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A pilot study

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ZNRD1-AS and *RP11-819C21.1* long non-coding RNA changes following painful laser stimulation correlate with laser-evoked potential amplitude and habituation in healthy subjects: a pilot study

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Abstract

Long non-coding RNAs (lncRNAs) are a group of non-coding RNAs that act as regulators of gene expression; they are implicated in various human diseases and have been reported to be involved in the modulation of pain. We aimed to study whether: 1) lncRNAs modifications could be found in an experimental model of pain and 2) there was a correlation between lncRNA changes and laser evoked potential (LEP) amplitude/laser-pain rating.

LEPs were recorded from 11 healthy subjects to both left hand and perioral region stimulation. Three consecutive averages were calculated for each stimulation site in order to investigate the LEP amplitude habituation. Blood samples were obtained immediately before LEP recording (pre-pain) and 30-minutes after the recording of the last LEP average (post-pain). Eighty-four lncRNAs, involved in autoimmunity and human inflammatory response, were screened. The criteria used for lncRNAs analysis were fold change >2 and $p<0.05$.

By Real-Time PCR, we identified 2 lncRNAs up-regulated at the post-pain time, as compared to thepre-pain time : *RP11-819C21.1* (fold change=8.2; p=0.038) and *ZNRD1 antisense RNA 1 non-protein coding* (*ZNRD1-AS*) (fold change=6.3; p=0.037). The *ZNRD1-AS* up-regulation was directly correlated with the N1 amplitude, while the *RP11-819C21.1* increase after pain showed a correlation with the reduced N2/P2 amplitude and laser-pain habituation.

This is the first study showing lncRNA changes in a human experimental phasic pain model. The correlation between lncRNA changes and LEP amplitude and habituation suggests that *RP11-819C21.1* and *ZNRD1-AS* could be involved in the pathophysiology of painful diseases characterized by abnormal excitability of the cerebral cortex.

Introduction

Many studies on the animal models of neuropathic pain showed that specific genes has a key role in the molecular regulation of pain pathways (Apkarian et al., 2006; Arruda et al., 1998; Ortega-Legaspi et al., 2011). Thus, understanding how genes are modulated in pain conditions can provide important information for the underlying pathophysiology and a better pharmacological therapy.

Non-coding RNAs (ncRNAs) are post-transcriptional regulators with the ability to modify the expression of a multitude of mRNA targets. Among ncRNAs, microRNAs (miRNAs) are small non-coding RNA (with nucleotide lengths = 20), are the best studied and are now recognized to modulate gene expression at post-transcriptional level in many human diseases (Bartel 2009). In a rat model of inflammatory pain, several miRNAs were down-regulated in neurons of the ipsilateral trigeminal ganglion, suggesting that this decrease facilitated the development of inflammation and allodynia (Bai et al., 2007). Furthermore, miRNA-183 cluster was found down-regulated in the dorsal root ganglia (DRG) neurons of L5 spinal nerve ligation (SNL) rat model of chronic neuropathic pain (Aldrich et al., 2009). These data suggest that miRNA-183 family can regulate several genes implicated in chronic neuropathic pain modulation (Aldrich et al., 2009; Bai et al., 2007).

Unlike miRNAs, long non coding RNA (lncRNA) are a heterogeneous group of non-coding RNAs of 200 nucleotides in length (Ip and Nakagawa 2012). They regulate gene expression through interaction with histones or altering chromatin structure with a 'duplex' formation that can promote stability, alter splice profiles and mask the binding sites of specific miRNAs (Geisler and Coller 2013; Ip and Nakagawa 2012). LncRNAs are produced through the Polymerase II and can act in both *cis* and *trans*, with both nuclear and cytoplasmic localization (Ponting et al., 2009). They are implicated in various human diseases such as cancer, cardiovascular and neurodegenerative diseases. In Alzheimer's disease, up-regulation of a conserved non-coding antisense transcript for β-secretase1 (*BACE1-AS*) induces an increase in the levels of BACE1 protein, an enzyme involved in the production of β-amyloid (Faghihi et al., 2008). In patients with relapsing-remitting multiple sclerosis, we have recently shown the specific over-expression of 3 circulating lncRNAs (NEAT1, TUG1 and RN7SK RNA), which are involved in different inflammatory pathways (Santoro et al., 2016).

The first investigated lncRNA in neuropathic pain is an endogenus voltage potassium channel (Kv) *Kcna2* antisense (AS) RNA (Zhao et al., 2013). In a SNL rat model, Zhao et al. documented

over-expression of *Kcna2* AS RNA after nerve injury through the transcriptional activator myeloid zinc finger protein 1 (MZF1), with consequent reduction of the *Kcna2* mRNA expression and protein levels. The microinjection of the adeno-associated virus carrying *Kcna2* sense RNA into the injured DRG reduced the up-regulation of *Kcna2* AS RNA restoring Kv1.2 expression and alleviating neuropathic pain. Moreover, transcriptome analysis in the DRG of rats showed an increase of three lncRNAs involved in diabetic neuropathic pain: *uc.48+* (Wang et al., 2016; Wu et al., 2018), *BC168687* (Liu et al., 2017; Liu et al., 2018) and *NONRATT021972* (Liu et al., 2016). In diabetic rats, the intrathecal or intravenous injection of each corresponding small interference RNA (siRNA) reduced diabetes-induced mechanical allodynia and thermal hyperalgesia (Wang et al., 2016; Liu et al., 2017; Liu et al., 2016). Functionally, knockdown of *uc.48+, BC168687* and *NONRATT021972* attenuated the activation of $P2X_3$, $P2X_7$, phosphorylated extracellular signal-regulated kinase (p-ERK) 1/2 and tumor necrosis factor-α (TNF-α). *NONRATT021972* was also found to be increased in the serum of patients with type 2 diabetes mellitus (Yu et al., 2017). Recently, RNA sequencing analysis during the progression of neuropathic pain (NP) in a spared nerve injury (SNI) model showed a specific expression profile of lncRNAs at different stages during neuropathic pain (Zhou et al., 2017). These dysregulated lncRNAs regulate genes that encode for receptor binding, structural molecular activity, calcium ion binding, transporter activity, protein and lipid binding. In the contest of peripheral nerves, microarray-based analysis in rat DRG after sciatic nerve injury revealed a down-regulated lncRNA *uc.217* in regenerative DRG neuronal outgrowth (Yao et al., 2015). Silencing of *uc.217* expression could significantly promote neurite outgrowth in cultured DRG neurons suggesting a regulatory involvement of this lncRNA in the plasticity of peripheral nerves.

Altough these studies suggest an involvement of lncRNAs in neuropathic pain, their specific functions are still elusive. A reliable laboratory tool for assessing human nociceptive pathways is laser evoked potential (LEP) recording (Cruccu et al., 2010). Laser stimulation excites the free nerve endings (A-δ and C fibres) in the superficial skin layers, without any involvement of Aβ fibres (Bromm and Treede 1984), and generates the LEPs by Aδ-fiber input (Valeriani et al., 2012). LEP recording represents the most reliable neurophysiological method to assess the nociceptive pathways (Valeriani et al., 2012). This technique has proved very useful in investigating many neuropathic pain conditions, so that the European Federation of Neurological Societies Panel on Neuropathic Pain states that "Laser-evoked potentials are useful for assessing function of the Aδ fibre pathways in patients with Neuropathic Pain (grade A)" (Cruccu et al.,

2010). In the present study, laser stimulation of the skin was used to induce phasic pain in healthy subjects. Moreover, LEPs were recorded as a correlate of the processed pain nociceptive related response.

Study Objectives

Based on these premises, the aims of the study were: i) to evaluate the expression profiles of 84 lncRNAs, validated or predicted to regulate the expression of genes for acute-phase response, autoimmunity, humoral immune response, inflammatory responses, and innate and adaptive immunity in plasma of healthy subjects before and after pain induced by laser stimulation; ii) to investigate any possible correlation between the pain-induced lncRNA regulation and the processing of the nociceptive input, as expressed by neurophysiological and psychophysiological values.

Materials and Methods

Subjects

Eleven healthy volunteers (7 females and 4 males with 35±14.6 years of age) were recruited. Exclusion criteria were pregnancy, steroid therapy, drugs use, infection within 1 month from the study, any painful disease, including migraine, and neuropathies.

The design of the study was approved by the Local Ethical Committee (Fondazione Don Gnocchi, Milan, Italy). Written informed consent was obtained from all volunteers after a detailed explanation of the study's aims and procedures also regarding their future use for research purposes.

LncRNAs analysis

Whole blood samples were collected from volunteers by venous punctures immediately before the laser stimulation (pre-) and after 30 minutes from the last laser stimulation (post-). Since this represents the first study investigating non-coding RNAs' modifications after experimental painful stimulation in humans, our choice to consider an interval of 30 minutes between the painful stimulation end and plasma collection could not be based on previous data. It depended on two main reasons. 1) Time interval to collect the plasma did not have to be too close to the painful stimulation, in order to avoid a "plateau" effect of the lncRNAs expression. 2) Time interval did not have to be too long, in order to avoid the loss of the laser stimulation effect on the expression

of the lncRNAs. EDTA-tubes were used for plasma isolation: samples were centrifuged at 1.500 g for 15min at 4 °C. After, the supernatant containing plasma was centrifuged again at 14.000 g for 15 min at 4 °C and stored at -80°C. Total RNA, extracted from plasma using miRNeasy Mini kit (QIAGEN, Germany), was subjected to complementary DNA (cDNA) synthesis using RT^2 PreAMP cDNA Synthesis Kit (QIAGEN, Germany) according to the manufacturer's instructions. cDNA samples were pre-amplified with RT^2 lncRNA PreAMP Primer Mix that contained specific set of primers to target genes of Human RT^2 lncRNA Inflammatory Response & AutoimmunityPCR Array (QIAGEN, Germany). Following pre-amplification, we eliminated residual primers with Side Reaction Reducer (QIAGEN, Germany) at 37 °C for 15 min with heat inactivation at 95 °C for 5 min. qRT-PCR was performed with RT^2 SYBR® Green qPCR MasterMix (QIAGEN, Germany) into the wells of RT^2 lncRNA PCR Array Human Inflammatory Response & Autoimmunity (QIAGEN, Germany) which contains pre-dispensed gene-specific primer pairs (for the complete list of genes see Supporting information - Appendix S1 - list of genes).

Thermal cycling conditions: holding stage at 95 °C for 10 min (enzyme activation), 40 cycles of each PCR step [(denaturation) 95 °C for 15 s and (annealing/extension) 60 °C for 1 min].

For data analysis, the CT values of pre- and post- stimulation were exported to an Excel file and uploaded into web portal at https://www.qiagen.com/dataanalysiscenter. CT values were normalized using as reference genes *Ribosomal protein, large, P0* (*RPLP0*, NM_001002), *ZNFX1 antisense RNA 1* (*ZFAS1*, NR_003604), and *Growth arrest-specific 5* (*non-protein coding*) (*GAS5*, NR_002578) as previously described (Santoro et al., 2016).

Fold change/regulation is calculated using ΔΔCT method where ΔCT is calculated between gene of interest and an average of reference genes, followed by ΔΔCT calculations [ΔCT(post-)-ΔCT(pre-)]. After, fold change is calculated using $2^{-\Delta\Delta CT}$ formula. Fold-change values > 1 indicates a positive- or an up-regulation, and the fold-regulation is equal to the fold change.

Fold-change < 1 indicate a negative or down-regulation, and the fold regulation is the negative inverse of the fold-change. We selected significant changes ($p < 0.05$) that were at least two fold up- or down-regulated as compared to pre-group. The p values were calculated based on a Student's t test of the replicate 2^ (−ΔCT) values for each gene in the pre- group and post- group. The data analysis was plotted with volcano plot, that combines a p value (y-axis) with the fold change (x-axis) that allows to identify genes with both large and small expression changes statistically significant.

Bioinformatic analysis of lncRNA-miRNA interactions

The prediction analysis of lncRNA-miRNA interactions was performed, using starBase v.2 (http://starbase.sysu.edu.cn/starbase2/index.php) software a web-based tool that predicted miRNA-lncRNA interactions by scanning lncRNA sequences overlapping with CLIP-Seq peaks for potential microRNA targets (Li et al., 2014; Yang et al., 2011).

LEP recording

The LEP recordings were performed in all subject after the baseline blood sample collection, randomizing the sequence of the stimulation sites. During LEP recording, the subjects lay on a couch in a warm and semi-dark room. Cutaneous heat stimuli were delivered by a Nd:YAP laser [Neodimium:Yttriume-Aluminiume-Perovskite; 1.34 μm wavelength; 5 mm beam diameter (ElEn®, Florence, Italy)]. LEPs were recorded after stimulation of the skin over the dorsum of the left hand, and over the left perioral region of the face (trigeminal nerve branches 2 and 3). We decided to obtain LEPs to stimulation of both somatically (hand) and trigeminally (face) innervated areas for 2 main reasons: 1) the distribution of the Aδ nociceptors, whose activation generates LEPs, shows a proximal-distal gradient, the highest density occurring in the trigeminal region (Agostino et al., 2000), and 2) the length of the peripheral nociceptive fibers is lower in the trigeminal region, making a possible impact of peripheral factors on the LEP amplitudes less relevant. A red helium-neon (He-Ne) laser beam visualized the stimulation site. To avoid the sensitization of the nociceptors, the location of the impact on the skin was slightly shifted between 2 successive stimuli. A fixed intensity of stimulation was set at 2.5 X sensory threshold to record LEPs (recording intensity, RI). In all LEP recordings RI was judged as moderately painful by all patients. The interstimulus interval varied randomly between 8 and 12 seconds. All subjects underwent a standard recording session with three scalp electrodes placed at Cz, Fz, and T4 positions of the 10-20 International System. The reference electrode was placed at the nose and the ground at the forehead (Fpz). The eye movements and eyeblinks were monitored by an electro-oculography (EOG) electrode above the right eyebrow. Signals were amplified, filtered (bandpass 0.3-70 Hz), and stored for off-line average and analysis. The analysis time was 1000 milliseconds with a bin width of 2 milliseconds. An automatic artifact rejection system excluded from the average all trials contaminated by transients exceeding ± 65 mV at any recording channel, including EOG. In order to assess the habituation of LEP amplitudes and latencies, we used an

experimental design which was previously validated and proved useful in discovering brain abnormalities in different painful conditions (de Tommaso et al., 2005; Di Clemente et al., 2013; Valeriani et al., 2003; Valeriani et al., 2005). Three consecutive blocks of responses were obtained for each stimulation site. Each block consisted of 25 sweeps. The consecutive blocks were separated by a 5-minutes time interval. The stimulation sites were changed after 10-minutes intervals. After each block of stimuli subjects were asked to rate the average pain according to a 101-points visual analogue scale (VAS), in which 0 corresponds to "no pain" and 100 to "the most intense pain one may conceive".

In order to ensure us that the attention level of our subjects did not change across the whole experiment, they were asked to count the number of the received laser stimuli silently. Any recording with a counting mistake higher than 10% would not be considered for further analysis.

The whole LEP recording lasted around 1 hour.

LEP analysis

LEP labeling and analysis in the present study are issued from previous literature on LEPs (Valeriani et al., 1996; Wu et al., 1999; Spiegel et al., 2000; Valeriani et al., 2000; Garcia-Larrea et al., 2002). In all our subjects, the negative N1 response was identified in the T4 recording, contralateral to the stimulation. At about the same latency, the positive P1 potential was recorded by the Fz lead. Because in labelling the N1 a certain difficulty may be caused by noise, the N1 amplitude was calculated offline by referring the temporal electrode contralateral to the stimulated side (T4) to the Fz lead (Kunde and Treede 1993). The N2 and P2 peak latencies were measured at Cz electrode. The peak-to-peak N2/P2 amplitude was measured at the same lead.

Statistical analysis

Both latencies and amplitudes of the N1, N2 and P2 components and VAS scores were submitted to Shapiro-Wilk test for normality. Since all of them overtook the test, the habituation was investigated by a series of Student's paired t-tests with Bonferroni's correction, which compared the values obtained at the second and third repetition with those at the first repetition. Moreover, habituation indexes were calculated by dividing the LEP amplitudes and VAS values of the second (N1 II/I, N2/P2 II/I, and VAS II/I) and third repetition (N1 III/I, N2/P2 III/I, and VAS III/I) by those of the first repetition, respectively. Once identified the lncRNAs significantly up or down regulated, the fold changes of these lncRNAs, calculated with $2^{-\Delta\Delta CT}$ method, were correlated with

the N1 and N2/P2 amplitudes and VAS values in each repetition and their habituation indexes by Pearson correlation. The level of statistical significance was fixed at 0.05.

Results

Expression analysis of LncRNAs

In order to evaluate the expression levels of lncRNAs in post- group vs. pre-group samples, we analyzed 84 lncRNAs, validated or predicted to regulate the expression of pro-inflammatory and anti-inflammatory genes and microRNAs. For data analysis, we eliminated the lncRNAs with expression levels that were too low (the CT cut-off was set to 35 cycles in both pre- and post-groups) so as to make their real-time quantification unreliable. We identified two lncRNAs that were significantly up-regulated after LEP stimulation compared to pre-group: *RP11-819C21.1* fold change = 8.2 ($p = 0.038$) and *ZNRD1 antisense RNA 1* (*non-protein coding*) (*ZNRD1-AS1*, NR_026751) fold change = 6.3 ($p = 0.037$) (Figure 1A).

We further evaluated the best lncRNA between *RP11-819C21.1* and *ZNRD1-AS1* normalizing the expression with standard housekeeping *beta-2-microglobulin* (*B2M*, NM_004048). In this case, only *RP11-819C21.1* showed a significant ($p = 0.039$) up-regulation (fold change = 9.4) between post-group and pre-group (Figure 1B).

Moreover, using starBase v.2 software we found an interaction of *RP11-819C21.1* with 24 miRNAs (Supporting information - Table S1) and *ZNRD1-AS1* with 40 miRNAs (Supporting information - Table S2). Both lncRNAs showed *miR-19a* and *miR19b* as common target.

Neurophysiological and psychophysical findings

Laser-pain rating did not vary significantly across the repetitions to both hand and face stimulation ($p > 0.05$).

The N1, N2, and P2 LEP latencies did not vary across the repetitions at all stimulation sites ($p > 0.05$) (Table 1). No significant habituation of the N1 amplitude was found to both hand and face stimulation ($p > 0.05$). On the contrary, the N2/P2 amplitude decrease at the third repetition, as compared to the first one, was marginally significant and significant to hand ($p = 0.07$) and face ($p = 0.005$) stimulation, respectively (Figures 2 and 3).

Correlation analysis showed only three significant interactions. First, the N1 amplitude in the first repetition to face stimulation was positively correlated with the *ZNRD1-AS1* lncRNA increase ($R^2 = 0.41$, $p = 0.03$), meaning that the subjects with the higher lncRNA increase showed the larger N1

amplitude (Figure 4A). Second and third, the III/I habituation indexes of the N2/P2 amplitude and VAS score to face stimulation were positively correlated with the *RP11-819C21.1* lncRNA increase, meaning that the subjects with the higher lncRNA increase showed the lower N2/P2 amplitude ($R^2 = 0.48$, $p = 0.01$, Figure 4B) and laser-pain rating ($R^2 = 0.45$, $p = 0.02$, Figure 4C) habituation.

Discussion

This is the first study showing lncRNAs modifications in a human model of phasic experimental pain. The main features were: 1) an up-regulation of both the *ZNRD1-AS1* and *RP11-819C21.1* lncRNAs after experimental cutaneous pain induced by laser stimulation of the skin, and 2) a correlation of the up-regulated lncRNAs with the N1 amplitude and N2/P2 amplitude and laser-pain rating habituation to trigeminal nerve stimulation.

LncRNAs modifications induced by pain

LncRNAs are involved in several physiological and pathological processes through modulation of the gene expression at the transcriptional, post-transcriptional and epigenetic levels (Cesana et al., 2011; Gupta et al., 2010; Tsai et al., 2010). About 50% of the over 10.000 lncRNAs reported are expressed in the central nervous system (CNS) suggesting a prominent role in this contest (Derrien et al., 2012; Knauss and Sun 2013). In according with these data, many studies documented a functional importance of lncRNAs in brain disorder such as Alzheimer's or Huntington's disease (Faghihi et al., 2008; Modarresi et al., 2012). In the contest of neuropathic pain modulation, microarray assay on sciatic nerve injury model showed a dysregulation of 105 lncRNAs in DRG and one of these, lncRNA BC089918, inhibited neurite outgrowth of DRG neurons (Yu et al., 2013). Moreover, Zhao and collaborators documented over-expression of *Kcna2* asRNA after nerve injury in DRG ipsilateral of rat model SNL (Zhao et al., 2013). They further identified in the promoter region of *Kcna2* AS RNA a binding motif for the transcriptional activator myeloid zinc-finger protein 1 (MZF1) that controls the expression of *Kcna2* AS RNA, *Kcna2* mRNA and the Kcna2 protein. Over-expressing or blocking *Kcna2* AS RNA in DRGs *in vivo* led to alleviated or attenuated neuropathy-induced mechanical, cold, and thermal sensitivity in rats (Zhao et al., 2013).

In the present study, we analyzed 84 lncRNAs validated or predicted to regulate the expression of

pro-inflammatory, anti-inflammatory genes, and microRNA in plasma samples from 11 healthy volunteers before and after painful laser stimulation of both hand and face skin. Our data showed that *RP11-819C21.1* and *ZNRD1-AS* were significantly up-regulated (fold change >2 and $p < 0.05$) after pain. In particular, *RP11-819C21.1* up-regulation was particularly solid, since it was retained with different normalization method. Moreover, the bioinformatic analysis showed a predicted interaction of *RP11-819C21.1* with 24 miRNAs and *ZNRD1-AS1* with 40 miRNAs. It is particularly interesting that both lncRNAs showed *miR-19a* and *miR19b* as common target, since these miRNAs are involved in the modulation of multiple potassium channel α subunits and auxiliary subunits in DRG neurons of L5 SNL model of neuropathic pain (Sakai et al., 2017). Our data agree with Zhao and collaborators' hypothesis, according to which the up-regulation of *Kcna2* antisense RNA reduces *Kcna2* mRNA expression leading to diminished K^+ current in the injured DRG of rat model SNL (Zhao et al., 2013). The relationship between pain and K^+ channel dysfunction is largely supported by previous literature (Li and Toyoda 2015). In various animal models of neuropathic and chronic pain, the down-regulation of various K^+ channels is feature of the neuropathic remodelling within the injured/degenerating peripheral nociceptive (and sometimes in non-nociceptive) afferents (Cao et al., 2010; Kim et al., 2002; Rose et al., 2011). Furthermore, Klein et al. (Klein et al., 2012) showed that a voltage-gated potassium channel (VGKC)-complex autoimmunity can be a syndromic manifestation of chronic idiopathic pain.

LncRNA modifications and LEPs

In our subjects, we investigated the excitability of the nociceptive pathway by calculating the habituation of the LEP amplitudes. The phenomenon of habituation consists in a reduction in amplitude of a sensory response to repetitive stimuli (Coppola et al., 2013). This can be due to either peripheral or central factors. In the present study, receptor fatigue, that could lead to a progressive LEP amplitude reduction, can be excluded, since the laser pulses were delivered to close by different skin areas during LEP recording. Although reduced LEP habituation was demonstrated in a model of peripheral sensitization using topic application of capsaicin (Hüllemann et al., 2015), in the present study no peripheral sensitization was induced. Reduced EP habituation to painful stimuli can be also interpreted as a marker of abnormal nociceptive processing and/or cerebral cortex excitability, as in migraine (Valeriani et al., 2003), fibromyalgia (de Tommaso et al., 2011, 2014), and cardiac syndrome X (Valeriani et al., 2005), that are diseases characterized by *pain sine materia*, and in neuropathic pain conditions, such as cervical

radiculopathy (Hüllemann et al., 2017). The experimental design used to study LEP habituation was validated previously (de Tommaso et al., 2005; Di Clemente et al., 2013; Valeriani et al., 2003; Valeriani et al., 2005). In our subjects, the N2/P2 amplitude recorded at the third repetition was lower than that at the first one to both hand and face stimulation. On the contrary, no habituation was found for the N1 amplitude. In previous studies, N1 amplitude habituation was reported inconsistently (Ferraro et al., 2012; Valeriani et al., 2003). This can be due to the different physiological meaning of N1 component and N2/P2 vertex potential. Indeed, while the N1 response is generated in the opercular cortex (Garcia-Larrea et al., 2003; Valeriani et al., 1996; Valeriani et al., 2000) and is related to the sensory component of pain (Lorenz and Garcia-Larrea 2003), the N2/P2 potential mainly originates from the midcingulate gyrus (Garcia-Larrea et al., 2003; Valeriani et al., 1996; Valeriani et al., 2000) and is related to the affective/motivational aspect of pain sensation (Lorenz and Garcia-Larrea 2003). However, the inconsistent N1 habituation can be merely due to technical difficulties in its recording, since the N1 lower amplitude, as compared to that of N2/P2 component, makes it difficult to recognize small variations.

In our subjects, the N2/P2 amplitude ratio between the third and first repetition to face stimulation showed a positive correlation with the *RP11-819C21.1 lncRNA* increase after experimental pain. This result, strengthened by the parallel behaviour of the laser-pain rating, means that the subjects who showed a lower N2/P2 amplitude and laser-pain habituation had also a higher *RP11-819C21.1* up-regulation. This suggests that this lncRNA can be involved in the physiological processes subtending habituation to painful stimuli and nociceptive cortex excitability. The other lncRNA (*ZNRD1-AS1*), which was found up-regulated in our subjects after experimental pain, showed a positive correlation with the N1 amplitude recorded in the first repetition to face stimulation.

This means that the subjects with a higher N1 amplitude showed the larger up-regulation of this lncRNA, which could be engaged in the codification of the perceptive qualities of pain sensation.

It is to be underlined that the in our study the up-regulated lncRNAs showed correlations only with LEP parameters to face stimulation. This can be due to two main reasons. First, LEPs to face stimulation can be a more objective marker of pin-prick pain sensation due to higher density of Aδ mechano-heat receptors in this area, as compared to the other body regions (Agostino et al., 2000). Second, LEP amplitudes to face stimulation could express the brain excitability changes to phasic

painful stimuli more reliably than those to hand stimulation, due to the lower length of the activated peripheral fibers.

Although the exact meaning of the correlations between LEPs and lncRNAs is difficult to be hypothesized, it is interesting to observe that both lncRNAs up-regulated after experimental cutaneous pain showed a direct correlation with LEP parameters (increased N1 amplitude and reduced N2/P2 habituation) suggesting augmented excitability of the nociceptive cortex. This point of view is particularly intriguing if we consider the possible involvement of both lncRNAs in the modulation of K^+ channels function (see above), which plays a key role in the excitability of the neural structures (Takeda et al., 2011; Tulleuda et al., 2011). In this light, we can suppose that the lncRNAs found up-regulated in our study can play a role in the activation of K^+ channels to phasic painful stimuli. Moreover, this physiological mechanism could be dys-regulated in clinical conditions characterized by chronic neuropathic and non-neuropathic pain. If this were so, the identification of the factor(s) involved in this dys-regulation could have a crucial therapeutic role.

Limitations of the study

The present study has some limitations that should be considered. First, a limited number (11) of healthy subjects was included. Since this is the first study investigating the modifications of a group of lncRNAs after experimental pain, we could not perform a power study. However, in the present study the lncRNA levels after pain were compared with the corresponding levels before pain in each individual subject. From this point of view, since every subject was a control of himself/herself (lncRNA after pain vs. lncRNA before pain), the negative impact of the small sample size on our results is reduced. Moreover, this is a pilot study, whose findings need confirmation in larger samples. Second, while previous experimental studies suggesting lncRNAs' role in pain used models of tonic pain, such as SNL, SNI, and diabetes (see the Introduction), in the present study phasic pain was induced by laser pulses. Since phasic pain is less ecological than tonic pain, which is far more similar to pain in clinical conditions, the translational value of our results in painful diseases is to be demonstrated. However, laser stimuli allowed us to record brain responses that can be assumed as an objective marker of nociceptive sensation in the present experimental setting. Third, our study was based on a neurophysiological measure, which shows a certain inter-individual variability (LEP values), and a biological measure whose consistency between subjects is still to be demonstrated. Although this must be considered in interpreting the present results, the design of our experiment can reduce the impact of the inter-individual

differences in lncRNA levels. Indeed, the lncRNA variations induced by pain were calculated by subtracting lncRNA levels measured after pain from those before pain in each individual subject. Fourth, we did not include a group of control subjects in whom the lncRNAs were measured in two blood samples collected 90 minutes from each other without any painful stimulation. However, we think that the lncRNAs' modifications observed in our study are related to pain for two main reasons. 1) The correlation between the lncRNAs' modifications and some LEP parameters makes it unlikely that these changes do not have to do with the laser stimulation. 2) A previous study showed that while the lncRNAs' expression has a lower inter-individual reproducibility, it is stable within the same subject (Kornienko et al., 2016). Since in our experiment every subject was a control of himself/herself, we can be confident that the lcnRNAs' modifications observed after painful laser stimulation were not found by chance.

Conclusions

In conclusion, our study documented for the first time that both *RP11-819C21.1* and *ZNRD1-AS* lncRNAs are up-regulated after experimental pain induced by painful phasic laser stimulation of the skin. Moreover, while the *ZNRD1-AS* increase was directly correlated to the N1 amplitude, the *RP11-819C21.1* up-regulation was higher in subjects showing a lower N2/P2 amplitude and laser-pain rating habituation. Further studies will have to clarify the physiological meaning of these lncRNAs in pathological conditions characterized by abnormal habituation to repetitive painful stimulation, such as migraine and fibromyalgia.

Author contributions

Massimo Santoro: design of the study, data collection, data analysis and interpretation, manuscript drafting and revision, final approval of the version to be published.

Catello Vollono: data collection, data analysis and interpretation, manuscript drafting and revision, final approval of the version to be published.

Costanza Pazzaglia: data collection, data analysis and interpretation, manuscript revision, final approval of the version to be published.

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Rocco Giordano: data collection, data analysis, manuscript drafting, final approval of the manuscript.

Luca Padua: manuscript revision, final approval of the version to be published.

Lars Arendt-Nielsen: data analysis and interpretation, manuscript revision, final approval of the manuscript.

Massimiliano Valeriani: design of the study, data analysis and interpretation, manuscript drafting and revision, final approval of the version to be published.

Legends for illustrations and tables

Figure 1. The volcano plot representation of lncRNAs plasma levels in post-group vs. pre-group. Statistical significance versus fold-change was showed on the y- and x-axes, respectively.
Yellow circle indicated the up-regulation, blue circle the down-regulation, and black circle unchanged regulation. A) Values were normalized to the average of expression levels of *RPLP0*, *ZFAS1*, and *GAS5*. B) Values were normalized to expression levels of *B2M.*

Figure 2. LEPs recorded in one representative subject to hand (left) and face stimulation. N2/P2 potential, recorded from Cz electrode, is shown in the upper 3 rows (I, II, and III repetition), while the N1 response, recorded by T4-Fz derivation, is shown in the lower 3 rows (I, II, and III repetition).

Figure 3. The histograms show the N1 (left) and N2/P2 (right) amplitude modifications along successive repetitions (I, II, and III) to hand and face stimulation. While the N2/P2 amplitude shows a clear habituation, the N1 amplitude does not change across the repetitions.

Figure 4. The figure shows the direct correlation between the N1 amplitude in the first repetition to face stimulation with the *ZNRD1-AS1* lncRNA increase after experimental pain (A) and the direct correlation between the III/I habituation index of the N2/P2 amplitude (B) and laser-pain rating (C) to face stimulation with the *RP11-819C21.1* lncRNA up-regulation.

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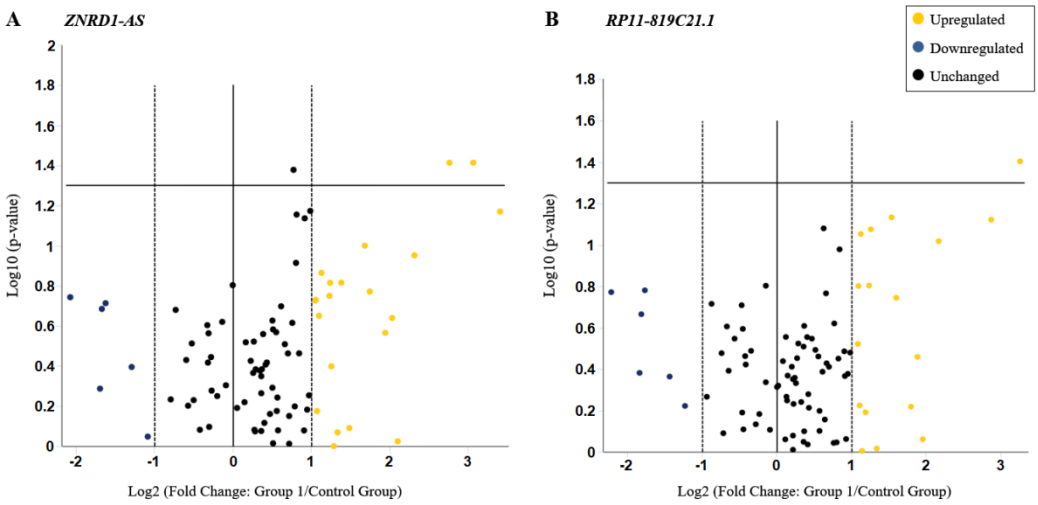
Table 1 - LEP amplitudes and latencies

			Latencies (ms)			Amplitudes (μV)	
		VAS	N1	N2	P2	N1	N2/P2
Face stimulation	I repetition	44.1±17.6	129±28.1	168.1±28.5	285.1±52.5	8.1±4.8	34.5±16
	II repetition	40.9±15.5	131.0±30.8	167.6±32.9	280.3±54.2	8.9±4.7	31.9±18
	III repetition	39.5±14.9	128.3±27.4	162.2±29.4	282.5±51	7.7±3.5	29.2±13.9
Hand stimulation	I repetition	40.9±16.9	158.8±16.7	205.7±17	336.6±45	4.4±2.9	34.8±13.2
	II repetition	38.9±14.4	156±18.6	208.4±19.8	331.9±36.7	4.9±3.4	30.9±15
	III repetition	37±11.2	161.2±23.3	209.2±21.7	334.2±36	4±2.2	27.4±14.3



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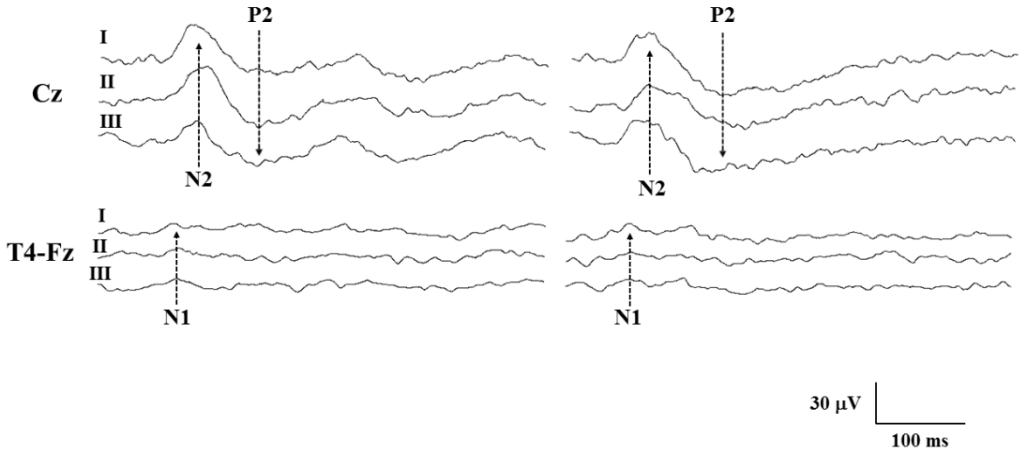

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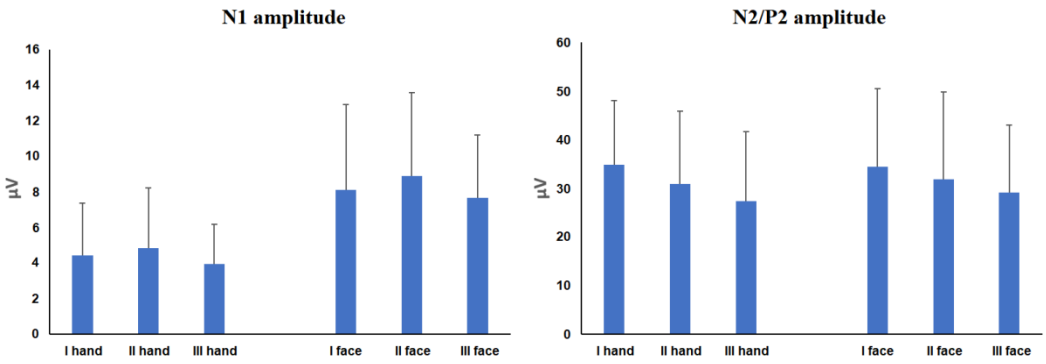

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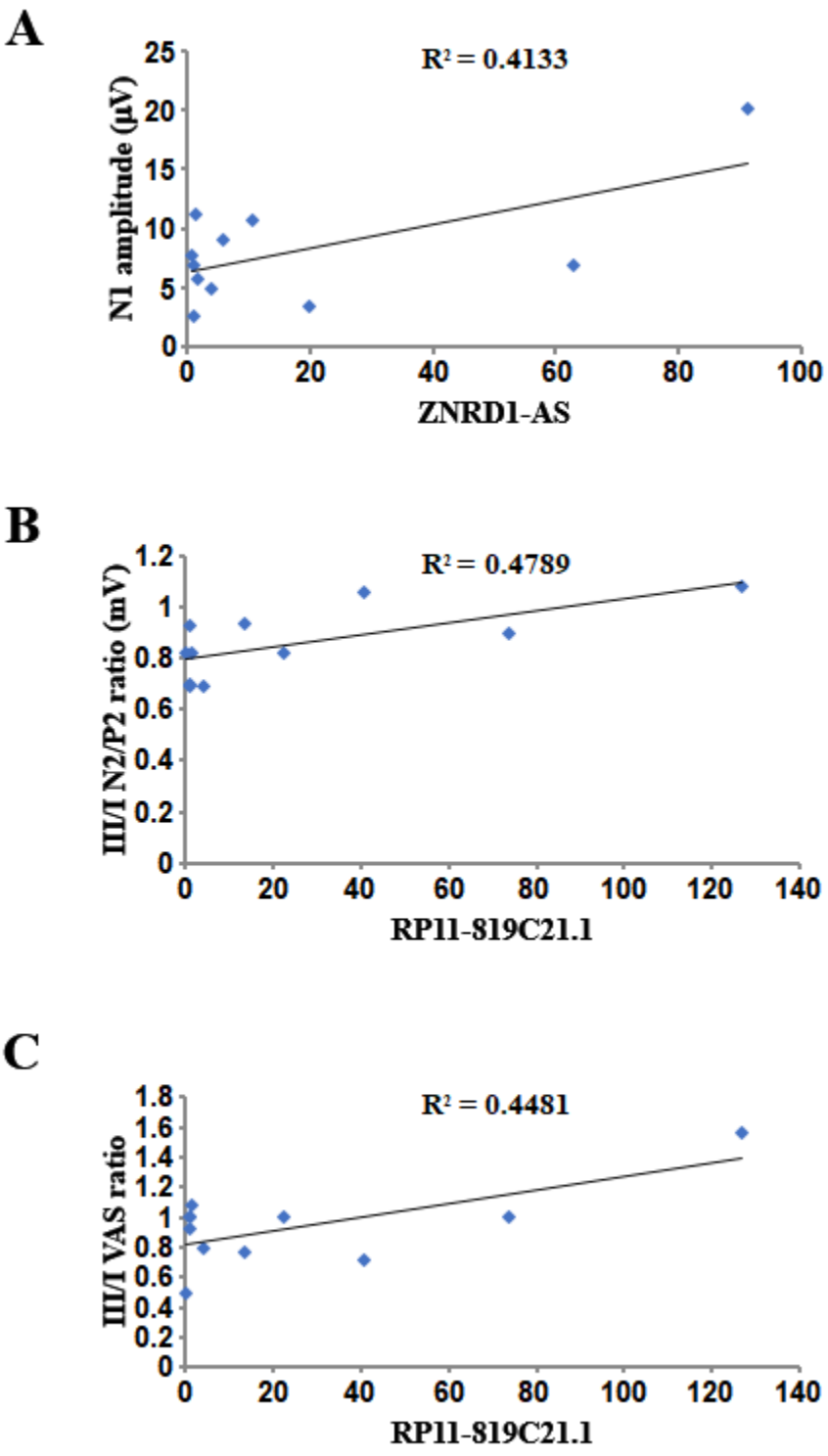

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