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Visual reminders of death enhance nociceptive–related cortical responses and event-related alpha desynchronisation

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Highlights

- We tested whether images conveying death-related vs. threat-related content had a specific effect on pain perception and cortical activity measured by EEG.
- We found increased amplitude of nociceptive P2 potential and oscillatory theta activity associated with death-related images, but no change in pain ratings.
- We found increased oscillatory alpha desynchronisation associated with death-related images but no significant difference in visual evoked potentials amplitude.

Abstract

Previous research suggests that prompting individuals to think on their own mortality affects their perception of painful somatic stimuli and related brain activity. Grounded on the assumption that reminders of mortality may recruit threat-defence mechanisms similar to the ones activated by painful nociceptive stimuli, we hypothesize that the effects exerted by linguistic reminders of death on pain perception and brain activity would be elicited by passive observation of death-related pictures vs. more generic threat-related pictures. Results showed an increase of the laser evoked P2 amplitude and oscillatory theta activity when participants observed death-related images. However, no change in pain ratings was found. Moreover, observation of death-related content was linked to increased oscillatory alpha desynchronisation but not to variations of visual evoked potentials amplitude. Our findings indicate that pairing potentially noxious stimuli with death-related images exerts a preferential modulation of nociceptive and visual cortical representations.

Keywords: alpha, EEG, laser evoked potentials, reminders of mortality, terror management theory, theta, visual evoked potentials.

Introduction

"*I'm not afraid of death; I just don't want to be there when it happens*". This famous quote by movie director Woody Allen offers us a sophisticated and light-hearted example of the human desperate attempt to cope with the unavoidable awareness of our own finiteness. Humour is one of the manifold cultural means humans use to cope with the fear of death, that Ernst Becker (1973) defined as the most powerful motivator of human behaviour. Becker compellingly disclosed how humans developed cultures, beliefs and symbolic systems aimed to minimize or even deny such, largely unconscious, primal fear. Building upon Becker's work, the Terror management theory (TMT) posits that in order to cope with existential anxiety, humans strive to defend their own cultural worldviews and self-esteem (Solomon et al., 2004). TMT theorists proposed a dual-process model according to which different cognitive defences are initiated to cope with the mental consequences of death reminders. According to the model, 'proximal' defences (i.e. explicit, voluntary, conscious) imply either distraction from or rationalisation of death content. In contrast, 'distal' defences (i.e. implicit, automatic, unconscious) imply development of self-esteem and cultural beliefs that help to buffer death-related anxiety (Greenberg et al., 2000; Pyszczynski et al., 1999).

Although the TMT and dual defence model received large support by experimental evidence, there is still substantial criticism about the assumptions of the model and alternative plausible models have been proposed (Pyszczynski et al., 2015 for a recent review). For example, some studies have shown that other types of psychological threats such as thoughts of being uncertain (van den Bos and Miedema, 2000) or not having control (Fritsche et al., 2008) can cause effects similar to reminders of mortality. Yet, there seems to be still a larger body of empirical work advocating for unique and specific effects of thinking about death (Hayes et al., 2010 and Burke et al., 2010 for a metanalytic assessment).

Highly relevant to the present study are alternative models that emphasise the biological implausibility of a unique and specific threat mechanism of action of death cognition compared to other threats (McGregor, 2006; Tritt et al., 2012).

The few studies that investigated possible brain correlates of death reminders have thus far endorsed the TMT predictions. And yet, as the knowledge underlying the neurological underpinnings of death cognition is still in its infancy, it is conceivable that these models may be integrated and reconciled with TMT rather than seen as radical alternatives. For example, TMT scholars do not seem to disagree on the idea that existential anxiety mechanisms may originate from a simpler and more general anxiety system apt to respond to different physical and psychological threats (Pyszczynski et al., 2015).

Neuroimaging studies reported specific effects of death-related cues that may be explained by activation of both proximal and distal defence mechanisms (see Valentini et al., 2015 for a critical discussion). Importantly, previous neurophysiological studies used single words or entire sentences as operational means to induce awareness of mortality in experimental volunteers (e.g., Han et al., 2010; Klackl et al., 2013; Quirin et al., 2012). At variance with these studies that did not explicitly require reflection on the idea of death, we recently provided evidence that death cognition based on classical mortality salience manipulation (Rosenblatt et al., 1989), where healthy individuals are asked to reflect on the ideas and emotions originating from thinking about their own death, is associated with higher intensity and threat ratings for nociceptive painful stimuli (Valentini et al., 2014; Valentini et al., 2015).

The empirical evidence that putative cognitive and emotional processes triggered by reminders of death are able to influence the perception of painful stimuli and related cortical processing adds to the multifaceted evidence of top-down cognitive and emotional effects on pain (Wiech, 2016). Subjective and neural correlates of the experience of pain

are sensitive to complex contextual information (e.g. placebo and nocebo effects; Carlino et al., 2014) and to ongoing anxiety or other aversive/negative emotional states (e.g. Rhudy and Meagher, 2000; Yoshino et al., 2012). Psychological appraisal-laden processes have been recently suggested to account for placebo phenomena (Ashar et al., 2017). By analogy, appraisal of threatening psychological information may account for cognitive and emotional effects exerted by reflection on one's own death.

It is worth noting that studying the relationship between reminders of mortality and brain representation of painful thermal stimuli is grounded on the homeostatic/motivational value of painful stimuli and meaning of death for humans. In most circumstances, nociceptive inputs represent a higher threat to the integrity of the body as compared to non-nociceptive inputs (e.g. Schrooten et al., 2012; Van Damme et al., 2004). Likewise, most cultures attach to the idea of death concerns about body alterations (e.g. pallor, algor, rigor, livor, putrefaction) as well as sufferance and despair in significant others (Hoelter, 1979). According to the TMT, the human body is a prominent mediator of existential anxiety as it serves as a perpetual reminder of the inevitability of death (Goldenberg et al., 2000). As a result, the reminders of death could trigger an aversive motivational and emotional state able to interact with signals of threat to the body via the appraisal-laden processes. Studies indicate that reminders of mortality significantly increase denial of similarities between humans and other animals as well as disgust for bodily products (Goldenberg et al., 2001). When individuals are primed to associate the physical aspects of sex with animalistic behaviour, mortality reminders seem to reduce the appeal for sex (Goldenberg et al., 2002). Threats associated with the animal nature of our body seem to hinder the attitude toward healthy behaviours. For instance, female volunteers tend to avoid breast self-examination following reminders of their own mortality (Goldenberg et al., 2006). In this vein, all the sensory events inherently related to the representation of the body may establish a close functional association with the

representation of death in the brain. More specifically, the elaboration of threatening bodily stimuli and the experience of pain can well be another proxy of this subtle relationship between existential anxiety and the body. Despite the lack of research on the relationship between physical pain and existential anxiety, clinical evidence hints at increased experience of pain in presence of fear of death or death anxiety in terminally ill patients (Grumann and Spiegel, 2003; LeMay and Wilson, 2008). The more general relationship between anxiety and pain is germane to the purported effects of reminders of mortality. Indeed, anxiety entertains a bi-directional relationship with pain (Gonzalez et al., 2011). For example, anxiety can increase dental pain perception (van Wijk and Hoogstraten, 2009) whereas chronic pain exacerbates pain-related fear and anxiety (Asmundson and Katz, 2009).

If reminders of mortality were to be processed as threatening psychological events and their neural, subjective and behavioural effects were relying on threat-defence mechanisms similar to the one activated by painful nociceptive stimuli then we should expect a top-down deployment of the attentional focus on the nociceptive stimulus triggered by reminders of mortality. Such cognitive effect would be associated with an affective top-down bias able to increase the motivational relevance of the ongoing nociceptive input for the body homeostasis, and in turn heighten subjective experience and magnitude of brain activity.

In the current study, we addressed whether visual cuing of death-related content (thus not requiring explicit reflection on the idea of death) could trigger effects on pain perception and brain activity recorded by means of electroencephalography (EEG) akin to those observed following explicit mortality salience induction (Valentini et al., 2014; Valentini et al., 2015). In fact, it is surprising that no study so far investigated whether images representing death-related content may induce prominent effects on brain activity compared to similar negative valence and arousing images but conveying a different

meaning. This hypothesis would be supported by the evidence that pictures can gain fast and automatic access to the activation of semantic representation than words (e.g. Carr et al., 1982), and are better remembered than words (e.g. Hockley, 2008).

Accordingly, pictures representing scenes with death-related meaning could modulate visual brain activity as well as activity associated with painful nociceptive stimuli. The rationale of using visual material rests on the effective role of affective pictures in triggering perceptual and emotional responses (e.g. Bradley and Lang, 2000; Codispoti et al., 2001). Indeed, there is large consensus on the effectiveness of standardised pictures databases (such as the International Affective Picture System, IAPS; Lang et al., 2008) to investigate subjective and cortical correlates of emotional states in healthy laboratory volunteers (Cuthbert et al., 2000; Hajcak et al., 2013). Only few studies thus far attempted to discriminate differential effects of other stimulus parameters, besides valence and arousal, such as picture content (e.g. Bernat et al., 2006; Rhudy et al., 2008). These studies revealed that some categories, such as pictures containing erotic and threatening information, exert a preferential modulation of participant's behaviour or physiological responses, due to their higher motivational relevance. However, to the best of our knowledge no study has investigated whether there is a specific modulation within the same dimension of valence. Therefore, we attempted to discriminate brain responses during the observation of affective pictures with negative valence but conveying different meaning.

We recorded EEG activity associated with different intensity of laser painful stimuli while healthy participants observed death- vs. other types of threat- related affective pictures (matched for valence and arousal ratings). In the conditioning phase, we established an association between death- or threat-related scenes (i.e. conditioned stimulus - CS) and high and low painful laser stimuli (i.e. unconditioned stimuli - US). In the subsequent testing phase, the observation of the same stimuli was associated with a

moderately painful stimulus that served as an index of any perceptual and neural criterion shift induced by the visual stimuli. Experimental designs based on associative learning and verbal suggestions of pain change have been largely adopted by scholars investigating placebo and nocebo phenomena (e.g. Colloca et al., 2008; Jensen et al., 2015). This approach was meant to disclose the modulation exerted by the image content within each level of the painful conditioned stimulation (high vs. low intensity) on subjective reports of pain, laser evoked potentials (LEPs) and nociceptive-related theta (3–8 Hz) oscillatory amplitude. Specifically, we expected to observe increased magnitude of the nociceptive-related variables for both low and high intensity of pain when painful stimuli were conditioned with death-related images. However, in the context of aversive unpleasant CS and US this effect would have been more markedly observed for the association “death image — low painful stimulus” because during the test phase, not only moderate pain trials would be associated with negative and arousing images, but they would also be on average more painful than expected. Accordingly, previous research showed that expectations for decreased pain, but not increased pain, affect perception (Atlas et al., 2010; Koyama et al., 2005).

Concerning visual-related brain responses during the observation of emotional pictures, we analysed the visual evoked potentials (VEPs) and event-related desynchronisation (ERD) in the alpha frequency band, time locked to the onset of the image. Previous research showed that VEPs are sensitive to both physical and semantic features of visual stimuli (Keil et al., 2002; Rozenkrants et al., 2008).

While some authors suggested that alpha ERD is a sensitive index of attentional/motivational modulation induced by affective pictures (De Cesarei and Codispoti, 2011), there is a surprisingly small number of studies on the effect of affective pictures on alpha ERD compared to the study of the more traditional VEPs (reviewed in Olofsson et al., 2008). Such shortage of information is particularly relevant in light of the

functional significance of oscillatory activity within the alpha frequency range (8–13 Hz). Modulation of cortical alpha oscillatory activity is meant to reflect inhibitory processes (Klimesch et al., 2007), and current consensus is that variations in alpha amplitude are closely associated with focused and anticipatory attention (Foxe and Snyder, 2011; Weisz et al., 2011; Klimesch, 2012).

We hypothesized that learning the association between images with death content and laser painful stimuli of different intensity could induce a greater perception of pain and increased amplitude of late LEPs compared to images with more generic threat content. Likewise, we hypothesized that late latency VEPs (particularly the late positive potential; LPP) would be increased in amplitude while EEG alpha oscillations would be further decreased (i.e. increase of ERD) following death-related scenes compared to non-death related, threatening scenes.

Materials and methods

Participants

Eighteen right-handed healthy subjects (9 females) between 20 and 34 years of age (mean±SD, 24.9±3.5) participated in the study. All had normal or corrected-to-normal vision and were naïve as to the purpose of the experiment. None had a history of neurological or psychiatric disease or conditions that could potentially interfere with pain sensitivity (e.g. drug intake or skin diseases). All gave written informed consent, were unaware about the purposes of the study and were fully debriefed about it at the end of the experiment. The experimental procedures were approved by the Fondazione Santa Lucia ethics committee and were in accordance with the standards of the Declaration of Helsinki.

Nociceptive and visual stimuli

The nociceptive heat stimuli were pulses generated by an infrared neodymium yttrium aluminium perovskite (Nd:YAP) laser with a wavelength of 1.34 μm (Electronical Engineering, Florence, Italy). Laser pulses, each lasting 4 ms, selectively and directly activate the Aδ and C-fiber nociceptive terminals located in the superficial layers of the skin (Cruccu et al., 2003). The laser beam was transmitted via an optic fiber and its diameter was set at approximately 6 mm (≈ 28 mm^2) by focusing lenses. Laser pulses were delivered on a square area (5x5 cm) defined on the left hand dorsum prior to the beginning of the experimental session. He-Ne laser indicated the area to be stimulated. To prevent increases in baseline skin temperature and fatigue or sensitization of the nociceptors, the position of the laser beam was changed after each pulse. An infrared thermometer (precision ±0.3 °C) was used to measure the temperature of the stimulated skin area before and during the experiment. The average temperature across subjects during the experiment was 34.3±0.7 °C. Participants were first familiarized with ten nociceptive stimuli of low-energy delivered to the right hand dorsum. The energy of the stimulus was then adjusted using the ascending and descending method of limits. Stimulus intensity was increased in steps of 0.5 Joules (J) starting from an energy value that is commonly perceived as a warm sensation in most volunteers until a pricking/burning painful sensation was reported. Then, intensity was decreased in 0.5 J steps until no painful sensation was reported any more. Next, the procedure was repeated with a second ascending and descending series of 0.25 J steps. The series were then narrowed around the most often reported pain threshold intensity until the participant associated the same stimulus intensity with a pain sensation 50±10% of times. Importantly, the experimental design implied the use of 3 stimulation energies that participants evaluated using a 101 point electronic visual analogue scale (VAS) ranging from 0 (no pain) to 100 (worst imaginable pain). Mean low (3.0±0.3 J) and high (3.9±0.4 J) conditioning energies elicited a sensation ranging from no pain to low pain (mean 19.25;

range 1–30) or moderate to high pain (mean 56.69; range 50–80) respectively. Moderate test energy (mean 3.5±0.4 J) elicited a sensation of moderate pain (mean 34.46; range 31–49).

Visual stimuli were extracted from the IAPS database (Lang et al., 2008). We first chose 22 images representing death-related and threat-related content and displaying no statistical difference in valence and arousal normative ratings. Then we submitted the selected images to a survey in which 88 respondents judged the images according to their power in evoking a sense of threat, brevity of life, fear of death, disgust, anxiety, sadness, anger and puzzlement on a 1 to 5 scale (“Not at all”, “Slightly”, “Moderately”, “Very”, “Extremely”). According to these preliminary results (see supplementary Table 1), we excluded 3 images with ratings of disgust over 3 from the final set of stimuli. We selected from the remaining stimuli, 4 images evoking high sense of brevity of life, and fear of death (Death, D) and 4 evoking high threat ratings but low death-related ratings (Threat, T). Both picture categories were not different in valence and arousal scores (supplementary Table 2). The E-Prime© software (Schneider et al., 2002) was used to control the onset/offset of both visual and nociceptive stimuli. The pictures were displayed on a 22 inch monitor, with a refresh rate of 60Hz and resolution of 800x600 pixels.

EEG Recording

EEG recordings were obtained by sixty tin electrodes (Electro-Cap International - ECI) placed according to the positions of the 10─20 International System. Two surface electrodes were positioned for the horizontal electro-oculographic (HEOG) recording. The reference was at the nose and the ground at AFz. Electrodes impedance was kept below 5 kΩ. The EEG signal was amplified and digitized at 1000 Hz.

Design and experimental procedure

After nociceptive stimuli calibration and EEG cap montage, five recording blocks were performed. During the first conditioning block (Fig. 1, left), the picture content (D or T) was paired with either low intensity eliciting low pain sensations (Low - L) or high intensity eliciting high pain sensations (High - H) in 64 trials. In the subsequent test block (Fig. 1, centre) D and T pictures used during the conditioning block were paired to a laser pulse with an intensity eliciting moderate (M) sensations of pain during the stimulus calibration phase (48 trials). The third extinction block consisting of 16 trials (Fig. 1, right) served to extinguish the association between picture content and pain intensity established in the first conditioning block (e.g., H intensity paired with D content while L intensity paired with T content). Following a second conditioning block where the association between picture content and pain intensity was reversed (e.g., H intensity paired with T content while L intensity paired with D content), a second test block was administered. The order of the associations was counterbalanced across participants. During the conditioning blocks only the 80% of the pictures were paired with a nociceptive stimulus whereas the remaining 20% were unpaired to prevent the participant's awareness of the conditioning procedure, and thus increase the effectiveness of the conditioning schedule (Lattal, 2010). During the extinction phase, the laser energy was set to moderate energy level (3.5±0.4 J; mean rating of pain 34.46) and the participant was instructed to provide ratings of pain only in trials were the laser stimulus was delivered. Each block lasted between 5 and 18 min (see Fig. 1), and there was a 5 min pause between blocks. Participants were comfortably seated in a temperature-controlled room (25 °C) with their hands resting on a table, ≈40 cm from the body midline and ≈60 cm from the computer monitor. A wooden frame blocked the sight of their left arm and the laser device. Participants were asked to relax and fixate the centre of the computer screen placed in front of them. The background of the computer screen was black throughout the experiment. Each trial (Fig. 1, B) started with two fixation crosses with the first (white) indicating trial onset (2 s), and the second

(yellow) preceding visual and nociceptive stimuli (variable between 5 and 8 s). While the onset of the moderate laser pulse was jittered randomly after the onset of the image (0.5 and 3 s) in the conditioning and test block, it occurred randomly (0.5 and 2 s) during the white fixation cross in the extinction block. Three seconds after the end of the image participants were asked to rate pain intensity by moving a mouse with the right hand and positioning a pointer on the electronic VAS, within 15 s from its appearance on the screen.

Data Analysis

Psychophysics

Ratings collected in the test session in response to moderate painful stimuli were submitted to full factorial analysis of variance (ANOVA) to analyse the effect of the relationship between 'image content' (D, T) and 'pain intensity association' (H, L) established in the conditioning phase. Planned t-test comparisons were also computed to directly test the relationship between the two levels of 'image content' within each level of 'pain intensity association'. Statistical analyses were performed using IBM SPSS 21. The level of significance was set at $P < 0.05$.

EEG pre-processing

The continuous EEG data were pre-processed with EEGLAB (Delorme and Makeig, 2004). Single participant data belonging to the test blocks were merged in a unique file and the power line-related sinusoidal artefacts (50-100 Hz) were removed using Cleanline (http://www.nitrc.org/projects/cleanline). Data were then band-pass filtered from 0.1 to 100 Hz (filter order 2) and re-sampled at 250 Hz. Data were segmented into epochs using a time window ranging from 1 s before to 3 s after the stimulus (total epoch duration: 4 s) and baseline corrected using the mean of the entire epoch (Groppe et al., 2011). Epoched data were merged and further processed using independent component analysis (ICA;

Jung et al., 2000) to subtract EOG and muscle-related artefacts, aided by the semi-automatic approach offered by Adjust (Mognon et al., 2011). Data resulting from the ICA were re-referenced to the average of all electrodes and segmented again into 3 s epochs for each sensory modality separately and baseline corrected using 0.5 s before the stimulus onset (-1 to 2 s).

Laser evoked potentials and theta oscillatory activity

Epochs belonging to the same experimental condition (DH, TH, DL, TL) were averaged and time-locked to the onset of the nociceptive stimulus. This yielded four average waveforms, one for each experimental condition. Post-hoc comparisons were specified according to planned contrasts between DH and TH or DL and TL conditions. The N1 laser evoked potential (LEP) was measured at the central electrodes contralateral to the stimulated side (C4) referenced to Fz (Hu et al., 2010). It was defined as the negative deflection preceding the N2 wave, which appears as a positive deflection in this montage. The N2 and P2 waves were measured at the vertex (Cz) referenced to the common average. The N2 wave was defined as the most negative deflection after stimulus onset. The P2 wave was defined as the most positive deflection after stimulus onset.

Time-frequency decomposition parameters were set to capitalise on the representation of the theta frequency range (3-8 Hz). We computed a Morlet wavelet in which the initial spread of the Gaussian envelope was set at 0.15 and the central frequency of the wavelet at 3 Hz. The transform expressed the oscillation amplitude as a function of time and frequency, regardless of its phase (Hramov et al., 2015). Theta event-related amplitude was sampled from the spectrograms obtained at the Cz electrode where this response is maximally expressed. For each estimated frequency, results were displayed as an event-related increase or decrease in oscillation amplitude relative to a pre-stimulus reference interval (-0.6 to -0.2 s before the onset of the laser stimulus).

We computed a whole-waveform ANOVA and t-test with correction for multiple comparisons, by means of the cluster-level randomization (Maris and Oostenveld, 2007) to identify differences between amplitudes across the experimental conditions. Cluster-level randomization allowed us to control the Type-1 error rate involving multiple comparisons and was carried out as follows. We firstly selected the points represented by F/t values lower than $P<0.05$ to identify the groups (clusters) of contiguous points that show a significant effect. An estimate of the magnitude of each cluster was then obtained by calculating the sum of the F/t values comprising each cluster. Afterwards, random permutation (1000 times) of the differences between data-points/pixels at the cluster level within each individual was used to obtain a reference distribution. This distribution is obtained by randomly swapping the conditions within participant and calculating the maximum cluster level test statistic. For each cluster a threshold of significance is found around the value $Z>2$ standard deviations from the mean. Then, for each cluster, a value corresponding to F/t and P (two tailed) was obtained and the statistical significance ascribed only to differences lower than 0.05 at the end of the permutation process.

Visual evoked potentials and alpha event-related desynchronisation

The aim of the analysis of visual-related responses was to identify the brain activity associated with the processing of the two classes of visual stimuli (D and T) regardless of the conditioned painful stimulus. This yielded two average signals, one for each experimental condition. Both VEPs and alpha ERD were averaged according to a region of Interest (ROI) at the level of the parietal and occipital electrodes (P1, P2, P3, P4, Pz, POz, PO3, PO4, PO7, PO8, O1 and O2).

Time-frequency decomposition parameters were set to capitalize on the representation of the alpha frequency range (8-13 Hz). We computed a Morlet wavelet in which the initial spread of the Gaussian envelope was set at 0.15 and the central frequency of the

wavelet at 9 Hz. For each estimated frequency, results were displayed as an event-related change relative to the average amplitude of a reference interval prior to the onset of the visual stimulus (-0.6 to -0.2 s). For both VEPs and alpha ERD we computed t-test with correction for multiple comparisons on the oscillatory activity recorded during D and T conditions, according to the approach described in the previous paragraph (Maris and Oostenveld, 2007).

Results

Pain ratings

All nociceptive stimuli were detected by participants and perceived as painful. We found no main effect of 'pain intensity association' (F_{68}=0.02; p=0.89) as well as no effect of 'image content' (F_{68}=0.004; p=0.95). The interaction between 'pain intensity association' and 'image content' was not significant (F_{68}=0.28; p=0.60). Planned t-test comparisons confirmed that mean differences between the two types of pictures for each 'pain intensity association' condition (H, L) were not significant (DH vs. TH: t_{17}=0.51; p=0.62; DL vs. TL: t_{17}=-0.59; p=0.56). Ratings in the four conditions are represented in Fig. 2.

LEPs and theta event-related oscillations

Grand average waveforms and topographies of main N2-P2 LEPs during the test phase are shown in Fig. 3. The moderate intensity nociceptive stimuli delivered during the test phase elicited maximal N2 and P2 waves at the scalp vertex (electrode Cz). Although there was no main effect of 'pain intensity association' and 'image content' on N1, N2, P2 LEPs amplitude, ANOVA revealed a significant interaction (F_{17}=12.21; P_{corr}=0.003) within 344-470 ms post-stimulus interval (left graph), compatible with the time course of the ascending part of the P2 wave. Post-hoc t-tests revealed that the interaction was driven by a greater LEPs amplitude for death-related compared to threat-related pictures when the

stimulus intensity was low during the conditioning session (t_{17}=3.90; P_{corr}=0.001). The time course of the effect was consistent with the time interval identified by the ANOVA (335-484 ms, right graph).

Grand average spectrograms and topographies of the oscillatory activity measured at Cz electrode are reported in Fig. 4 (left panel). The ANOVA revealed a main effect of 'pain intensity association' (F_{17}=12.80; P_{corr}=0.002) and 'image content' (F_{17}=11.29; P_{corr}=0.005), but no interaction (F_{17}=4.85; P_{corr}=0.51). The main effect of 'pain intensity association' was accounted for by greater theta magnitude for images previously associated with high painful stimuli while the effect of 'image content' was accounted for by greater theta magnitude during observation of death-related pictures (Fig. 4, top right panel).

VEPs and event-related alpha oscillations

Grand average waveforms of VEPs during the test phase are shown in Fig. 5. Whole waveform t-test of the parietal-occipital signal revealed no difference between death- and threat-related pictures (Fig. 5 lower panel; max t value = -2.40 at 316 ms).

Grand average spectrograms of the oscillatory activity measured at the level of the parietal-occipital ROI, and topographies of significant modulations observed during the two image conditions (death and threat) are displayed in Fig. 6 (left panel). Differences were accounted for by a significant increase in alpha ERD (t_{17}=-4.80; P_{corr}=0.0001) during death- than threat-related images (right panel). The difference showed a regional peak at the left hemisphere (300 ms, 11 Hz, P3 electrode).

Discussion

We employed a classic conditioning procedure (Fig. 1) to assess whether the passive observation of images depicting either death- or threat-related scenes differentially affects

subjective reports of pain and the amplitude of nociceptive LEPs. In addition, we investigated whether the two image categories differentially influenced both VEPs and a well-known marker of visual excitability, i.e. alpha-band desynchronisation. Based on the assumption that during the conditioning phase more attentional resources are recruited by images representing death content compared to equally unpleasant and arousing but death-unrelated images, we predicted higher pain ratings, greater LEPs and alpha ERD magnitude in the test phase.

Results show that visual reminders of death did not bring about a significant increase in pain ratings (Fig. 2) but did induce increased amplitude of nociceptive evoked potentials particularly when these images were coupled with low painful stimuli in the conditioning phase (Fig. 3, right). Moreover, we found greater nociceptive-related theta synchronisation during visual reminders of death (Fig. 4). Similarly, we found greater alpha desynchronisation following death- than threat-related pictures over parieto-occipital electrodes (Fig. 6). However, this finding was not replicated by the analysis of VEPs amplitude (Fig. 5).

Previous research showed that pleasant pictures can reliably inhibit pain and nociception whereas unpleasant pictures enhance pain and nociception, through a descending central modulation of spinal reflexes (Rhudy et al., 2007; Rhudy et al., 2008). The lack of modulation of the subjective experience of pain may seem at odds with this evidence and with our previous studies (Valentini et al., 2014; 2015). The lack of a conditioning effect on the painful experience may be explained by a competition between visual reminders of death and painful stimuli for attentional and motivational resources. The change of nociceptive intensity between the conditioning (high and low) and the test (moderate) may have caused larger habituation to the visual stimuli, which were repeated at the single item level between the two blocks. Therefore, it may well be that painful laser stimuli acquired greater salience and motivational relevance compared to the images

displayed on the screen in the test phase. This interpretation would explain why differences between the two conditions did single out at the implicit processing level (i.e. EEG) and not at the explicit experiential level (i.e. psychophysics). Indeed, independently from the type of images delivered in the conditioning phase, participants were expecting either low or high intensity in the test phase. However, they rather received a moderate stimulus that violated their expectancy. Such violation likely determined the activation of compensatory elaboration that led to overestimation or underestimation of the moderate stimuli depending on the previous conditioned association. As clearly shown in Fig. 2, participants tended to feel a similar amount of pain in the test phase for death and threat images associated with either low or high painful stimuli in the conditioning phase. Although subjective ratings did not reveal increased perception of pain for the violation of the expectation of lower pain (i.e. low pain conditioning) as suggested by previous studies (Atlas et al., 2010; Koyama et al., 2005), we observed a pattern of EEG nociceptive-related responses that indeed hints at neural modulations contingent upon pain processing.

A methodological difference in the procedure used to elicit pain may have contributed to bring about the inconsistency between the present and our previous studies. We previously adopted a repetition suppression design and tested the mortality salience effect on the second stimulus out of each pair of stimuli without any other co-occurring sensory stimulus (Valentini et al., 2014; 2015). Here we used a multisensory context in which single painful stimuli were coupled to each experimental visual condition.

A more likely explanation for the perceptual discrepancy between studies concerns the impersonal connotation of the visual stimuli. Indeed, all the death-related (as well as threat-related) images did not convey cues about the participants' own mortality, unlike the classical mortality salience procedure (Rosenblatt et al., 1989). It is thus possible that the mere exposure to affective images used in the present study may not trigger the same

explicit contemplative processes activated by the mortality salience manipulation used in our previous research. In this regard, there seems to be metanalytic support to the notion that longer delays between reminders of death and the measurement of the dependent variable are crucial in increasing the effect-size of the distal effects (Martens et al., 2011), particularly when compared to meaning and certainty threats (Steinman and Updegraff, 2015).

And yet, recent studies showed that even such impersonal cues can trigger a specific modulation of brain activity, regardless of whether this may have been interpreted as reflecting the effects of proximal or distal defences (e.g. Han et al., 2010; Klackl et al., 2013). In line with these findings, our participants showed increased LEP P2 amplitude during the observation of death-related images previously associated with low painful nociceptive stimuli (Fig. 3, right). This heightened amplitude may be triggered by the interaction between the specific attentional-motivational modulation induced by death cues and the violated expectation of low painful nociceptive stimuli associated with the moderate painful test stimuli.

Studies show that novelty (Legrain et al., 2003; Legrain et al., 2009; Valentini et al., 2011) and salience (Ronga et al., 2013) of nociceptive stimulation are major determinants of the magnitude of nociceptive event-related potentials. However, these responses can also reflect top-down attentional and emotional modulations acting on the contextual relevance of the nociceptive representation, and associated with the meaning attached to the sensory event (e.g. Valentini et al., 2013). Importantly, the current study was not designed to disentangle the precise role of bottom-up and top-down attentional modulations on P2 LEPs (e.g. Legrain et al., 2002) but rather to identify the broader attentional engagement of multimodal brain structures involved in the detection of bodily threatening stimuli (Legrain et al., 2011). Interestingly, previous research suggests that at least some neural generators of the P2 wave (namely, mid-anterior cingulate cortex,

primary and supplementary motor areas) are involved in motor response preparation and selection during elaboration of salient nociceptive stimuli (Legrain et al., 2012), thus suggesting a preferential sensitivity of this evoked response for cognitive and affective modulation, such as the one involved in our study.

Theta oscillations revealed a general increase in magnitude in response to moderate painful test stimuli during observation of death-related images, regardless of whether these images were previously associated with low or high painful stimuli. The variability across the four different conditions may account for the absence of interaction, even though a trend similar to that observed for the P2 wave can be noticed from the data distribution (Fig. 4, right). Related to this issue, one can note that, as we previously discussed (Valentini et al., 2014), the theta oscillatory activity mostly reflects synchronised activity which only partially represents specific time-locked nociceptive evoked potentials (particularly the N2 and P2 waves).

The increase of alpha band desynchronisation observed at the parieto-occipital region further supports the notion of a greater attentional-motivational activation associated with death-related images. Current consensus is that alpha ERD reflects cortical activation associated with suppression of task-irrelevant neuronal processing and increased attentional focus on task relevant events (Klimesch, 2012 for a review). We argue that the alpha ERD modulation exerted by death-related images may be explained either by i) higher attentional load allocated as result of a motivational modulation exerted by the more meaningful content of the death-related pictures or by ii) an active attempt to disengage attention from the affective content of the image. Our findings are compatible with other studies investigating the effect of affective pictures observation on alpha band activity using EEG (Uusberg et al., 2013). In the same vein, Onoda et al. (2007) investigated event-related power changes of alpha activity during observation of affective images taken from the IAPS database using magnetoencephalography. The authors found

augmented alpha ERD during the period anticipating the negative but not the positive images, an effect that was maximal at the occipital region. One of the most well-established finding in the literature on affective picture processing is that emotionally arousing (pleasant and unpleasant) pictures elicit larger magnitude of electrical (Cuthbert et al., 2000) and hemodynamic brain activity (Lane et al., 1999) than neutral pictures. This finding may reflect the engagement of attentional resources by emotional stimuli. We expand on previous research by showing that different meaning/content of images with comparable or even same valence (namely, unpleasant negative valence pictures) can exert divergent modulation of an important index of visual attentional processing such as alpha ERD.

It is worth noting that, as already suggested by other authors, LPP and alpha ERD may rely on different brain mechanisms and index different processes (Ferrari et al., 2015; Uusberg et al., 2013). The effect we observed in the alpha band was comprised in an earlier latency (i.e. 200-400 ms post image onset; peak at 300 ms) which would be more consistent with the latency of a late P3 potential (350-600 ms) than a slow LPP (600-800 ms post-stimulus). Yet, even if non-significant, topographical representation of VEPs shows greater amplitude during observation of threatening images at late but not at very late latencies (Fig. 5, top). This relationship seems to vanish during the long lasting LPP which is meant to provide a sensitive measure of effects associated with affective pictures processing (reviewed in Olofsson et al., 2008).

Concerning the specific characterisation of the emotional states purportedly triggered by the two different categories of images we are aware that different specific negative emotions may be activated by the two sets. Indeed the whole threat category of the IAPS database included pictures that represent attack, violence, food contamination, and dangerous animals, that may result in emotions of fear, anger, or disgust (Bradley et al., 2001). Conversely, the death category was less variable and included images that can

trigger fear, disgust, and sadness. This is the reason why we (i) have matched all the images for valence and arousal, (ii) avoided attack images that were facing directly the observer (and thus being possibly interpreted as a direct threat to the onlooker), (iii) ensured that the death-related pictures were rated significantly higher in terms of sense of brevity of life, and (iv) ensured that there was no difference in reported disgust between the two categories. While the two categories of stimuli might have induced different discrete or blended emotions, we tend to interpret our results on the basis of the meaning conveyed by the two types of images. The enhanced brain activation associated with death-related content would be in agreement with previous research carried out by TMT scholars. Arndt et al. (1997) reported that subliminal exposure to the word *death* (compared to the negative control word *pain*) led to more positive evaluations of people who praised participants' cultural worldview and more negative evaluations of those who challenged it. In a subsequent study, the same authors reported an increase of facial electromyography when the word *death* was primed subliminally, while leaving conscious experience of negative affect unaltered (Arndt et al., 2001). These findings were interpreted as indicating that unconscious emotional processes are involved during the subliminal elaboration of death-related information. In other words, these effects were interpreted as an index of distal defences according to the dual process model put forth by TMT (Greenberg et al., 2000). More recent research reported an increase of the late positive potential during supraliminal observation of death-related relative to unpleasant death-unrelated words (Klackl et al., 2013). These neural effects were interpreted as an index of proximal defences. However, we recently demonstrated that both self-report measures of affect and self-esteem personality trait correlate with EEG measures (Valentini et al., 2015), thus supporting the notion that neural activity may reflect both ongoing proximal and distal defence processes.

Altogether, our findings suggest a preferential cortical processing of images representing death content compared to images depicting threatening scenes that do not directly involve death-related content. Using visual reminders of death allowed us to extend the study of the neural correlates of processing death related stimuli beyond the classical induction of mortality salience mind-set, thus paving the way to new paradigms based on more implicit processing of visual content such as subliminal priming, continuous flash suppression, or fast periodic rate stimulation. Future studies may be able to capture parallel antagonistic processes of inhibition and excitation coupled with the different meaning of the images that could account for effects associated with more general threat processing and more specific death-related processing. Accounting for both general and specific aspects of threat and death cognition will foster the integration of biological theories of existential threat (Tritt et al., 2012) with the most recent version of TMT (Pyszczynski et al., 2015).

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Conflict of Interest

None declared.

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Figure captions

Fig. 1. Experimental design. Electroencephalography and ratings of pain were recorded during 3 phases. During the preliminary conditioning phase (left), single nociceptive laser pulses were delivered to the left hand dorsum using either low intensity eliciting low pain sensations (Low, blue) or high intensity eliciting high pain sensations (High, red). The two stimulus intensities were coupled to either death- or threat-related images (bottom left). Low and high energy stimuli were paired to both D and T stimuli in separate blocks and the order of the pairing was counterbalanced across participants. Conversely, during the test phase (center), the laser energy was set to moderate intensity that elicited moderate sensations of pain regardless of the image content. In both the conditioning and test block, each trial started with a white fixation cross displayed on a black screen for 2 seconds, followed by a second yellow fixation cross (variable range: 5-8 s). The onset of the laser pulse was jittered with respect to the onset of the image. During the extinction phase, the laser energy was set again to moderate intensity and the participant was instructed to provide ratings of pain only in trials were the laser stimulus was delivered (right). Here the laser stimulus was delivered before the observation of the image and only in few trials. At the image offset participants were asked to rate pain intensity using a digital visual analogue scale (VAS). The within-subject design allowed us to estimate the modulatory effect of image content on electroencephalographic activity and the experience of pain.

Fig. 2. Box-plots of pain ratings (y axis) during the four different conditions (x axis) in the test block. Median is depicted in red, 25^{th} and 75^{th} percentiles in blue, and whiskers representing extreme values are in black. There was no difference in pain ratings following moderate nociceptive laser pulses with death-related previously associated either with

high or low nociceptive energy (DH and DL) compared to threat-related images previously associated either with high or low nociceptive energy (TH and TL).

Fig. 3. Grand average waveforms and scalp topographies of the N2-P2 LEPs recorded at the vertex electrode (Cz, white disk) during the test phase. The ANOVA with corrections for multiple comparisons revealed a significant interaction between 'pain intensity association' and 'image content' (left graph) in the 350-480 ms post-stimulus interval, a time-course compatible with the P2 wave and its ascending positivity. Post hoc t-tests revealed that the interaction is explained by greater positive amplitude during the observation of death-related compared to threat-related pictures previously associated to low intensity painful stimuli in the conditioning phase. Topographies of the activity within the significant range are displayed in the insets.

Fig. 4. A. Grand average spectrograms and scalp topographies of the theta oscillatory magnitude recorded at the vertex electrode (Cz, white disk) during the test phase in the four different conditions. B. Box-plots of peak amplitude (y axis) during the four different conditions (x axis) in the test block. Median is depicted in red, 25th and 75th percentiles in blue, and whiskers representing extreme values are in black. Topographies show the significantly greater increase of theta synchronisation during observation of death- than threat-related images regardless of the pain intensity previously associated with each condition (bottom).

Fig. 5. VEPs recorded at the parietal-occipital ROI during the test phase. T-test did not detect an effect of 'image content' (bottom). Single participant average (middle) and grand

average (top) waveforms display the variability and central tendency of the VEPs in the two experimental conditions. Scalp topographies represent the maximal activity in the late (350-600 ms) and very-late (600-800 ms) latency range post-stimulus interval.

Fig. 6. The effect of image content on grand average time-frequency representation of oscillatory activity at the parietal-occipital ROI during the test phase (left). Significant amplitude differences were detected in the alpha band (8-13 Hz) and were accounted for by a significantly greater increase of alpha desynchronization during death- than threat-related images (top left). T-test revealed that the difference was maximal in the 200-400 ms post image onset and on the left hemisphere (top right, inset; peak at 300 ms, 11 Hz, P3 electrode).