] observed that rats kept on soy diet rich in phytoestrogens were less likely to develop tactile allodynia and mechanical hyperalgesia after spinal nerve ligation, however, the effect was less evident on thermal hyperalgesia. **Tall *et al.*** [54] on the other hand, found that male sex hormone suppressed the development of thermal hyperalgesia after CCI of sciatic nerve in gonadally intact male rats compared with castrated males. However, they did not find differences as regard touch evoked allodynia after CCI between gonadally intact male and female rats and ovariectomized female or castrated male. Their findings showed that male and female rats exhibited discordant thermal hyperalgesic response after CCI but the expression of mechanical allodynia was less variable among genders. This could be explained by the fact that mechanical allodynia and thermal hyperalgesia are mechanistically distinct using 2 different pathways: Aβ fibers for touch evoked allodynia and C-fibers for the thermal, and that nociceptive input is differentially modulated by sex steroids[54]. **Ito *et al.*** [20] found that the effect of estrogen therapy to suppress thermal hyperalgesia was dependent on the timing of its administration. Estrogen therapy given late by the week 15 after ovariectomy was less effective to alleviate thermal hyperalgesia due to downregulation of estrogen receptors in the spinal cord shortly after ovariectomy.

The mechanisms of action by which estrogen modulate neuropathic pain remained largely unanswered, there appears to be a contribution from both central and peripheral components [55]. Peripherally, within the dorsal root ganglia, estrogen activates non genomic, genomic and paracrine regulatory pathways. These functional changes affect the activity of various pain effectors and result additionally in survival and regeneration of spinal neurons [56]. Centrally, estrogen triggers neurochemical changes that modulate pain responses. For instance, estradiol influences opioid neurotransmission through the mu opoid receptor [57]. Estrogen also modulates GABA ergic neurons.[58]

Since CGRP was reported to enhance nocicpetive transmission at the spinal cord level [34], the effect of

estrogen on spinal cord CGRP expression and levels were determined in the current work. We found that both CGRP expression and levels were significantly higher in groups II (CCI) and III (OVX) rats and IV (OVX+CCI) compared to sham operated rats and its expression and levels were decreased by estrogen therapy compared with its level in the spinal cord in untreated rats. Our findings suggest that estrogen modulation of mechanical hyperalgesia could be through modulation of spinal cord level of CGRP.

Previous studies either support or contradict the possible modulation of CGRP by sex steroids [59-61]. CGRP was detected in medial preoptic area (MPOA) of rats and was shown to be 25 folds more abundant in female than male rats in this area. MPOA is implicated in sex specific function and behavior in rats [59]. It was found that neither ovariectomy nor estrogen therapy affected CGRP level in MPOA in rats, however, it could be modulated by male gonadectomy[59,60]. In the peripheral nervous system, CGRP is found mainly in small neurons of DRG of lumbar spinal cord and was found to be significantly lower in female than male rats [61]. The expression of CGRP in DRG small neurons was enhanced by ovariectomy and was shown to be down- regulated by estrogen,[61] which is consistent with our findings. On the other hand, **Noguchi *et al.*** [62] found that ovariectomy had no effect on CGRP level in DRG or lumbar spinal cord. Moreover, 17-β estradiol did not affect the level of CGRP in the spinal cord in ovariectomized rats.

Other mechanism by which estrogen can affect or modulate neuropathic pain could be through modulation of immune functions [63]. Estrogen receptors α and β are found on organs of immune system.[64] Activation of each receptor differentially contributes to granulocyte-induced inflammation, T-lymphocyte proliferation, suppression of NK cell cytotoxicity and B-lymphocyte suppression [64].

In the present work, level of IL-6 was increased in lumbar spinal cord after ovariectomy in group III and after CCI in ovariectomized rats in group IV (OVX+CCI) relative to sham operated rats. It was decreased after estrogen therapy in group V (OVX+CCI/17-β estradiol) relative to untreated group (OVX+CCI), but was still higher than control level (group I). Also PWF% was decreased by estrogen therapy. Our findings suggest that estrogen possibly attenuated mechanical hyperalgesia through decreased IL-6 level in lumbar spinal cord, thus suppressing the inflammatory reactions contributing to neuropathic pain. In consistence with our findings, previous studies [65,66], reported that estrogen suppressed brain inflammatory reaction by blocking macrophage activation and subsequent cytokine release.

Considerable interrelationship between CGRP and IL-6 suggest their importance in the pathogenesis of neuropathic pain [67, 68]. Following sciatic nerve ligation, increased CGRP level in injured nerve trunk was involved in the upregulation of IL-6 in invading macrophages and the abnormal reaction to pain stimuli [67]. Perineural injection of CGRP antagonist (CGRP 8-37), not only attenuated thermal hyperalgesia but also reduced IL-6 in injured nerves, suggesting a strong link between CGRP, IL-6 and thermal hyperalgesia [67]. *In vitro*, in cell culture derived from injured nerves, CGRP induced IL-6 release in a concentration dependent effect.[67] On the other hand, **Opree & Kress**[68] reported that cytokines such as IL-6, IL-1B and tumor necrosis factor α (TNFα) were involved in the development of heat hyperalgesia in isolated skin flap from rat hindpaw through evoked release of CGRP from nociceptive afferents.

TENS is a non invasive treatment used in physiotherapy practice to promote analgesia in acute and chronic inflammatory conditions.[25] The mechanism of analgesia induced by TENS are not well defined, and the literature on the subject remains controversial.[25]

In the present study, TENS therapy resulted in significant decrease of PWF% on post CCI days 14 and 21 associated with significant reduction of expression of CGRP and decrease in levels of CGRP and IL-6 in lumbar spinal cord compared to untreated group (OVX+CCI). Our findings suggest that TENS possibly attenuated mechanical hyperalgesia through decreased levels of IL-6 and CGRP in lumbar spinal cord.

The present findings are consistent with **Somers *et al.***[25] showing that TENS delivered from the same day of CCI of sciatic nerve was effective to attenuate thermal and mechanical hyperalgesia relative to untreated rats with CCI. Other results are also consistent with the present study [69], which suggests that the anti hyperalgesic effect of TENS may be partly mediated via inhibition of inflammation as evidenced by alteration of IL-6 levels. Thus, Repeated TENS may be useful for treating chronic pain clinically. Other studies attributed the anti hyperalgesic effects of TENS therapy to release of endogenous opioids[70] or to reduction of the level of excitatory amino acids aspartate and glutamate in dorsal horn of spinal cord.[71]

Conclusion

Increased spinal cord CGRP and IL-6 could be involved in the mechanism of mechanical hyperalgesia after CCI of sciatic nerve and ovariectomy. Administration of estradiol or TENS significantly attenuated hyperalgesia in ovariectomized rats after CCI surgery, possibly by decreasing spinal cord levels of CGRP and IL-6. The findings suggested that TENS could be considered as an alternative to hormonal therapy for CCI-induced neuropathic pain in ovariectomized rats.

Acknowledgement

We acknowledge Doctor Gihan Abdel Latif, Lecturer of Physical Medicine, Faculty of Medicine, Alexandria University for her assistance in the methodology of transcutaneous electric nerve stimulation.

Corresponding author
Gihan Sharara
Department of Biochemistry Faculty of Medicine, Alexandria University

5. References

1. Akada Y, Mori R, Matsuura K, Suzuki K, Kato K, Kamiya M, Naba H, Kurokawa M, Ogihara T, Kato Y. (2006): Pharmacological profiles of the novel analgesic M58996 in rat models of persistent and neuropathic pain. J Pharmcol Sci; 102: 205-12.
2. Marchand F, Perretti M, McMahon SB. (2005): Role of the immune system in chronic pain. Nat Rev Neurosci; 6: 521-32.
3. Sommer C, Kress M. (2004): Recent findings on how proinflammatory cytokines cause pain: peripheral mechanisms in inflammatory and neuropathic hyperalgesia. Neurosci Lett;361:184-7.
4. Levite M. (2000): Nerve-driven immunity. The direct effects of neurotransmitters on T-cell function. Ann NY Acad Sci; 917: 307-21.
5. Bennett GJ, Xie YK (1988): A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain; 33:87-107.
6. Hiruma H, Saito A, Ichikawa T, Saito A, Ichikawa T, Kiriyama Y, Hoka S, Kusakabe T, Kawakami T. (2000): Effects of substance P and calcitonin gene related peptide on axonal transport in isolated and cultured adult mouse dorsal root ganaglion neurons. Brain Res; 883: 184-91.
7. Sun RQ, Lawand NB, Willis WD. (2003): The role of calcitonin gene related peptide (CGRP) in the generation and maintenance of mechanical allodynia and hyperalgesia in rats after intradermal injection of capsaicin. Pain; 104: 201-8.
8. Sun RQ, Tu YJ, Lawand NB, Yin-Yan J, Lin Q, Willis WD. (2004):Calcitonin gene-related peptide receptor activation produces PKA-and PKC-dependent mechanical hyperalgesia and central sensitization. J Neurophysiol; 92: 2859-66.
9. Suzuki S, Brown CM, Wiss PM. (2006): Mechanisms of neuroprotection by estrogens. Endocrine; 29(2): 209-15.
10. Maria Angeles Arevalo, Maria Santos-Galindo, Natalia Lagunas, Inigo Azcoitia and Luis M Garcia-Segur. (2011): Selective estrogen receptor modulators as brain therapeutic agents. Journal of Molecular Endocrinology; 46: R1–R9.
11. Ji Y, Murphy AZ, Traub RJ. (2003): Estrogen modulates the visceromotor reflex and responses of spinal dorsal horn neurons to colorectal stimulation in the rat. J Neurosci; 23(9): 3908-15.
12. Frot M, Feine JS, Bushnell MC. (2004): Sex differences in pain perception and anxiety. A psychological study with topical capsaicin. Pain; 108: 230-6.
13. Vanderhorst-VG, Gustafsson JA, Ulfhake B. (2005): Estrogen receptor alpha and beta on immunoreactive neurons in the brainstem and spinal cord of male and female mice: relationship to monoaminergic, cholinergic, and spinal projection systems. J Comp Neurol; 488: 152-79.
14. Yezierski R (2012). The effects of age on pain sensitivity: preclinical studies. Pain Med.;2:S27-36.
15. Sluka KA, Vance CGT, Lisi TL. (2005): High frequency, but not low frequnecy, transcutaneous electical nerve stimulation reduces aspartate and glutamate release in the spinal cord dorsal horn. J Neurochem; 95: 1794-801.
16. Wall PD, Sweet WH. (1967): Temporary ablation of pain in man. Science; 155: 108-9.
17. Sluka KA, Deacon M, Stibal A, Strissel S. (1999): Spinal blockade of opioid receptors prevents the analgesia produced by TENS in arthritic rats. J Pharmacol Exp Ther.; 289: 840-6.
18. Radhakrishnan R, Sluka KA. (2003): Spinal muscarinic receptors are activated during low and high frequency TENS-induced antihyperalgesia in rats. Neuropharmacology; 45: 1111-9.
19. Aloisi AM, Ceecarelli I, Herdegen T. (2000): Gonadectomy and persistant pain differently affect hippocampal c-fos expression in male and female rats. Neurosci Lett.; 281: 29-32.
20. Ito A, Takeda M, Furue H, Shibata K, Hori M, Sagai H, Sakurada T. (2004): Administration of estrogen shortly after ovariectomy mimics the anti-nociceptive action and change in 5-HT_1A-like receptor expression induced by calcitonin in ovariectomized rats. Bone; 35: 697-703.
21. Zhao X, Wang X, Zheng S, Xu J. (2004): Gonadectomy affects brain derived neutrophic factor in rats after chronic constriction nerve injury. Acta Pharmacol Sin; 25(3): 286-92.
22. Blackburn-Munro G, Jensen BS. (2003): The anticonvulsant retigabine attenuates pain behaviours in animal models of persistent and neuropathic pain. Eur J Pharmacol; 460: 109-16.
23. Tal M, Bennett GJ. (1994): Extraterritorial pain in rats with a peripheral mononeuropathy: mechano-hyperalgesia and mechano-allodynia in the territory of an uninjured nerve. Pain; 57: 375-82.
24. Attal N, Jazat F, Kayser V, Guilbaud G. (1990): Further evidence for pain related behaviours in a model of unilateral peripheral mononeuropathy. Pain; 41: 235-51.

25. Somers DL, Clemente FR. (2006): Transcutaneous electric nerve stimulation for the management of neuropathic pain: The effects of frequency and electrode position on prevention of allodynia in a rat model of complex regional pain syndrome type II. Phys Ther; 86: 698-709.
26. Chomczynski P, Sacchi N. (1987): Single-step method of RNA isolation by acid guanidinium thiocyanatephenol- chloroform extraction. Anal Biochem.; 162:156-159.
27. Kashihara Y, Sakaguchi M, Kuno M. (1989): Axonal transport and distribution of endogenous calcitonin gene-related peptides in rat peripheral nerve. J Neuroscience; 9(11): 3796-802.
28. Porskmann T, Kiessig ST. (1992): Enzyme immunoassay techniques, An overview. Journal of Immunological Methods; 150: 5-21.
29. Hirano T, Akira S, Taga T, Kishimoto T. (1990): Biological and clinical aspects of interleukin 6. Immunol Today; 11(12): 443-9.
30. Lowry O. H., Rosenbrough N. J., Farr A. L., and Randall R. (1951): Protein determination using Folin-Ciocalteu Reagent. J Biol Chem; 193: 265–278.
31. Naik AK, Tandan SK, Dudhgonkan SP, Jadhav SH, Kataria M, Kumar D. (2006): Role of oxidative stress in pathophysiology of peripheral neuropathy and modulation by N-acetyl-L- cysteine in rats. Eur J pain; 10(7): 573-9.
32. Rowbotham MC. (2001): Efficacy of opioids in neuropathic pain. In: Hansson PT, Fields HL, Hill RG, eds. Neuropathic pain: Pathophysiology and treatment. IASP press,; 203-11.
33. Casals-Díaz L, Vivó M, Navarro X (2009): Nociceptive responses and spinal plastic changes of afferent C-fibers in three neuropathic pain models induced by sciatic nerve injury in the rat. Exp Neurol.; 217(1):84-95.
34. Bird GC, Han JS, Adwanikar H, Willis WD, Neugebauer V. (2006): Pain-related synaptic plasticity in spinal dorsal horn neurons: role of CGRP. Mol pain; 2: 31-43.
35. Furue H, Katafuchi T, Yoshimura M. (2004): Sensory processing and functional reorganization of sensory transmission under pathological conditions in the spinal cord dorsal horn. Neurosci Res; 48: 361-8.
36. Malon JT, Maddula S, Bell H, Cao L. (2012): Involvement of calcitonin gene-related peptide and CCL2 production in CD40-mediated behavioral hypersensitivity in a model of neuropathic pain. Neuron Glia Biol.; 1-12.
37. Sun RQ, Lawand NB, Lin Q, Willis WD. (2004): Role of calcitonin gene related peptide in the sensitization of dorsal horn neurons to mechanical stimulation after intradermal injection of capsaicin. J Neurophysiol; 92: 320-6.
38. Ebersberger P, Banegap H. (2000): Differential effect of CGRP and CGRP8-37 upon responses to N-methyl-aspartate. Neuroscience; 99: 171-8.
39. Veronica A Peotta, Robert M Weiss, Kathy A Zimmerman, Yongjun Lu, Andrew F Russo, Francois M Abboud and Mark W Chapleau. (2010): Consequences of Enhanced CGRP Receptor Signaling on Cardiac Function and Mortality Post-Myocardial Infarction. FASEB J.; 24 : 1037.4
40. Jang JH, Nam TS, Paik KS, Leem JW. (2004): Involvement of peripherally released substance P and calcitonin gene related peptide in mediating mechanical hyperalgesia in a traumatic neuropathy model of the rat. Neurosci lett; 360: 129-32.
41. Long-Chuan Yua, Jun-Feng Houa, Feng-Hua Fub, Ying-Xin Zhanga. (2009): Roles of calcitonin gene-related peptide and its receptors in pain-related behavioral responses in the central nervous system. Neuroscience & Biobehavioral Reviews; 33(8): 1185–1191
42. Dongmei Wanga, Tingjun Chena, Yun Gaoa, Rémi Quirionb, Yanguo Hong. (2012): Inhibition of SNL-induced upregulation of CGRP and NPY in the spinal cord and dorsal root ganglia by the 5-HT2A receptor antagonist ketanserin in rats. Pharmacology Biochemistry and Behavior; 101 (3): 379–386.
43. Parkitna JR, Korostynsk PM, Kaminska-chowaniec D, Ohara I, Mika J, Przewlocka B. (2006): Comparison of gene expression profiles in neuropathic and inflammatory pain. Journal Physiol Pharmacol; 57: 401-14.
44. Hirose K, Iwakura N, Orita S, Yamashita M, Inoue G, Yamauchi K, Eguchi Y, Ochiai N, Kishida S, Nakamura J, Takaso M, Ishikawa T, Arai G, Miyagi M, Kamoda H, Aoki Y, Hiwatari R, Kakizaki J, Kunishi T, Kono M, Suzuki T, Toyone T, Takahashi K, Kuniyoshi K, Ohtori S. (2010): Evaluation of behavior and neuropeptide markers of pain in a simple, sciatic nerve-pinch pain model in rats. Eur Spine J.; 19(10):1746-52.
45. Eduardo E. Benarroch. (2011): CGRP Sensory neuropeptide with multiple neurologic implications. Clinical Implications of Neuroscience Research; 77 (3): 281-287.
46. Zhang YP, Fu ES, Sagen J, Levitt RC, Candiotti KA, Bethea JR, Brambilla R. (2011): Glial NF-κB inhibition alters neuropeptide expression after sciatic nerve injury in mice. Brain Res.;1385:38-46.
47. Tanaka T, Kishimoto T. (2012): Immunotherapeutic implication of IL-6 blockade. Immunotherapy; 4(1):87-105.
48. Liou JT, Liu FC, Mao CC, Lai YS, Day YJ. (2011): Inflammation confers dual effects on nociceptive processing in chronic neuropathic pain model. Anesthesiology Mar;114(3):660-72.

49. St-Jacques B, Ma W. (2011): Role of prostaglandin E2 in the synthesis of the pro-inflammatory cytokine interleukin-6 in primary sensory neurons: an in vivo and in vitro study. J Neurochem.; 118(5):841-54.
50. Ma W, Quirion R. (2005): Up-regulation of interleukin-6 induced by prostaglandin E_2 from invading macrophages following nerve injury: an in vivo and in vitro study. J Neurochem; 93: 664-73.
51. Joachim Scholz, Clifford J Woolf. (2007): The neuropathic pain triad: neurons, immune cells and glia. Nature Neuroscience; 10: 1361 – 1368.
52. Sanoja R, Cervero F. (2005): Estrogen dependent abdominal hyperalgesia induced by ovariectomy in adult mice: a model of functional abdominal pain. Pain; 118(1-2): 243-53.
53. Shir Y, Campbell JN, Raja SN, Seltzer Z. (2002): The correlation between dietary soy phytoestrogens and neuropathic pain behavior in rats after partial denervation. Anesth Analg; 94: 421-6.
54. Tall JM, Stuesse SL, Cruce LR, Crisp T. (2001): Gender and the behavioral manifestations of neuropathic pain. Pharmacol Biochem behav; 68: 99-104.
55. Song RX, Zhang Z, Samten RJ. (2005): Estrogen rapid action via protein complex formation involve estrogen receptor alpha and src. Trends Endocrinol Metab; 16: 347-53.
56. Hucho TB, Dina OA, Kuhn J, Leviene JD. (2006): Estrogen controls PKC epsilon-dependent mechanical hyperalgesia through direct action on nociceptive neurons. Eur J Neurosci; 24: 527-34.
57. Smith YR, Stohler CS, Nichols TE, Buller JA. Koeppe RA. (2006): Pro-nociceptive and antinociceptive effects of estradiol through endogenous opioid neurotransmission in women. J Neurosci; 26: 5777-85.
58. Blurton-Jones M, Tuszynski MH. (2006): Estradiol-induced modulation of estrogen receptor-beta and GABA within the adult neocortex: a potential trans-synaptic mechanism for estrogen modulation of BDNF. J Comp Neurol; 499: 603-12.
59. Herbison AE. (1992): Identification of a sexually dimorphic neural population immunoreactive for CGRP in rat medial preoptic area. Brain Res; 591(2): 289-95.
60. Yuri K, Kawata M. (1993): Time course analysis of changes in CGRP and met-enkephalin immuno-reactivity in female rat preoptic area after estrogen treatment. Neuroscience; 55(4): 1067-74.
61. Zheng LF, Wang R, Xu YZ, Yi XN, Zhang JW, Zeng ZC. (2008): Calcitonin gene-related peptide dynamics in rat dorsal root ganglia and spinal cord following different sciatic nerve injuries. Brain Res.; 1187:20-32.
62. Noguchi M, Ikarashi Y, Yuzurihara M, Kase Y, Takeda S, Aburada M. (2003): Effects of 17 β estradiol and the Japanese herbal medicine Keishi-bukuryo-gan on the release and synthesis of calciotonine gene related peptide in ovariectomized rats. J Pharmacol Sci; 93: 80-86.
63. Jyoti Puri, Larry L. Bellinger, Phillip R. Kramer . (2011): Estrogen in cycling rats alters gene expression in the temporomandibular joint, trigeminal ganglia and trigeminal subnucleus caudalis/upper cervical cord junction. Journal of Cellular Physiology; 226 (12):3169–3180.
64. Leventhal L, Brandt MR, Cummons TA, Piesla MJ, Rogers KE. (2006): An estrogen receptor β-agonist is active in models of inflammatory and chemical induced pain. Eur J Pharmacol;553:146-8.
65. Vegeto E, Belcredito S, Etteri S, Ghisletti S. (2003): Estrogen receptor alpha mediates the brain anti-inflammatory activity of estradiol. Proc Natl Acad Sci USA; 100(16): 9614-9.
66. Vegeto E, Belcredito S, Ghisletti S, Meda C, Etteri S. (2006): The endogenous estrogen status regulates microglia activity in animal models of neuroinflammation. Endocrinology;147(5):2263-72.
67. Ma Y, Quirion R. (2006): Increased calcitonin gene related peptide in neuroma and invading macrophages is involved in the upregulation of interleukin-6 and thermal hyperalgesia in rat model of mono-neuropathy. J Neurochem; 98: 180-92.
68. Oprée A, Kress M. (2000): Involvement of proinflammatory cytokines, tumor necrosis factor α, IL1β and IL-6 but not IL-8 in the development of heat hyperalgesia: Effects on heat-evoked calcitonin gene related peptide release from rat skin. J Neurosci; 20(16): 6289-93.
69. Gim GT, Lee JH, Park E, Sung YH, Kim CJ, Hwang WW, Chu JP, Min BI. (2011): Electroacupuncture attenuates mechanical and warm allodynia through suppression of spinal glial activation in a rat model of neuropathic pain. Brain Res Bull; 86(5-6):403-11.
70. Hingne PM, Sluka KA. (2008): Blockade of NMDA receptors prevents analgesic tolerance to repeated transcutaneous electrical nerve stimulation (TENS) in rats. J Pain; 9(3):217-25.
71. Somers DL, Clemente FR. (2003): Relationship between dorsal horn neurotransmitter content and allodynia in neuropathic rats treated with high frequency transcutaneous electric nerve stimulation. Arch Phys Med Rehabil; 84(11): 1575-83.

5/2/2012