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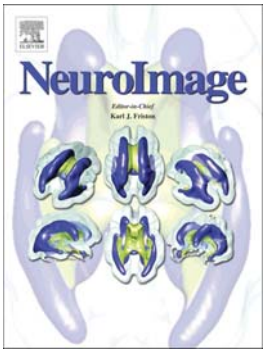

Evidence of trace conditioning in comatose patients revealed by the reactivation of EEG responses to alerting sounds

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TITLE PAGE

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ABSTRACT

Trace conditioning refers to a learning process occurring after repeated presentation of a neutral conditioned stimulus (CS+) and a salient unconditioned stimulus (UCS) separated by a temporal gap. Recent studies have reported that trace conditioning can occur in humans in reduced levels of consciousness by showing a transfer of the unconditioned autonomic response to the CS+ in healthy sleeping individuals and in vegetative state patients. However no previous studies have investigated the neural underpinning of trace conditioning in the absence of consciousness in humans.

In the present study, we recorded the EEG activity of 29 post-anoxic comatose patients while presenting a trace conditioning paradigm using neutral tones as CS+ and alerting sounds as UCS. Most patients received therapeutic hypothermia and all were deeply unconscious according to standardized clinical scales. After repeated presentation of the CS+ and UCS couple, learning was assessed by measuring the EEG activity during the period where the UCS is omitted after CS+ presentation. Specifically we assessed the 'reactivation' of the neural response to UCS omission by applying a decoding algorithm derived from the statistical model of the EEG activity in response to the UCS presentation. The same procedure was used in a group of 12 awake healthy controls.

We found a reactivation of the UCS response in absence of stimulation in eight patients (five under therapeutic hypothermia) and four healthy controls. Additionally, the reactivation effect was temporally specific within trials since it manifested primarily at the specific latency of UCS presentation and significantly less before or after this period. Our results show for the first time that trace conditioning may manifest as a reactivation of the EEG activity related to the UCS and even in the absence of consciousness.

Keywords: learning, trace conditioning, coma, consciousness, EEG, multivariate analysis, auditory stimuli.

Highlights:

- Evidence of trace conditioning can be found in comatose patients
- Learning an association can manifest as a reactivation of the EEG activity
- This association is temporally specific
- Healthy controls can also show the same reactivation effect
- Trace conditioning may not require consciousness

1. INTRODUCTION

Trace conditioning refers to the ability of learning the association between a neutral conditioned stimulus and a salient unconditioned stimulus when they are separated by a temporal gap. This learning phenomenon is of paramount importance for ensuring species survival, avoiding danger and optimizing reward (Bekinschtein et al., 2011).

The investigation around the minimal neural resources underlying trace conditioning in humans has led to controversial results. In particular, it remains elusive whether the awareness of the contingency between the conditioned and unconditioned stimulus is a necessary condition for such a learning phenomenon to occur and last over time. On one side, in awake healthy individuals, the ability of the human brain of creating a trace of temporally separated sensory stimuli has shown a strong dependency upon subjects' awareness of the contingency (Carter et al., 2003; Christian and Thompson, 2003; Clark and Squire, 1998). On the other hand, recent studies provided evidence that trace conditioning in humans can occur under reduced consciousness level: in some vegetative patients, repetitions of a sequence of neutral tones and air puffs triggered an anticipatory eye-blink response after the tone (Bekinschtein et al., 2009b); a repeated presentation of neutral tones followed either by pleasant or unpleasant odors elicited a differential sniff response after the presentation of the sound alone in healthy individuals during deep sleep (Arzi et al., 2012). However consciousness level assessment remains uncertain in these previous studies: diagnosis of disorders of consciousness (i.e. vegetative or minimally conscious state patients) is affected by a considerable degree of misclassification (Godbolt et al., 2012; Schnakers et al., 2009), whereas consciousness level in healthy individuals during sleep as assessed by the global sleep scoring (Iber et al., 2007) is not sensitive to the presence of local

wake activity (Dang-Vu et al., 2008; Le Van Quyen et al., 2010), which might provide critical information on the consciousness depth during sleep (see discussion in Arzi et al. 2012). In addition, in anesthetized individuals, trace conditioning has not produced any significant result (Bekinschtein et al., 2009b). Taken together, it remains open whether trace conditioning can be observed in the absence of consciousness. Furthermore, to the best of our knowledge, no studies have investigated the neural correlates of trace conditioning in altered state of consciousness (see also Kim et al. 2012; Lancioni et al. 2014 for learning assessment based on behavioral and autonomic responses).

Here we aim at testing the occurrence of trace conditioning based on comatose patients' EEG activity using an auditory protocol. Specifically, we used a differential trace conditioning paradigm with auditory stimuli presenting CS+ coupled with UCS on the majority of the trial and CS+ uncoupled on intermixed unexpected trials. Importantly the series comprised a control condition with a tone that was never associated to UCS, i.e. CS-. During the presentation of the paradigm, we recorded EEG activity of post-anoxic comatose patients. All of them were deeply unconscious based on standardized clinical tests and some of them under sedation and therapeutic hypothermia (TH) treatment (Bernard et al., 2002; The hypothermia after cardiac arrest study group, 2002). We assessed trace conditioning by applying a single-trial decoding analysis testing the reactivation of the neural activity associated by the UCS when the UCS is expected but not presented after CS+ (Chouiter et al., 2015). In order to obtain reference performances in awake conditions, the same analyses were performed on a control group of healthy participants. Investigating the neural correlates of trace conditioning overcomes the difficulty of measuring autonomic responses to the conditioned stimulus in a deep unconscious state. Moreover, the use of EEG allows assessing the specificity of the association by comparing

two different levels of expectation states, i.e. after CS+ and after CS-. The high EEG temporal resolution provides insight about the temporal specificity of the reactivation effect within trial. Finally, by testing the occurrence of the reactivation phenomenon along the duration of the experiment, we were able to unravel the appearance and persistence of the learning effect over time.

2. METHODS

2.1. Population

2.1.1. Comatose patients

We included data from 29 comatose patients in acute anoxic-ischemic coma (Glasgow Coma Scale < 6 at hospital admission; Teasdale & Jennett, 1974) following cardiac arrest, most of them from cardiac etiology (25/29; 86%). All patients were admitted to the Intensive Care Unit between March and October 2014 (5 women; mean age: 68 ± 2). Twenty-one were recruited from Lausanne University Hospital and 8 from Valais Hospital in Sion. Signed consent was obtained from patients' families according to a protocol approved by the Vaud and the Valais Ethics Committee.

Among these patients, 20 (2 women; mean age: 66 ± 2) were treated with a standard cooling procedure using therapeutic hypothermia at 33-34°C. At Lausanne University Hospital, this procedure was applied for the first 24 hours after coma onset through ice packs, intravenous ice-cold saline fluids and the Arctic Sun® system surface cooling device (Medivance, Louisville, CO, USA). Midazolam (0.1 mg/kg/h) and fentanyl (1.5 µg/kg/h) are given for sedation-analgesia and vecuronium (0.1 mg/kg boluses) in case of shivering. At Valais Hospital, hypothermia was maintained for 18 hours using the Coolgrad® intravascular cooling device (Zoll, Chelmsford, MA, USA), sedation was applied with disoprivan (2mg/kg/h) and fentanyl (50 µg/h); and norcuron (0.1 mg/kg boluses) was given in case of shivering. Patients suffering myoclonus or epileptic seizures received intravenous, nonsedating anti-epileptic treatment (valproate, levetiracetam). Return to normal temperature (normothermia; NT) after TH was controlled at a rate of 0.5°C increase per hour until 37°C and achieved within 8 hours. The remaining 9 patients

(3 women; mean age: 73 ± 4) did not receive TH protocol for clinical reasons and were treated only with sedative drugs if needed.

Patients were recorded at one or several occasions in the course of their coma, depending on their clinical evolution and the EEG machine's availability. Hypothermic conditions occurred only on the first day of coma, while normothermic conditions happened on the first day in patients not treated with therapeutic hypothermia and for all patients from the second day on. A total of 43 recordings were performed, 19 during TH and 24 in NT (7 recordings on the first day, 13 on the second day, and 4 later). Twelve patients were recorded twice and one patient was recorded three times. During recordings, all patients were intubated and with eyes closed. At the moment of the recording, body temperature was collected and consciousness was assessed using the FOUR score (Wijdicks et al., 2005).

Decision to withdraw intensive care was discussed within 7 days after coma onset, based on the occurrence of specific clinical variables strongly associated with poor outcome (Rossetti et al., 2010a). Patients' outcome was assessed at 3 months in terms of survival (alive vs. deceased).

2.1.2. Healthy controls

We recorded EEG data from 12 healthy control participants (6 women; mean age: 26 ± 1 years) without history of neurological, psychiatric or hearing disease. The study obtained full approval from the Vaud Ethics Committee and subjects gave their informed consent to participate. Participants were installed on a comfortable chair in a quiet dimly lit room. During the presentation of the auditory trace conditioning paradigm, they were asked to relax and to fixate a point without paying attention to the auditory stimuli while recording EEG. At the end of the experiment, the explicit awareness of the association between CS+ and UCS was evaluated by

asking the participants whether they noticed any specific sequence in the presented stimuli. Identical experimental procedure and statistical analyses were performed for both patients and controls.

2.2.Stimuli

Two neutral tones of 100 ms duration, 16-bit stereo 44100 Hz digitization were used as CS+ and CS- respectively at 700 Hz and 900 Hz frequency. A linear envelope of 5 ms increase time at the beginning of the tone and 5 ms decay time at the end of the tone was applied to minimize clicks. Tones were further normalized according to the root mean square of their amplitude.

As UCS, we used three alerting sounds selected on the ratings of an independent group of healthy participants (see Supplementary material). Each of these three sounds was randomly chosen as UCS along the experiment to prevent the occurrence of an habituation.

2.3.Procedure and task

Three types of trials were presented randomly (Figure 1). Reinforced trials refer to the presentation of the CS+ followed by the UCS after a fixed inter-stimulus interval (ISI) of 800 ms (Figure 1, panel A). Non-reinforced trials designate the presentation of the CS+ without UCS; in these trials, the CS+ is followed by a silence of the same duration as the UCS (conditioned silence) after the same fixed ISI as in reinforced trials (Figure 1, panel B). Unpaired trials present the CS- alone and followed by a silence of the same duration as the UCS (neutral silence) after the same fixed ISI (Figure 1, panel C). Inter-trial interval (ITI) varies randomly between 1 and 2 seconds with steps of 0.1 seconds. In summary, our trace conditioning paradigm combined principles of differential conditioning by using two CS (CS+ and CS-), with a partial

reinforcement procedure by presenting CS+ in association with USC in 67% of trials comprising CS+ (reinforced trials) while CS+ alone occurred in 33% (non-reinforced trials).

The same sequence was used in four conditioning blocks with ten additional reinforced trials presented consecutively at the beginning of the first block in order to create the association. Presentation order was the same for all participants. A total of 206 reinforced trials, 100 non-reinforced trials and 296 unpaired trials were presented, such as to have a similar number of CS+ (in reinforced and non-reinforced trials) and CS- presentation (unpaired trials). Each block lasted approximately 8.2 min for a total experiment time of 33 minutes. Auditory sequences were presented at 90 dB using specialized ER4 Etymotic earphones (Etymotic Research, Inc.) while recording 19 channels EEG.

2.4.EEG acquisition and pre-processing

EEG recordings were performed using a 19 electrodes montage following the international 10-20 system (Viasys Neurocare, Madison, WI, USA) with a sampling rate of 1024 Hz and an online reference to the Fpz electrode.

Peri-stimulus epochs were extracted for the duration of the UCS, conditioned silence and neutral silence (900 to 1800 ms latency as in Figure 1 panel A) with a 100 ms baseline before onset (total epoch duration: 1000 ms). An artifact rejection criterion of ±100 μV was applied offline at all 19 electrodes. Data were re-referenced offline to the common average reference and were 0.18–40 Hz band-pass filtered and 50 Hz notch filtered.

2.5.Univariate statistical analysis of the EEG during sound omission

In this first analysis, we included preprocessed EEG trials extracted along the conditioned and neutral silence. Epochs were 1000 ms long, starting at 100 ms before the onset of each of these silence periods, as defined in Figure 1 (panels B and C). For each individual, we considered the minimum number of artifact-free trials between the two conditions. We carried out a cluster permutation test corrected for multiple comparisons at the single-patient and single-subject level ($p<0.05$) as described elsewhere (Maris and Oostenveld, 2007).

2.6.Multivariate decoding of single-trial EEG

We used a single-trial topographic analysis (STTA; Tzovara et al., 2012) to analyze EEG responses to sounds and the EEG activity in the absence of physical stimuli. This method is based on extracting time periods and prototypical voltage topographies discriminating most accurately between two conditions (Tzovara, Murray, et al. 2012). In this specific setting applied to comatose patients, the STTA has the advantage of being free of *a priori* hypotheses about the electrode location where a stimulus-related activity would be expected, and is independent of any inclusion criteria aside from having sufficient artifact-free trials. Moreover, because this method is based on voltage topographies, we can interpret an accurate performance as the result of the activation of different underlying neural generators between experimental conditions (Tzovara, Murray, et al. 2012).

The STTA is based on estimating the voltage topographies of the single-trial EEG by a Mixture of Gaussians model. One part of the dataset is kept aside from the analysis for final validation of the decoding performance (validation dataset). The rest of the dataset is divided in *n* splits and used to train the decoding algorithm on *n*-1 data splits and test on the remaining one. The training and testing is repeated *n* times in a way that the decoding is always evaluated on *n* non

overlapping test datasets. According to the maximum number of available artifacts-free trials for each condition, the value of *n* was 8 when 80 trials could be used for the training dataset and 7 when fewer trials were available. This procedure is repeated for several parameters values (i.e. number of Gaussians in the mixture) in the model in order to select the values maximizing the decoding performance. The final decoding value is computed on the validation dataset using trials that were never used either for training the model or for the model parameters selection. All values reported below refer to the validation datasets.

The decoding performance is measured as the area under the Receiver Operating Characteristic curve (AUC; Green & Swets, 1966). The significance of the decoding performance is then assessed by comparing its value to the chance level computed by randomly permuting the labels of the single trials used for training the algorithm and recomputing the mixture of Gaussians models 200 times for each recording. The AUC on the validation dataset was compared to the distribution of the AUC values based on these random permutations (Wilcoxon signed-rank test, $p < 0.001$).

2.6.1. Occurrence and specificity of the reactivation effect

We performed the EEG analysis in three steps.

1. *Auditory discrimination*. The first step identified recordings showing evidence of preserved auditory processing, i.e. showing significant auditory discrimination between sound and silence. Specifically, we trained the decoding algorithm to distinguish between the EEG activity elicited by the UCS vs. the EEG activity elicited by the neutral silence (Figure 1, panels A and C). The significance was assessed on a separate validation dataset (see previous section). Decoding results obtained at this stage provided the statistical models to be used in the second step.

2. *Reactivation effect*. The second step aimed at assessing the presence of a reactivation effect, defined as the reactivation of the EEG activity elicited by the UCS (in reinforced trials) at the corresponding silent period in non-reinforced trials ("conditioned silence"). We tested this hypothesis in recordings showing significant auditory discrimination (cf. step 1) by applying the above-mentioned statistical models to discriminate the EEG activity elicited by the conditioned silence (supposed to resemble to the one elicited by the UCS) from the EEG activity during the neutral silence (Figure 1, panels B and C). We considered that the reactivation effect was present when the decoding algorithm discriminated significantly ($p < 0.001$) conditioned silences and neutral silences at the beginning of the experiment (first 20 trials). This criteria was chosen according to previous studies showing that conditioning without consciousness may vanish fast in the time-course of the experiment (Raio et al., 2012).

3. *Temporal specificity*. In the third and last step, we assessed the temporal specificity of the reactivation effect, i.e. we checked that reactivation was specific to the precise time-window where the UCS is expected. We considered two silent intervals where no reactivation of the UCS is expected: before UCS onset (during ISI) and after UCS termination (during ITI). To this aim, we extracted epochs of 1000 ms duration (same duration as in previous analyses) spanning from 100 ms to 1100 ms latency for ISI and from 1800 ms to 2800 ms latency for ITI (Figure 1, panels B and C). The same decoding analysis (cf. step 2) was applied to these two intervals for the first 20 trials.

All analyses explained above (steps 2 and 3) were repeated for the last 20 trials of the experiment to check the persistence of the reactivation effect over time.

In order to assess the temporal specificity of the reactivation effect at the group-level, we performed a 3 x 2 repeated measure ANOVA with interval (ISI, UCS interval, ITI) and time within the experiment (beginning vs. end of the experiment) as within-subject factors for all recordings showing a reactivation effect. Two-tailed paired sample t-tests were applied post-hoc to identify the direction of effects. These tests were run using SPSS Statistics version 22.0 (IBM, 2013).

2.7.Topographic consistency test (TCT)

An additional topographic consistency analysis (Koenig and Melie-García, 2010) was performed in recordings showing a significant reactivation effect to quantify the degree of consistency of a given topography across trials. This test allows to assess statistically the presence of an evoked potential at each time frame based on a randomization analysis. It generates a non-parametric p-value corrected for multiple comparisons indicating the probability by which a given topography could be generated by chance. We applied it here at UCS interval (800-1800 ms latency comprising 100 ms baseline before onset) in reinforced, non-reinforced and unpaired trials (using the same number of trials) to investigate whether a consistent evoked response could be identified in the absence of any physical stimulus in non-reinforced trials. We considered the presence of an evoked response when a continuous period of at least 30 ms was significant ($p \leq 0.002$) on the TCT analysis.

2.8.Contingent Negative Variation analysis

The Contingent Negative Variation (CNV) is a well known EEG marker of event expectation occurring after specific temporal delay (Mento et al., 2013; Walter et al., 1964). The trace

conditioning paradigm designed in the current study is well suited for measuring such a component in passive conditions both in controls and patients. It complements the multivariate analysis with a more 'classical' picture of the neural correlates of temporal expectation as performed in previous related studies (Faugeras et al., 2012). The analysis of the CNV was carried out on the average EEG response to the tone in conditioned trials. Epochs were extracted starting at the onset of the tone and ending at the onset of the alarm with 100 ms baseline (-100-900 ms latency; Figure 1 panel A). An artifact rejection criterion of ±100 μV was applied offline at all 19 electrodes. Data were re-referenced offline to the common average reference and were 0.18–40 Hz band-pass filtered and 50 Hz notch filtered. These single trials were averaged for each subject and patient separately.

As previously reported, the CNV is maximally detected at the level of central electrodes. We therefore restricted our analysis to the evaluation of the CNV as measured at the Cz electrode for each individual separately. Specifically we fitted a linear regression model to the average EEG response to the tone and extracted the slope and the statistical significance of the model parameters. For each individual recording, significance level was set at $p < 0.005$. Analyses were carried out using the function fitlm available in the Statistics and Machine Learning Toolbox of Matlab 2015 (MATLAB, The MathWorks Inc., Natick, MA, 2000).

2.9.Clinical variables in relation to the reactivation effect

To assess whether the occurrence of a reactivation effect is indicative of survival or is influenced by clinical factors, we compared patients showing a reactivation effect vs. the others on several clinical characteristics. We performed two-tailed independent sample t-tests on continuous variables, including age, time to return of spontaneous circulation (time to ROSC), and

temperature at the moment of the recording. We tested categorical variables, such as survival at three months, etiology and FOUR score, using Fisher's exact test.

3. RESULTS

3.1.Univariate statistical analysis of the EEG during sound omission

A cluster-based permutation test revealed a significant difference between conditioned and neutral silences in only two out of the 43 recordings from comatose patients. Both recordings had been acquired in hypothermic conditions. These significant differences occurred during the period -100-132 ms and 319-574 ms at electrodes FP1 and 358-542 ms at electrode Fz in one patient. The second patient exhibited significant differences starting a -100-206 ms post-stimulus at O1 electrode; late effects starting at 432 ms were apparent at electrodes Fp2, O1 and O2.

No control subjects exhibited significant effect.

The paucity of the results obtained with univariate statistics encourages the use of a multivariate analysis for the investigation of reliable difference between the conditioned and unconditioned silences at the single individual level (for similar considerations in the comparison between univariate and multivariate statistic in hemodynamic studies, please see (Kahnt et al., 2014; Staeren et al., 2009) and (Tzovara et al., 2013, 2012b) for EEG studies).

3.2.Decoding results and specificity of the reactivation effect

3.2.1. Comatose patients

1. *Auditory discrimination.* The first step of the multivariate analysis identified significant auditory discrimination for UCS vs. neutral silence in 14 out of 29 patients, seven of them during TH and nine during NT (16 recordings; two patients showed significant results both during TH and NT). The average decoding performance across all significant results in the validation dataset was 0.67 ± 0.03 while chance level was 0.60 ± 0.02.

2. *Reactivation effect*. In the second step, nine of these 16 recordings (five in TH, two in NT during Day 1 and two in NT during Day 2) from eight patients reached our criteria for a reactivation effect (see Figure 2 for an exemplar patient; Table 1 and Figure 3 panel A1 for average significant results). We observed significant decoding results at the end of the experiment in five out of the nine recordings (two in TH; Table 1 and Figure 3 panel A1 for average significant results), suggesting a persistence of the reactivation effect over the duration of the experiment. The seven remaining recordings with auditory discrimination did not show a reactivation effect (mean decoding performance: 0.49 ± 0.01; Figure 3, panel A2: light grey bar at UCS interval).

As a control analysis, we also tested the reactivation effect in recordings without auditory discrimination, which provided very low decoding performance (0.46 ± 0.02; see Figure 4, panel A for an overview of auditory discrimination and reactivation effect results in patients with and without reactivation effect).

3. *Temporal specificity*. The third step of analysis, assessing the occurrence of the reactivation effect before (i.e. during ISI) and after (i.e. during ITI) the interval where the reactivation effect is expected (UCS interval) provided on average lower values, but few recordings showed significant decoding results (Table 1, "ISI" and "ITI" columns; Figure 3, panel A1). As a control analysis, we applied the same procedure to recordings showing significant auditory discrimination but no reactivation effect, which provided decoding performances around chance level (Figure 3, panel A2).

At the group level, the 3 x 2 ANOVA performed for the nine recordings with reactivation effect showed a main effect of interval ($F_{(2, 16)}$ = 8.49, p = 0.003), with UCS interval providing the

highest decoding values (mean: 0.60 ± 0.02) as compared to both ISI (mean: 0.48 ± 0.05; $t_{(8)}$ = 5.51, p = 0.001) and ITI (mean: 0.49 ± 0.02; $t_{(8)}$ = 2.67, p = 0.03) (see Figure 3, panel A1). No main effect of time within the experiment was found, although the decoding performance was lower at the end of the experiment than at the beginning of the experiment for all intervals except for ITI. Exact decoding values for the three intervals (ISI, UCS interval, ITI) and the two times (beginning / end of the experiment) in patients showing a reactivation effect can be found in Table 1. The same analysis was performed for recordings without reactivation effect (Figure 3, panel A2), which provided no significant results.

3.2.2. Healthy controls

Nine out of twelve (75%) recordings from healthy controls showed a significant auditory discrimination for UCS vs. neutral silence (average decoding value in the validation dataset: 0.78 ± 0.02; chance level: 0.74 ± 0.02). Among those, four recordings (44%) met our criteria for a reactivation effect (validation dataset: 0.62 ± 0.03; chance level: 0.51 ± 0.01). In three of those four recordings, the comparison was also significant at the end of the experiment, suggesting the persistence of the reactivation effect over time (see Table 1 and Figure 3, panel B1 for an overview of all decoding values and chance levels). The five remaining recordings did not show a reactivation effect (average decoding performance: 0.48 ± 0.01; Figure 3, panel B2). All results showing significant and non significant auditory discrimination along with the corresponding reactivation effects provide a qualitative indication of the gradient in the reactivation effect following the strength of the auditory discrimination (Figure 4, panel B). Only two of the 12 participants reported some explicit knowledge of CS+ and UCS association; one of them had a reactivation effect.

3.3.TCT results

In comatose patients with reactivation effect, the topographic consistency test applied at UCS occurrence revealed that the nine recordings except one showed at least one significant continuous period of 30 ms, demonstrating a consistent evoked response to the UCS. At the corresponding silent period in non-reinforced trials ("conditioned silence"), six recordings showed an evoked response and four in unpaired trials ("neutral silence"). However, two of the patients showing a reliable evoked response in both non-reinforced and unpaired trials exhibited significant results over longer lasting periods in non-reinforced trials in comparison to unpaired trials (see Figure 2 for the TCT results of an exemplar patient). All together, these results suggest a more reliable evidence of evoked-like activity in conditioned silences in comparison to neutral silences in comatose patients, consistently with the presence of a reactivation activity.

In the four healthy controls with reactivation effect, the TCT analysis showed large and continuous periods of significance for UCS occurrence (over time periods longer than 30ms), confirming a reliable evoked response to the alerting sound. At the corresponding silent period in non-reinforced trials, three of those recordings showed an evoked response. None of them displayed continuous periods of significance at the same latency in unpaired trials. These results strongly support the presence of evoked activity in conditioned silences in comparison to neutral silences in healthy controls.

3.4.CNV results

A significant negative CNV slope was observed in 12/43 (28%) recordings from comatose patients. This CNV was not more present in recordings from patients with reactivation effect (3/9, 33%) as compared to patients without reactivation effect (9/34, 26%; Fischer's exact test: p = 0.69) (see Figure 5 Panel A for an exemplar patient). At the group-level, when compared to a zero-centered distribution, the slopes did not differ neither in recordings from patients with a reactivation effect (mean = 4e-5 ± 3e-5; $t_{(8)}$ = 0.36, p = 0.73) or from patients showing no reactivation effect (mean = 9e-5 ± 5e-5; $t_{(33)}$ = 0.29, p = 0.77), suggesting no specific expectation at the group level (see Figure 5 Panel B for an overview).

In healthy controls, 10/12 recordings (83%) exhibited a significant negative CNV slope in the average EEG response to the tone in reinforced trials (see Figure 5 Panel A for an exemplar control), providing evidence of the temporal expectation of the unconditioned stimulus. All four recordings showing a reactivation effect had a significant negative CNV slope. At the group level, the slopes from controls differed significantly from a zero-centered distribution (mean = -9e-4 ± 1e-4; $t_{(11)}$ = -2.57, p = 0.03), revealing an expectation of the upcoming stimulus also at the whole group level (see Figure 5 Panel B for an overview).

3.5.Clinical factors in relation to the reactivation effect

The level of consciousness (FOUR score) of patients showing a reactivation effect was on average 2 ± 0.6 points for hypothermic recordings and 2.8 ± 1.1 for normothermic recordings; these values indicate a deep coma state (Wijdicks et al., 2005). Hypothermic patients showing a reactivation effect did not differ from hypothermic patients without reactivation effect on consciousness state or any other considered clinical variable (Table 2). In addition, survival at

three months was similar between the two groups, suggesting that the occurrence of a reactivation effect is not predictive of outcome. The same tests could not be performed in normothermia considering the reduced number of patients showing a reactivation effect on subsequent days (two on Day 1, two on Day 2). Of note, all survivors completely recovered consciousness (no patient in vegetative or minimally conscious state).

4. DISCUSSION

Our study aimed at detecting preserved learning capacity in the unconscious human brain. To this aim we recorded EEG in post-anoxic comatose patients during the presentation of an auditory trace conditioning paradigm and we tested the reactivation of the neural activity in response to an alerting sound during the interval where this stimulus is expected but not presented. We found evidence of reactivation in one third of the comatose patients based on significant decoding performance in classifying the spontaneous EEG activity following the CS+ vs. that following the CS-. In these patients, the reactivation phenomenon exhibited a high degree of temporal specificity as shown by a significantly better decoding performance at the latency where UCS is expected than at adjacent time periods. In other words, these patients developed an expectation not only of the occurrence of a specific event, but also of the timing of its occurrence.

In healthy controls, the reactivation effect was found in 4 out of 9 (44%) of the recordings with significant auditory discrimination (similar to Bekinschtein et al., 2009 where trace conditioning was found in 8 out of 16 (50%) controls) and persisted until the end of the experiment in all but one recording. Remarkably, these significant results were obtained despite the strict procedure we adopted for assessing the significance of the decoding performance, based on a hierarchical approach for including only individuals with significant auditory discrimination and on the assessment of the chance level by permuting data in the training datasets (as recommended in (Pereira et al., 2009)). Importantly, by adopting other widely accepted – but less conservative – method for assessing the significance of the decoding values (as for example based on a binomial

distribution of the classification output as in (Hausfeld et al., 2012)), all healthy subjects would have shown significant auditory discrimination.

Additional analyses on the same datasets acquired in comatose patients and healthy controls provided further evidence of the possibility of establishing an auditory association in the absence of consciousness. Specifically, the presence of an expectation of the UCS in healthy controls (while unaware of the stimulus contingency) was largely confirmed by the CNV analyses showing negative regression slopes in anticipation to the alerting sound. In patients, the same analysis provided inconclusive evidence, in agreement with previous literature (Faugeras et al., 2012) where only 9 out of 24 (37%) vegetative state patients showed such an effect with no significant association with the detection of global deviants.

The significance of the reactivation effect during the conditioned silence was further supported by the TCT analysis, providing evidence of the presence of an evoked activity despite the absence of any sensory stimuli. Specifically, the TCT revealed an evoked response in conditioned silences in the majority of healthy controls with significant reactivation effect. Remarkably, these same individuals did not show any evidence of evoked activity during the neutral silences further supporting the specificity of the reactivation effect in conditioned trials only. In patients, the presence of an evoked response was observed in the majority of recordings with trace conditioning and more specifically in conditioned trials in comparison to unpaired trials. Traditional ERPs analyses failed to show significant differential activity between the conditioned and unpaired silences both in comatose patients and in healthy controls, consistently with the higher sensitivity of multivariate analysis in comparison to univariate methods as shown in previous studies (Kahnt et al., 2014; Staeren et al., 2009; Tzovara et al., 2013, 2012b).

In the present cohort, all patients were in acute coma and with a very low score in clinical consciousness scales. Moreover, the majority of recordings showing a reactivation effect took place during the first day of coma (i.e. in seven out of nine recordings), five of them under hypothermia and sedation, a state of deep unconsciousness. In previous mismatch negativity and global auditory regularity detection paradigms (Tzovara et al., 2015a, 2013), we had already observed a higher decoding performance during the first days of coma in comparison to the following days. These data, interpreted as a possible consequence of the degeneration over time of brain functions after coma onset, offer a possible explanation to the current results by suggesting the integrity in early coma of those functions underlying violation detection paradigms in general. We speculate here that this preservation in the first day in comparison to later onsets could possibly be related to the degeneration of tissue properties of those regions that are most affected in hypoxic ischemia such as the hippocampus (Fortin et al., 2002; Halgren et al., 1980; Rutishauser et al., 2006), known to play a crucial role in violation detection paradigms (Kumaran and Maguire, 2006), trace conditioning (Cheng et al., 2008; Christian and Thompson, 2003; Degonda et al., 2005), or the formation of contingency awareness (Knight et al., 2009; Phelps and LeDoux, 2005).

An alternative explanation is a possible saturation of the conditioning over subsequent days, as some of these patients were recorded over two or more consecutive days. A decay of the trace conditioning over time is in accordance with previous literature focusing on fear conditioning at different levels of perceptual awareness of the conditioned stimulus (Raio et al., 2012): fear conditioning occurs no matter whether subjects could perceive the stimulus consciously or not, however its persistence over time was crucially dependent on whether the threat was consciously

perceived. Finally, the possibility that off sedation patients may have been more prone to environmental noise cannot be formally ruled out.

Considering that some patients received curare administration to prevent shivering during the first 24 hours, we cannot completely exclude that they were actually conscious but unable to answer commands. However, this only applies for recordings in the first day. In addition, considering that we included only patients in acute post-anoxic coma that were under pharmacological sedation, and showing clinical EEG alterations typical of comatose patients (Oddo and Rossetti, 2014; Rossetti et al., 2010a, 2010b), it seems extremely unlikely that residual consciousness accounts for the present results. In addition, none of the clinical descriptors could explain the difference between patients exhibiting trace conditioning and the rest (Table 2) and we found no straightforward relation between the occurrence of a reactivation effect and patients' outcome. Together with previous studies showing high decoding results on the first day of coma in auditory discrimination of local and global deviants (Tzovara et al., 2015a, 2013) as well as in the semantic categorization of sounds (Cossy et al., 2014), these results suggest that the degree of preserved cognitive functions in this very early stage of coma is not always informative of the severity of the clinical condition.

Overall, our study showed evidence of a reactivation effect in eight of 29 patients or nine of the 43 recordings performed. Although this number can seem low, it is consistent with results from other studies in the field of disorders of consciousness testing the integrity of various sensory and cognitive functions (Daltrozzo et al., 2007; Morlet and Fischer, 2014). In this clinical population, electrophysiological responses to auditory stimuli have been extensively investigated through

oddball paradigms allowing to explore the so called mismatch negativity component (MMN) which is typically identified in less than half of the tested patients (Fischer et al., 2004, 2000; Kotchoubey et al., 2005; Naccache et al., 2005). In more complex auditory paradigms (see for complete paradigm description and related discussion (Bekinschtein et al., 2009a; Faugeras et al., 2012, 2011; King et al., 2013; Naccache et al., 2015; Tzovara et al., 2015b)), multivariate decoding algorithm showed significant classification performance in response to auditory global deviants in 14% of vegetative state patients (King et al., 2013) and in 25% of recordings in comatose patients (Tzovara et al., 2015a). Evidence of semantic discrimination was also found in acute coma, with 14/38 recordings (37%) showing distinct EEG activity in response to human vs. animal vocalizations and 11/38 (29%) to living and man-made sounds (Cossy et al., 2014). Closer to our study, Signorino et al. (1995) elicited the P300 novelty component in 9/16 patients (56%) in acute coma using patients' own name in a conditioning procedure (Signorino et al., 1995). A limited number of significant patients is thus common in the field of disorders of consciousness and can be at least partially explained as variations in the severity of patients' medical condition.

Many previous studies investigating the extent of conditioning in unconscious condition were based on subliminal stimulus perception. By varying the degree of perceptual awareness of the CS+ through masking, Balderston et al. (2014) showed that unperceived conditioned stimulus could still elicit a conditioned response (Balderston et al. 2014; see also Esteves et al. 1994 for similar results). Along the same line, a continuous flash suppression paradigm provided further evidence of conditioning with subliminal stimuli (Raio et al., 2012). However the reliability of awareness threshold of subliminal stimuli is limited by the inherent variability of subjects'

perception sensitivity upon repeated presentation of the same stimulus, and might explain why other studies reported opposite results arguing for the crucial role of awareness in trace conditioning (Knight et al. 2003; Weike et al. 2007; Asli et al. 2009; see also Pessoa 2005 on the difficulty on an assessing perceptual threshold). Another approach used a concurrent task to diminish attentional resources and demonstrated a modulation of the degree of learning as a function of attention (Carter et al., 2003; Lovibond and Shanks, 2002); even in this case, the difficulty relies on the absence of a general consensus on the assessment of the dedicated attentional resources and its reliability. Our study relates more directly to other two recent papers showing learning in altered state of consciousness where conditioning was assessed via peripheral measurements (Arzi et al., 2012; Bekinschtein et al., 2009b).

Our study adds the extra value of assessing trace conditioning directly at the neural level, avoiding the measure of autonomic or behavioral responses that might be impaired in unconscious and sedated individuals. To the best of our knowledge, previous electrophysiological studies have exclusively investigated trace conditioning in conscious subjects. These previous studies have primarily emphasized an enhanced sensory processing for the CS+ compared to the CS- (Bröckelmann et al., 2011; Liu et al., 2012; Stolarova et al., 2006). This sensory enhancement appeared already at early latencies of stimulus processing irrespective of the sensory modality and implicates the activation of subcortical structures including hippocampus, insula and amygdala (Miskovic and Keil, 2012) and even when the conditioning is elicited by masked stimuli (Wong et al., 2004). Even though modulations in the processing of the CS+ indicate that an association has been established, these results do not provide information about the mechanisms allowing the storage and maintenance of the acquired representation after

the CS+ processing terminates. In our study, we made an explicit hypothesis that this learning phenomenon can be attested by the reactivation of the neural activity belonging to the expected stimulus, allowing also the investigation of the specificity of temporal contingency of the learned association.

Previous literature has provided ample evidence of modulation in neural activity within the sensory areas of the expected stimuli after presentation of a series of stimuli following specific rules (Kastner et al., 1999; Larsson and Smith, 2012; McNally et al., 2011; Summerfield et al., 2011). This evidence has been explained within the general framework of predictive coding. This theory formalizes the role of higher areas of the cortical hierarchy in formulating a prior probability of the incoming sensory stimuli by collecting information over time from sensory cortices. This flow of information is constantly updated over time from lower areas up to higher levels in the hierarchy in order to compare prior expectation with current evidence (Friston, 2005). In the particular case of a missing stimulus which was strongly expected based on previous experience, the brain can produce an activity strongly resembling the neural response to the missing stimulus as a manifestation of its internal expectation (Chouiter et al., 2015; SanMiguel et al., 2013) even when the expectation is built upon unperceived or task-irrelevant stimuli (Den Ouden et al., 2009). Along the same line, predictive coding has been suggested to hold even for unconsciously processed target stimuli (Vetter et al., 2014). Our study complements and extends these findings by showing that the prediction can take place in complete absence of consciousness based on single-patients' internal models of expectation of a relevant arousing stimulus.

Future experiments will investigate the level of generalization of the learned representation. The current evidence leaves unresolved whether patients had established an association between the CS+ and the UCS by keeping a memory trace of the physical features of those stimuli or whether this association was established along other dimensions of the UCS, such as emotional value, alerting features or semantic characteristics (i.e. being an alerting sound). Testing trace conditioning based on a larger variety of sounds would help clarifying the flexibility and the level of generalization that the brain can achieve in implicit learning without conscious perception of the incoming stimuli.

A further unresolved question relies on the dependency of the reactivation effect on having a fixed temporal interval between the CS+ and the UCS. It is possible that consciousness is required to adjust flexibly the probability of receiving salient stimuli over time based on the repeated presentation of the CS+ and UCS at variable temporal intervals.

Alternatively, learning with conditioning in the absence of consciousness might be only possible in a very repetitive and redundant experimental context both in terms of stimuli and temporal gaps. Further investigation of the learning capacity during coma will help defining the boundaries of the learned association in the absence of consciousness and will provide new insights on the preserved cognitive capacities in these patients as a function of time from coma onset.

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FIGURES CAPTIONS

Figure 1: Experimental design. The differential trace conditioning paradigm consisted of three types of trials, each of them starting with a tone (CS+ or CS-). In Reinforced trials (panel A), CS+ is followed by UCS, while in Non-reinforced trials (panel B) and Unpaired trials (panel C), the tone (CS+ and CS- respectively) is followed by a period of silence of the same length as the UCS. ISI stands for inter-stimulus interval and ITI for inter-trial interval.

Figure 2: Auditory evoked potentials (AEPs) for reinforced, non reinforced and unpaired trials (A, B and C respectively) in an exemplar patient showing a reactivation effect during hypothermia. Dashed lines indicate sounds onset and offset (CS+, CS-, UCS) and silent periods of interest (conditioned silence and neutral silence). 1) AEP across the electrodes montage for the whole trial duration and 2) Global Field Power (GFP) indicating the strength of the electric field; superimposed grey bars show the periods of significance of the TCT analysis.

Figure 3: Average decoding results when comparing non-reinforced and unpaired trials at UCS interval and during ISI and ITI in all comatose patients (panel A) and healthy controls (panel B) showing a significant auditory discrimination. Patients and controls are separated according to the presence of a reactivation effect (panels A1 and B1 respectively) or not (panels A2 and B2 respectively). In all panels, light grey bars indicate decoding performance at the beginning of the experiment and dark grey bars at the end of the experiment. The reactivation effect is presented with light grey bars at UCS interval. In comatose patients showing a reactivation effect (panel A1), repeated measures ANOVA showed a main effect of interval, with higher decoding values

for UCS interval compared to both ISI and ITI ($F_{(2, 16)}$ = 8.49, p = 0.003). No significant results were found for comatose patients without reactivation effect (panel A2). The same analyses were not performed in healthy controls considering the limited number of recordings (panels B1 and B2).

Figure 4: Summary of the average decoding results for auditory discrimination (black bars) and reactivation effect (white bars) in comatose patients (panel A) and healthy controls (panel B). Data are displayed separately according to the inclusion criteria defined for assessing trace conditioning. From left to right in both panels (in brackets are the number of individual recordings contributing to each average): average auditory discrimination and reactivation effects when they were both significant; average auditory discrimination and reactivation effect when only the first was significant; average values when auditory discrimination was not significant.

Figure 5: (Panel A) CNV regression line and superimposed EEG trace for Cz electrode in reinforced trials for two exemplar recordings with significant reactivation effect (healthy control in black and comatose patient in blue). Sounds onset and offset are indicated with dashed lines. Both recordings demonstrated a significant and negative CNV slope, suggesting an expectation of the UCS. (Panel B) Overview of CNV slopes calculated for all recordings from healthy controls and comatose patients (separated according to the presence or absence of a reactivation effect). Negative CNV slopes (reflecting an expectation of the upcoming stimulus) are displayed in green while positive CNV slopes are presented in orange. On average, healthy controls showed the more negative slopes (mean = -9e-4 ± 1e-4), followed by comatose patients with

reactivation effect (mean = 4e-5 ± 3e-5) and finally comatose patients without reactivation effect (mean = 9e-5 ± 5e-5).

TABLES

Table 1. Mean decoding results (SEM) for the recordings showing a reactivation effect, presented separately for hypothermic (TH) and normothermic (NT) patients and for healthy controls (CTL).

		ISI		**UCS interval**		**ITI**	
		Beginning	End	Beginning	End	Beginning	End
TH (n=5)	Decoding value	0.52 (0.02)	0.45 (0.03)	**0.62 (0.02)**	0.54 (0.04)	0.46 (0.03)	0.53 (0.03)
	Chance level	0.50 (0.01)	0.50 (0.00)	**0.50 (0.00)**	0.49 (0.01)	0.50 (0.00)	0.50 (0.00)
	Significant recordings	3	1	**5**	2	2	4
NT (n=4)	Decoding value	0.48 (0.06)	0.46 (0.07)	**0.67 (0.04)**	0.58 (0.07)	0.49 (0.05)	0.49 (0.07)
	Chance level	0.51 (0.00)	0.50 (0.01)	**0.51 (0.01)**	0.51 (0.01)	0.50 (0.01)	0.51 (0.01)
	Significant recordings	1	1	**4**	3	2	2
CTL (n=4)	Decoding value	0.51 (0.05)	0.60 (0.04)	**0.62 (0.03)**	0.57 (0.04)	0.53 (0.02)	0.51 (0.05)
	Chance level	0.51 (0.01)	0.50 (0.01)	**0.51 (0.01)**	0.52 (0.01)	0.50 (0.01)	0.50 (0.01)
	Significant recordings	2	4	**4**	3	2	2

TH therapeutic hypothermia; *NT* normothermia; *CTL* healthy controls; *ISI* inter-stimulus interval; *UCS* unconditioned stimulus; *ITI* inter-trial interval

Summary of the decoding results for the three within-trial intervals (ISI, UCS interval and ITI) and the two within-experiment times (beginning and end of the experiment) in all comatose patients (split according to the time of the recording, i.e. TH or NT) and healthy controls (CTL) showing a reactivation effect. Results of the reactivation effect are highlighted in bold. For each group of results, decoding value, chance level and number of patients providing significant results are indicated. All TH patients were recorded on Day 1. During NT, two patients were recorded on Day 1 and two on Day 2. One patient showed a reactivation effect for both TH and NT recordings.

Table 2. Comparison of clinical characteristics in patients showing significant reactivation effect or not, split by day and temperature.

	Patients with reactivation effect	**Patients without reactivation effect**	**p value (t value)**
Hypothermia (Day 1)	**n = 5**	**n = 14**	
Age (years)	71 ± 5	64 ± 3	0.29 (1.10)
Time to ROSC (min)	26 ± 8	21 ± 4	0.58 (0.56)
FOUR score ≤ 4	5 (100%)	14 (100%)	1 (Fischer)
Temperature (°C)	34 ± 0.2	34 ± 0.2	0.22 (-1.27)
Patients alive at 3 months	4 (80%)	10 (71%)	1 (Fischer)
Normothermia (Day 1)	**n = 2**	**n = 5**	
Age (years)	63 ± 8	75 ± 6	
Time to ROSC (min)	4 ± 1	24 ± 9	
FOUR score ≤ 4	2 (100%)	3 (60%)	
Temperature (°C)	35 ± 1.8	36 ± 0.2	
Patients alive at 3 months	1 (50%)	1 (20%)	
Normothermia (Day 2)	**n = 2**	**n = 11**	
Age (years)	77 ± 4	69 ± 3	
Time to ROSC (min)	19 ± 10	23 ± 5	
FOUR score ≤ 4	1 (50%)	7 (64%)	
Temperature (°C)	36 ± 0.7	37 ± 0.2	
Patients alive at 3 months	0 (0%)	6 (54%)	
Normothermia (Day N)[†]	**n = 0**	**n = 4**	
Age (years)		63 ± 8	
Time to ROSC (min)		14 ± 2	
FOUR score ≤ 4		3 (75%)	
Temperature (°C)		37 ± 0.4	
Patients alive at 3 months		1 (25%)	

ROSC return of spontaneous circulation; *FOUR* full outline of unresponsiveness (Booth et al., 2004).

† A total of four EEG recordings were performed in three patients. Two recordings took place on Day 3, one on Day 4 and one on Day 6.

Clinical description of comatose patients separated according to whether they showed a reactivation effect and to the day of recording. Although patients were recorded over several subsequent days, only one patient had significant results on both Day 1 (hypothermia) and Day 2 (normothermia). Temperature and FOUR score were collected at the moment of the EEG recording. Time between cardiac arrest and return to spontaneous circulation is indicated as ROSC.

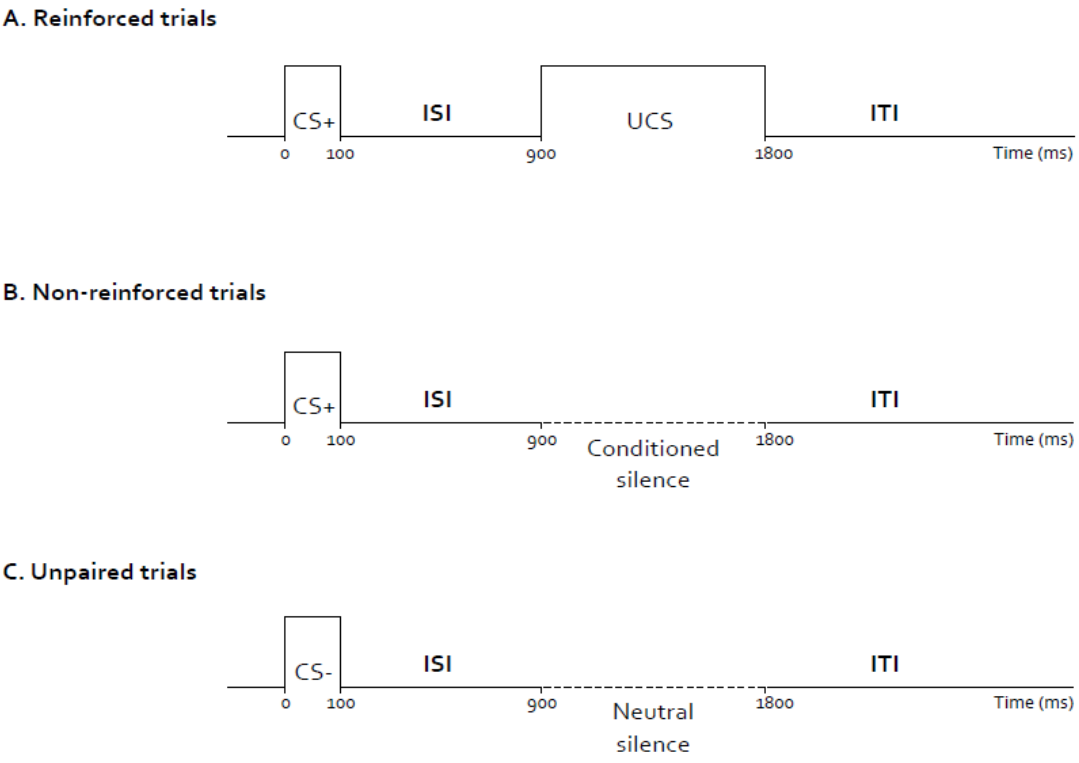


Figure 1

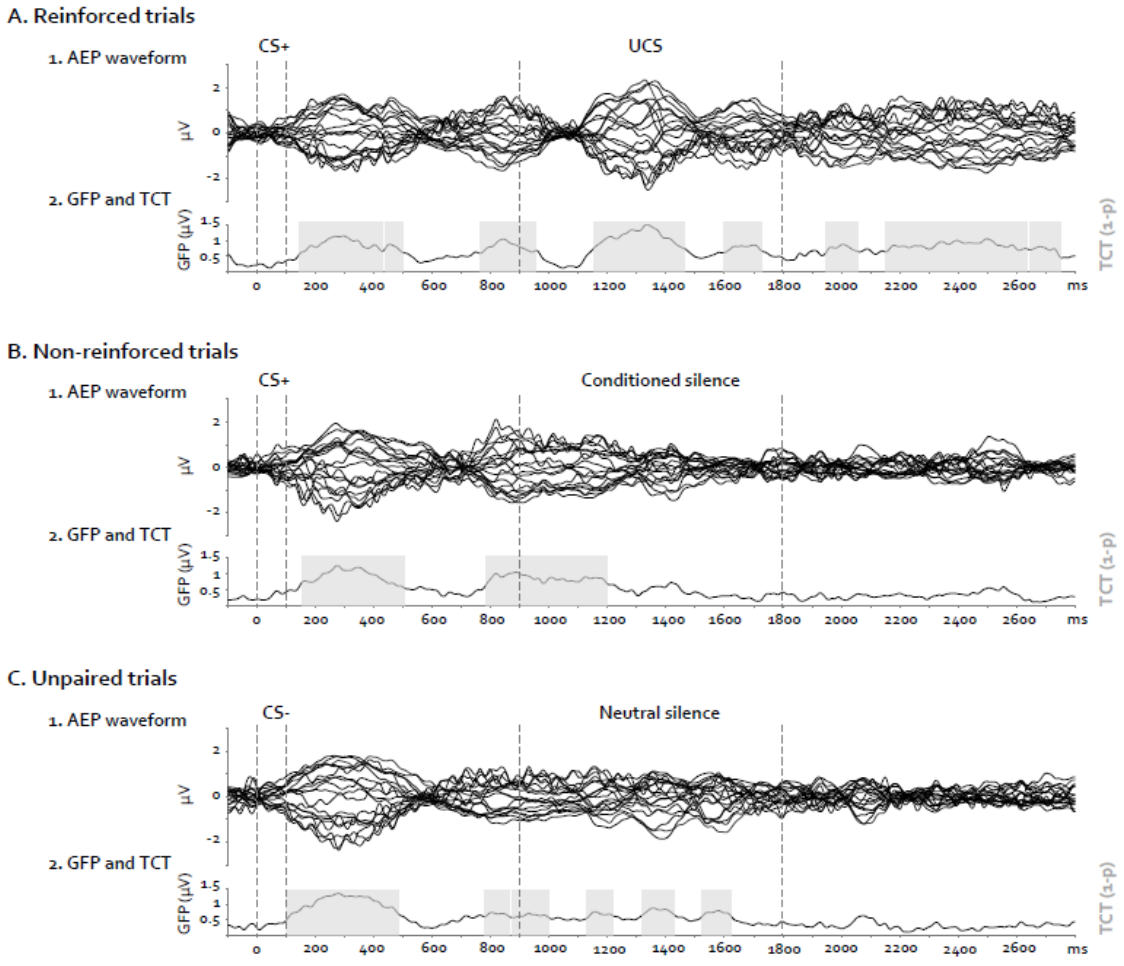


Figure 2

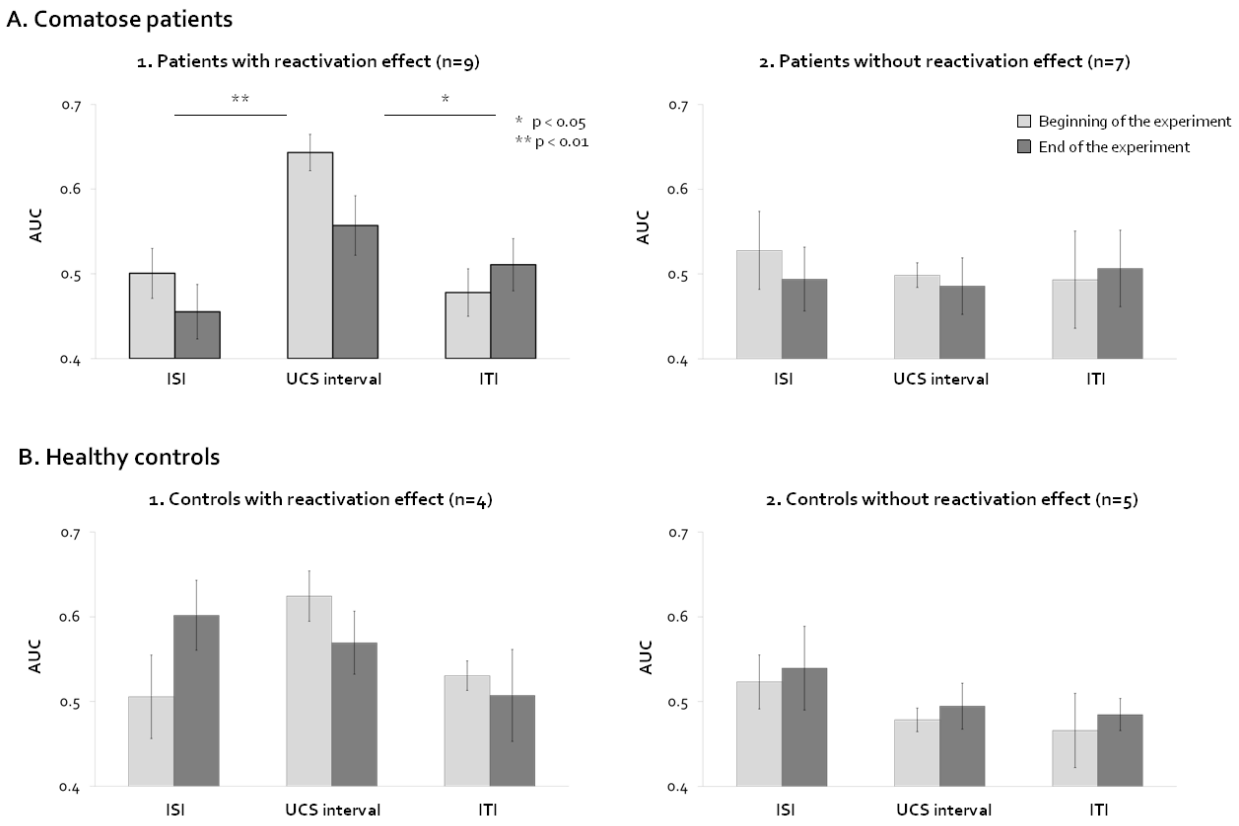
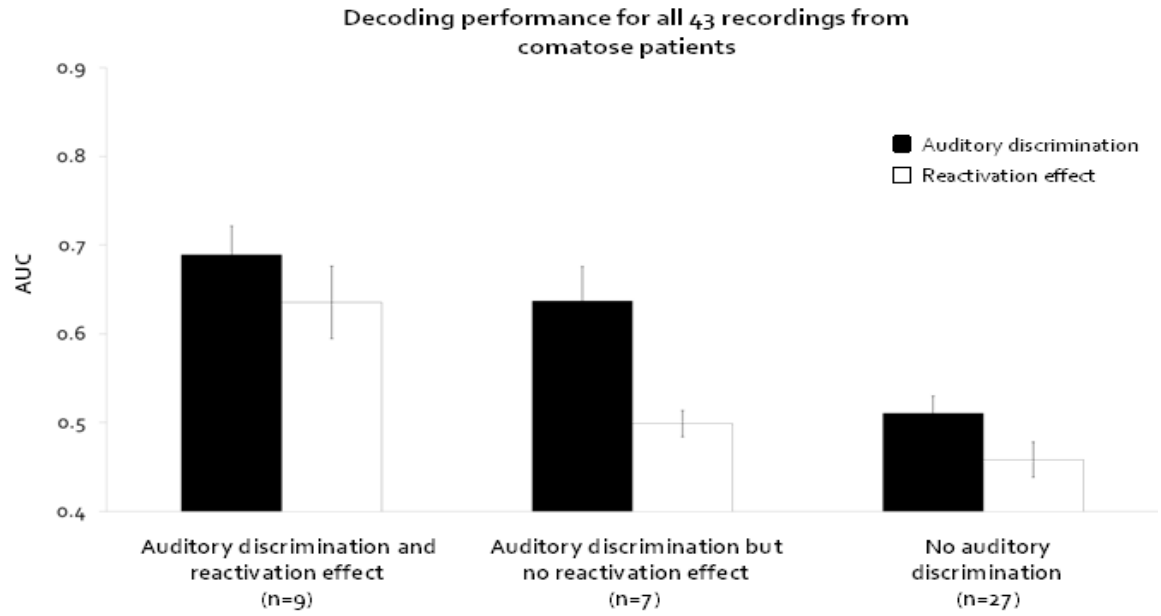


Figure 3

A. Comatose patients



B. Healthy controls

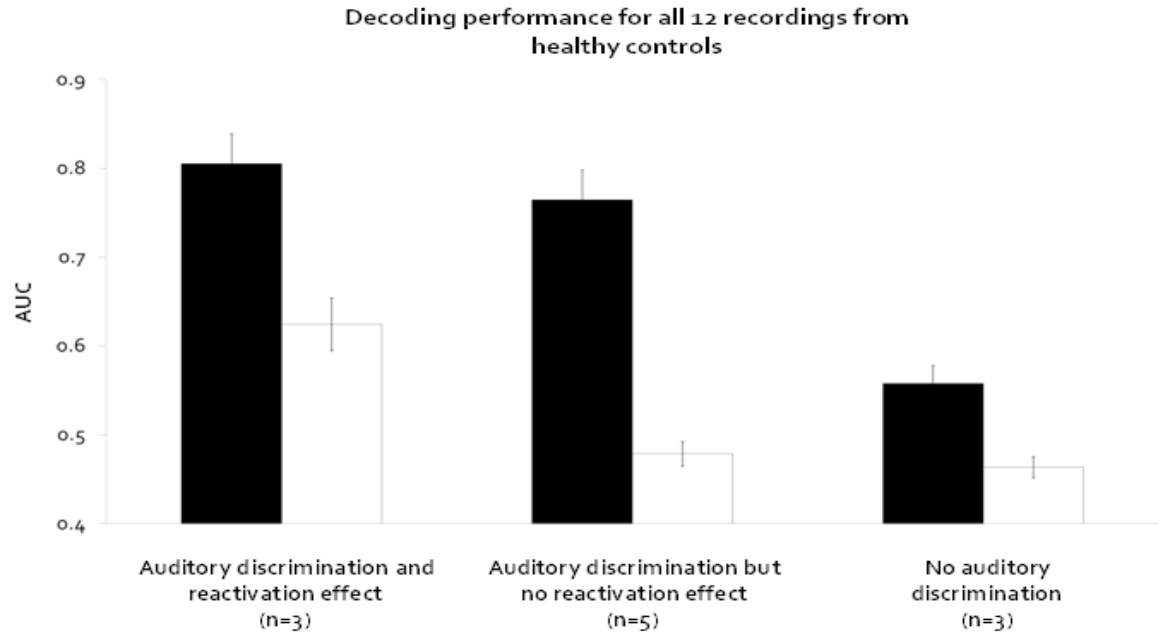


Figure 4

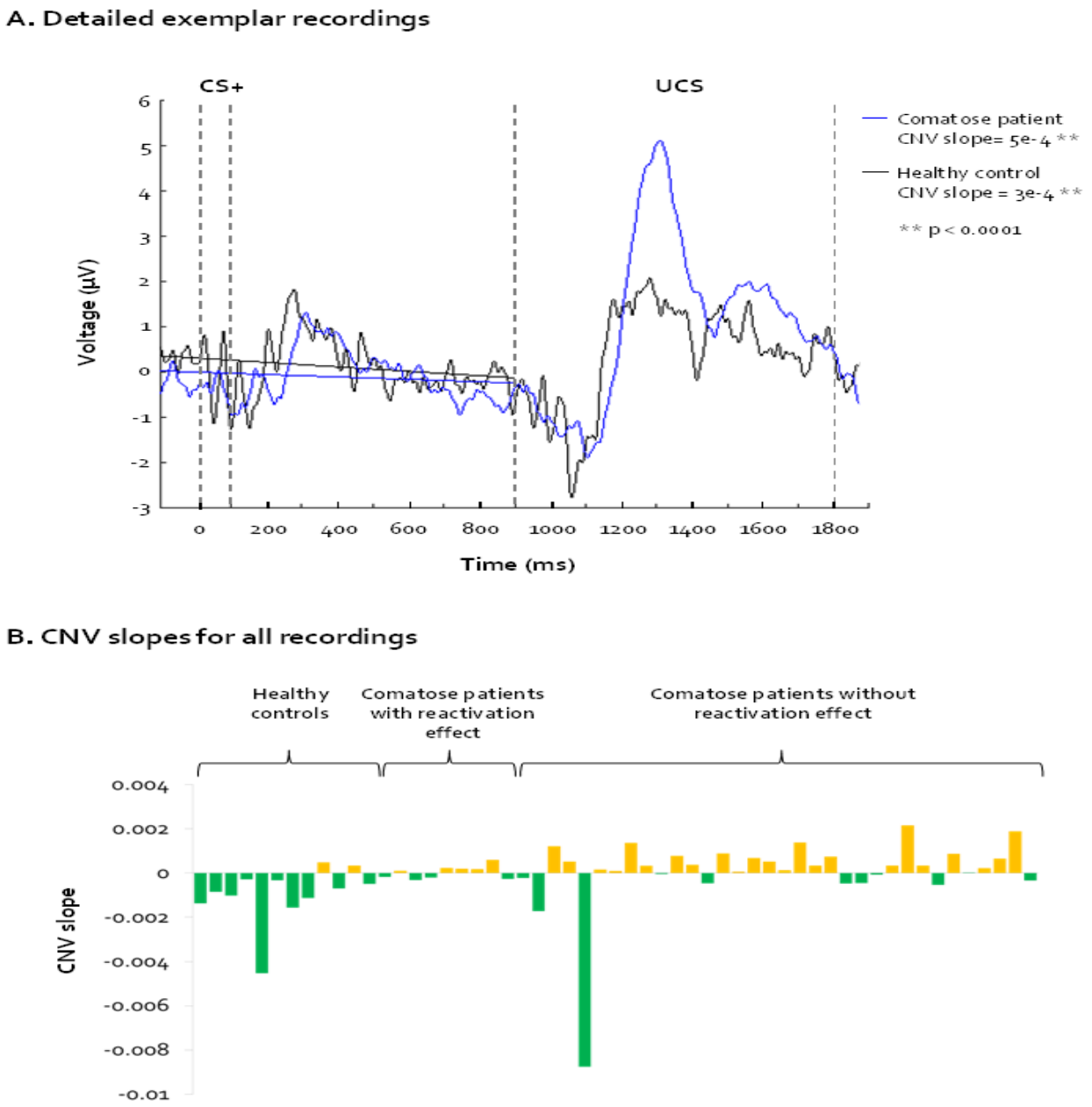


Figure 5