nature neuroscience

Classical conditioning in the vegetative and minimally conscious state

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Pavlovian trace conditioning depends on the temporal gap between the conditioned and unconditioned stimuli. It requires, in mammals, functional medial temporal lobe structures and, in humans, explicit knowledge of the temporal contingency. It is therefore considered to be a plausible objective test to assess awareness without relying on explicit reports. We found that individuals with disorders of consciousness (DOCs), despite being unable to report awareness explicitly, were able to learn this procedure. Learning was specific and showed an anticipatory electromyographic response to the aversive conditioning stimulus, which was substantially stronger than to the control stimulus and was augmented as the aversive stimulus approached. The amount of learning correlated with the degree of cortical atrophy and was a good indicator of recovery. None of these effects were observed in control subjects under the effect of anesthesia (propofol). Our results suggest that individuals with DOCs might have partially preserved conscious processing, which cannot be mediated by explicit reports and is not detected by behavioral assessment.

A pressing issue in neuroscience, with important theoretical and practical implications, is whether an objective manner or reliable test can be established to assess awareness without relying on explicit reports[1,2]. A specific implementation of the classical conditioning procedure, trace conditioning, is considered to be one of the best candidates for such a test[1,3]. Classical conditioning is a simple form of associative learning in which contingencies are established between a behaviorally important stimulus (unconditioned stimulus, UCS) and a closely paired neutral stimulus (conditioned stimulus). In the trace conditioning of the eyeblink response, the conditioned stimulus is a tone that is presented several hundred milliseconds before the UCS, which is an air puff to the cornea. The temporal demand imposed by the silent trace interval between both stimuli has been shown to engage a broad cerebral network, including the cerebellum, neocortex and hippocampus[4–6], and to require awareness of the contingencies between stimuli[7–9]. Subjects with bilateral mediotemporal lobe lesions fail to acquire the conditioning as well as normal subjects that did not show explicit knowledge of contingencies between stimuli[4,6].

DOCs[10] describe a heterogeneous group of individuals who have survived severe brain damage. The clinical assessment of these individuals is challenged with the task of behaviorally distinguishing those who demonstrate no evidence of awareness (vegetative state) from those who demonstrate inconsistent, but reproducible, evidence of awareness (minimally conscious state, MCS). Vegetative state patients typically emerge from coma and remain in a state in which they seem to be awake, but are unaware of themselves and/or their environment[11]. They have a preserved capacity for spontaneous or stimulus-induced arousal, but no evidence of purposeful (voluntary) behavior in response to visual, auditory, tactile or noxious stimuli[12,13]. The MCS differs from the vegetative state by the presence of inconsistent, but reproducible, purposeful behavior, which might include a response to command. In the upper bound of the DOC spectrum, an MCS patient who starts to communicate is reclassified as being severely disabled (SED)[14].

The neuroscience community has recently made great efforts to facilitate the assessment of individuals with DOCs by developing objective tools through which an individual's level of retained awareness can be assessed without requiring an overt movement on their part. Brain integrative processing, a proposed prerequisite of awareness[15,16], has been observed in MCS patients using functional imaging procedures presenting simple tones[17,18] and speech[19–23]. In the vegetative state, brain activations are mostly restricted to primary sensory cortices[17,18,24–26]. However, some exceptions have been found. Three vegetative state patients activated the inferior frontal gyrus and posterior superior temporal gyrus in response to speech stimuli[27], individuals in the vegetative state have shown differential responses to syntactic violations[20] and a single case report of an individual in the vegetative state showed specific brain activations in functional magnetic resonance imaging (fMRI) when asked to imagine playing tennis in her head or to imagine moving around her house[28].

Here, we sought to test whether individuals with DOCs have the capacity to learn trace conditioning to determine whether these individuals might exhibit partially preserved conscious processing, which cannot be mediated by explicit reports. We found that individuals

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Received 12 June; accepted 3 August; published online 20 September 2009; doi:10.1038/nn.2391

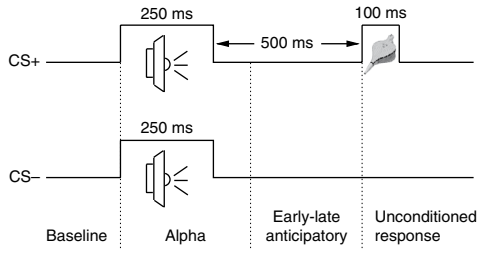

Figure 1 Stimulus design and different stages of the EMG response. The procedure consisted of a total of 140 conditioning trials (70 conditioned stimulus (CS+) and 70 unpaired tones (CS–)). Tones lasted for 250 ms. The conditioned stimulus was followed, after an interval of 500 ms, by an aversive stimulus, the UCS. We divided the EMG response from the eye into four temporal intervals: the period before tone onset (baseline, –400 to 0 ms), a short period that started with the onset of the tone and lasted for 300 ms (α response), a subsequent period (anticipatory interval) following this transient response and before the presentation of the puff (conditioned response) and the period following the onset of the air puff (unconditioned response). The EMG signal was rectified and normalized, on a trial by trial basis, to the s.d. of the baseline window for each trial. We refer to this as the nEMG, measured in s.d.

with DOCs were able to learn trace-conditioning associations, that learning was specific to stimulus and temporal contingencies, and that learning was a good indicator of recovery.

RESULTS

Subjects were trained with 140 trials of a trace-conditioning eye-blink response procedure (70 tones paired with an airpuff (conditioned stimulus) and 70 unpaired tones). Only the conditioned stimulus was followed, after an interval of 500 ms, by an aversive stimulus, the UCS (**Fig. 1**). The main group of the study, the DOC group, included 22 individuals with DOCs who met recognized criteria defining the vegetative state (12, 13), MCS or SED (14). We carried out the same experimental procedure in two control groups: a control to assess learning in subjects with intact conscious processing, the controlled consciousness group ($n = 16$), and a controlled unconsciousness group ($n = 12$), which was under the influence of the anesthetic agent propofol during standard endoscopic procedures. The controlled unconsciousness group was included in this study as a negative control, that is, a baseline for comparison in a group in which we expected no trace learning. It must be emphasized, however, that anesthesia leads to a broad state change that affects distinct factors, including a general amnesia that must be considered when comparing these groups[29].

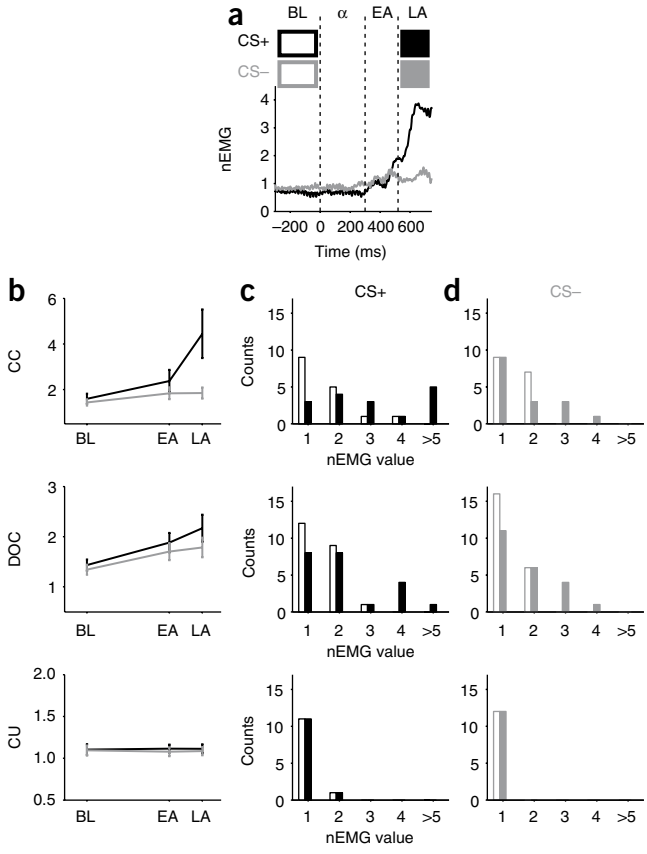

Figure 2 Learning during the anticipatory interval. ERRs of nEMG activity in response to conditioned stimulus (black) and unpaired tones (gray). (**a**) Example of a representative normal volunteer showing significant learning ($P < 0.05$). The conditioned stimulus response was larger in the anticipatory interval and this effect was more pronounced in the second half of this interval, close to the onset of air puff. (**b**) Average nEMG activity for each group (control consciousness (CC), DOC subjects and controlled unconsciousness (CU)) during three segments of the response: baseline and the early (EA) and late (LA) anticipatory intervals. The conditioned stimulus response was larger in the late anticipatory interval for the controlled consciousness and DOC groups. The response in the subjects was weaker (note the change of scale of nEMG). The controlled unconsciousness group showed no trace of anticipatory learning. (**c**,**d**) Individual contribution to the mean nEMG for each group. The numbers of subjects showing distinct values of nEMG activity (from 1 to 5) during the baseline (white) and late anticipatory interval (filled bars) are shown.

Learning during the anticipatory interval

We first measured the event-related response (ERR), averaging the normalized electromyographic (nEMG) response across all trials, for the conditioned stimulus and unpaired tones (**Fig. 2a**). The ERR reflects the magnitude and the specificity of learning. An increase in the anticipatory interval as compared with baseline is indicative of learning; if this increase is present for the conditioned stimulus, but not for the unpaired tones, then learning is specific. We examined the ERR of a subject who showed specific learning in the controlled consciousness group (**Fig. 2a**). The conditioned stimulus response ramped during the anticipatory interval. This effect was tuned close to the onset of the air puff, reflecting the temporal specificity of the contingency. These observations in a single subject serve as a guide for the subsequent analysis.

For each experimental group, we calculated the average nEMG activity during three segments of the response: baseline, early (first half) and late (second half) in the anticipatory interval. Averaged across the controlled consciousness group, the nEMG response increased during the anticipatory interval. This increase was specific to the conditioned stimulus and was more pronounced in the second half of the anticipatory interval (**Fig. 2b**). The DOC group showed the same pattern, although the main effect and the stimulus specificity were less pronounced. In contrast with these observations, we did not see any trace of learning in the controlled unconsciousness group. This observation summarizes our main finding: individuals with DOCs, as a group, showed learning during the anticipatory period, which was restricted to the few hundred milliseconds before the puff and thus reflected the temporal specificity of the contingency. When compared with the conscious control subjects, the effect size and the specificity were considerably reduced.

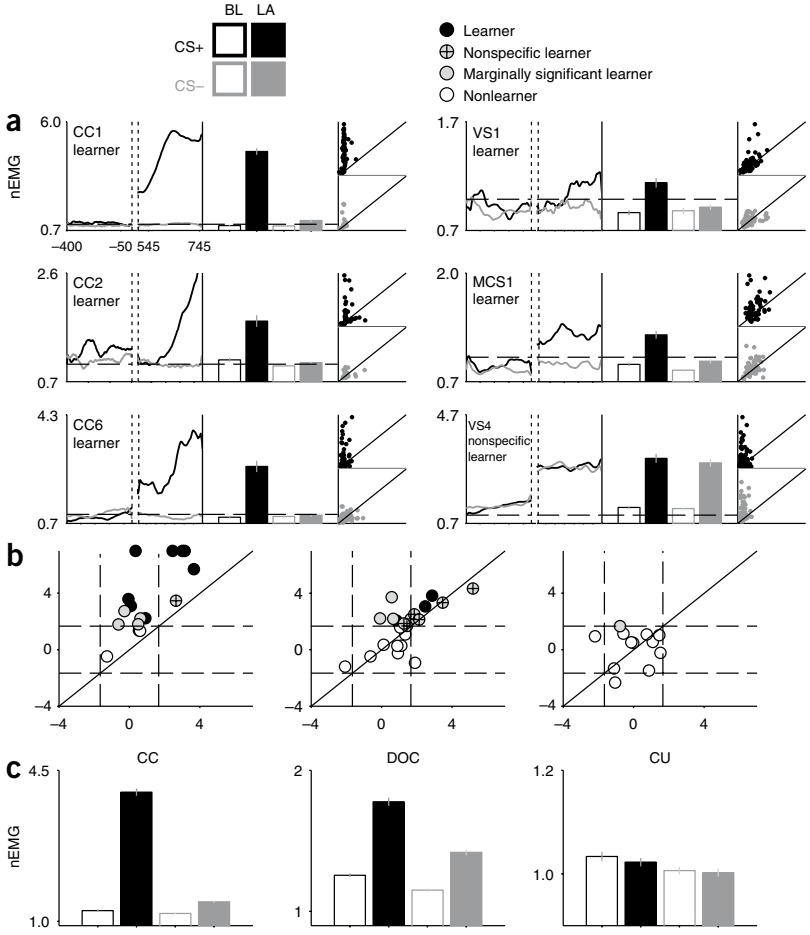

Figure 3 Single-subject measures of learning during the anticipatory interval. (**a**) Single-subject summary of nEMG responses for three healthy volunteers (left) and three DOC subjects (right). Each panel shows the ERRs of the nEMG during the baseline and late anticipatory interval (left). These two intervals are separated by a vertical dotted line. The average nEMG for the conditioned stimulus (black) and unpaired tones (gray) and for the baseline (white face–color) and late anticipatory interval (filled bars) is shown in the center of each panel. Error bars indicate standard errors. Learning is indicated by an increase in the anticipatory interval activity compared with baseline. If this increase is selective to the conditioned stimulus, then learning is specific. Scatter plots of nEMG activity during the late anticipatory interval (*y* axis) and nEMG activity during the baseline (*x* axis) for the conditioned stimulus (black) and unpaired tones (gray) are shown on the right side of each panel. Each dot above the diagonal indicates a trial in which activity during late anticipatory was higher than during the baseline. The figure shows two subjects with specific learning (top two panels) and a subject with nonspecific learning (bottom panel). (**b**) Comparison between late anticipatory and baseline (*t* values of paired *t* tests) for conditioned stimulus (*y* axis) and unpaired tones (*x* axis) for all subjects in the controlled consciousness (left), DOC (center) and controlled unconsciousness (right) groups. Learners are depicted as black circles, nonspecific learners as crossed white circles, marginally significant learners as gray circles and nonlearners as white circles (see text for details on learning criterion). Dashed lines indicate the *t* scores corresponding to *P* values of 0.05. (**c**) Learning group averages. Bars are colored as in **a** for the controlled consciousness (left), DOC (center) and controlled unconsciousness (right) groups. For a summary of results for each individual subject, see **Supplementary Figures 1–3**.

Single-subject measures of learning

Because individuals with DOCs form a heterogeneous population that is classified by the vegetative state and MCS criterion, but have a high rate of misdiagnosis[12,14], we conducted a subject by subject analysis to determine which subjects showed significant learning. On the basis of our previous results, we focused our analysis on the late anticipatory interval (200 ms before the presentation of the puff), where the effects were more pronounced. A comparison of the first segment of the anticipatory interval yielded similar results with an overall smaller effect size for all measures.

To quantify the individual contributions to the group results, we first measured nEMG activity for each subject for the conditioned stimulus and unpaired tones during baseline and the late anticipatory interval (**Fig. 2c**,**d**). In the controlled consciousness group, 6 out of 16 subjects had a nEMG response to the conditioned stimulus that was greater than three, that is, a threefold increase of activity during the late anticipatory interval, as compared with fluctuations before the onset of the trial. Only 1 out of 16 had a nEMG response to the unpaired tones that was greater than three. A similar result was found in the DOC group, where 5 out of 22 individuals had a nEMG response to the conditioned stimulus (and only one to the unpaired tones) that was greater than three. These data indicate that there was a great heterogeneity in the amount of learning (even in the control group), emphasizing the necessity of a subject by subject analysis.

For each subject, we carried out a 2 × 2 ANOVA with interval (baseline or late anticipatory) and stimulus (conditioned stimulus or unpaired tones) as main factors (see **Supplementary Tables 1–3** for the results of the 50 subjects involved in this study). We considered an effect as significant in a single subject if *P* value was below 0.05. With this criterion, nine of the 16 subjects in the controlled consciousness group showed a significant effect of interval, eight showed a significant effect of stimulus and six showed an interaction between these two factors ($P < 0.001$ for all cases; binomial probability of obtaining more than nine, eight or six positive results out of 16 tosses, each one of $P = 0.05$). Of the 22 subjects in the DOC group, 10 showed a significant effect of interval, 4 showed a significant effect of stimulus and 1 showed an interaction between these two factors ($P < 0.001$, $P = 0.004$ and $P > 0.1$; binomial probability of obtaining more than 10, 4 or 1 positive results out of 22 tosses, each one of $P = 0.05$). In the controlled unconsciousness group ($n = 12$), none showed a significant effect of interval, one showed a significant effect of stimulus and none of them showed an interaction between these two factors ($P > 0.1$ for all cases; binomial probability of obtaining 0 or 1 positive results out of 12 tosses, each one of $P = 0.05$). We determined a learning criterion from the ANOVA, in which we considered a subject to be a learner if he/she showed a significant effect of stimulus and interval. We considered a subject to be a nonspecific learner if he/she showed an effect of interval without reaching significance for the stimulus factor (**Fig. 3**).

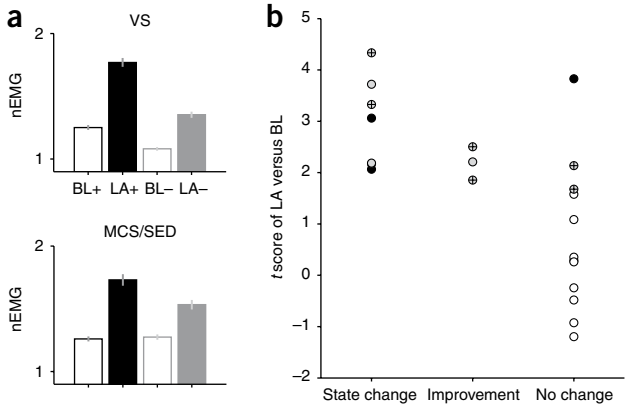

Figure 4 Learning, clinical measures and prediction of recovery. (**a**) The learning pattern did not show a significant difference between subgroups of DOC subjects (vegetative state (VS) and MCS) ($P > 0.05$). For both groups, we observed significant learning that was, at the group level, less specific than for control subjects (see also **Table 1**). (**b**) Learning was a good estimate of recovery probability. Each data point indicates (on the *y* axis) the *t* value of the conditioned stimulus response (late anticipatory versus baseline), measured for each individual subject. The *x* axis indicates a measure of recovery. Improvement, subjects who had higher scores in the CRS after several months; no change, subjects who showed no signs of improvement; state change, subjects who changed from vegetative state to MCS or SED and subjects who changed from MCS to SED. We observed a clear relationship between learning and recovery. Learners are depicted as black circles, nonspecific learners as crossed white circles, marginally significant learners as gray circles and nonlearners as white circles.

We then followed the ANOVA with *post hoc* specific tests comparing conditioned stimulus and unpaired tones nEMG activity in the different epochs. When specifically looking at the anticipatory-baseline comparison, we found that many subjects in the controlled consciousness and DOC groups showed a significant difference for the conditioned stimulus (late anticipatory-baseline) stimulus (13 out of 16 for the controlled consciousness and 13 out of 22 for the DOC group). Subjects passing this criterion and not reaching significance in the ANOVA test were referred to as being marginally significant learners. Considerably fewer subjects (5 out of 16 for the controlled consciousness and 7 out of 22 for the DOC group) showed a substantial difference between the late anticipatory interval and the baseline for the unpaired tones (late anticipatory-baseline). For the controlled unconsciousness group, only 1 subject out of 12 showed a difference for the conditioned stimulus (anticipatory-baseline).

To simultaneously visualize the amount of learning for all subjects in all groups, we plotted, for each individual subject, the *t* score of the difference between the anticipatory interval and the baseline for the conditioned stimulus versus the unpaired tones (**Fig. 3b**). As in our previous analysis, these data reflect the same pattern. Learning was significant and highly specific in the controlled consciousness group and learning was significant, although considerably less prominent, in the DOC group. Although learning was specific, the specificity of learning was considerably less than for the conscious control group. Virtually no learning was seen in the controlled unconsciousness group. This group effect was seen synthetically after averaging all subjects in each condition (**Fig. 3c**). We then set out to quantitatively study the differences in learning across groups and, in the DOC group, assess which clinical factors relate to the amount of anticipatory learning.

Comparison across different groups

The nEMG patterns for the vegetative state and MCS groups (**Fig. 4a**) showed virtually no differences and were identical to the two groups pulled together (**Fig. 3c**). To compare learning between the four groups of subjects (controlled consciousness, vegetative state, MCS/SED and controlled unconsciousness), we applied a Kruskal-Wallis one-way ANOVA for each of two learning contrasts defined previously. The conditioned stimulus late anticipatory-baseline showed a significant difference between groups ($\chi^2 = 16.78$, $P < 0.001$), whereas the same measure for unpaired tones was not significant ($\chi^2 = 6.58$, $P < 0.083$).

Specific comparisons between pairs of these measures revealed distinct patterns of conditioning between the controlled consciousness, DOC and controlled unconsciousness groups (**Table 1**). The controlled consciousness group showed marginally significantly higher learning than the vegetative state group for the conditioned stimulus (late anticipatory-baseline) comparison ($P < 0.05$). This difference was not significant for the unpaired tones comparison ($P > 0.1$). The comparison between the vegetative state and controlled unconsciousness groups showed significantly higher learning in the vegetative state for both contrasts ($P < 0.025$ for both). Notably, the unpaired tones (late anticipatory-baseline) contrast showed significant differences ($P < 0.025$) between the vegetative state and controlled unconsciousness groups, but not between control and anesthesia groups. This further supports our previous finding of lesser learning specificity in the vegetative state group and suggests that this does not result exclusively from a decreased response to the conditioned stimulus, but rather that individuals in the vegetative state fail to inhibit the anticipatory response to unpaired tones. The MCS/SED subjects also showed partial learning as a group, with a slightly different pattern than the vegetative state. None of the contrasts showed a difference between the MCS/SED and the controlled consciousness groups, although the conditioned stimulus (anticipatory-baseline) was close to significance ($P = 0.07$). The comparison with the controlled unconsciousness group showed an effect of both contrasts, indicating that, for the vegetative state group, there was significant, but not specific, learning ($P < 0.025$ for both). The comparisons between the MCS/SED and vegetative state group showed no significant differences ($P > 0.1$ for both).

In summary, this analysis confirmed our previous qualitative observations, that the control group showed the most significant and specific learning effect. The vegetative state and MCS groups were very similar and showed a significant effect of learning over the anesthesia group in the conditioned stimulus and unpaired tones contrast, revealing a less specific form of learning.

Learning and clinical measures

We then explored the relationship of learning in the DOC group with four relevant clinical markers, age, cortical atrophy score (CAS),

Table 1 Learning differences at the group level

	CS+ LA/BL	CS– LA/BL
CC versus CU	$z = -3.95$***	$z = -1.42$ ns
CC versus VS	$z = -1.93$*	$z = -0.81$ ns
CC versus MCS	$z = -1.70$ ns	$z = -1.10$ ns
VS versus MCS	$z = -0.17$ ns	$z = -0.03$ ns
VS versus CU	$z = -2.26$**	$z = -2.26$**
MCS versus CU	$z = -1.97$*	$z = -2.06$*

Each cell in the table shows the *z* value for the Mann-Whitney test. MCS group includes two SED subjects. CC, controlled consciousness group (volunteers); CU, controlled unconsciousness group (anesthetized subjects); VS, vegetative state group. ns, not significant ($P > 0.05$). *$P < 0.05$, **$P < 0.025$, ***$P < 0.001$.

Table 2 Cortical atrophy partially explains anticipatory activity (learning) in trace conditioning

	CS+ LA/BL	CS– LA/BL
Age	Beta = –0.037	Beta = –0.285
	t = –0.54	t = 0.84
	P = 0.601	P = 0.420
CAS	Beta = –0.773	Beta = –0.327
	t = –5.28	t = –1.33
	P = 0.001	P = 0.204
TFI	Beta = –0.277	Beta = –0.178
	t = –1.89	t = –0.67
	P = 0.081	P = 0.514
CRS	Beta = –0.131	Beta = –0.337
	t = –0.69	t = –1.34
	P = 0.505	P = 0.201

Beta, t and P values of the backward regressions are shown for each of the four clinical independent variables (age, CAS, TFI and CRS) scores in both conditioned stimulus late anticipatory-baseline and unpaired tones late anticipatory-baseline. CS+, conditioned stimulus; CS–, unpaired tones; LA/BL, late anticipatory-baseline.

time from ictus (TFI) and coma recovery scale (CRS) scores. In a correlation analysis, age and CAS significantly correlated with conditioned stimulus (late anticipatory-baseline; $r = -0.384$, $P = 0.039$ and $r = -0.640$, $P = 0.001$, respectively), but when placed in conjunction with the other clinical variables in a regression analysis, only CAS contributed to explain the variance of learning (**Table 2**).

Two backward regressions, one for late anticipatory-baseline in conditioned stimulus and the other for the control comparison late anticipatory-baseline unpaired tones, showed that CAS—which explains ~40% of the learning measure conditioned stimulus (anticipatory-baseline) variance independently of the regression model—and, to a lesser degree, TFI can partially predict learning in our group of patients. CRS scores and age do not significantly explain variance for conditioned stimulus (anticipatory-baseline) and none of the independent measures (TFI, CAS, CRS and age) showed significant values for unpaired tones.

We performed two different analyses to evaluate the predictive power of learning measures to discriminate between groups, one for clinical diagnosis (vegetative state or MCS/SED) and the other for cause of injury (traumatic brain injury (TBI) or non-TBI). A binary logistic regression was undertaken to assess the power of conditioned stimulus late anticipatory-baseline to classify subjects in two groups. Conditioned stimulus late anticipatory-baseline contrast failed to accurately classify the subject population in the vegetative state or MCS/SED groups. The model incorrectly classified 2 out of 11 individuals in the vegetative state and 4 out of 9 non–vegetative state subjects, leaving the model with an accuracy of 72.7% ($\chi^2 = 3.61$, $P = 0.057$). This is consistent with our previous observation that learning in vegetative state and MCS was not significantly different. This same measure was used to differentiate between TBI and anoxic/hypoxic events as the cause of the DOC. The model distinguished between TBI and non-TBI subjects with 82% accuracy. The contribution from conditioned stimulus late anticipatory-baseline contrast significantly increased the accuracy of the model ($\chi^2 = 4.52$, $P = 0.033$). The mode correctly classified 11 out of 12 TBI subjects, but 3 out of 10 non-TBI subjects were incorrectly assigned (those who showed some degree of learning, VS2, VS6 and MCS3).

Finally, we explored whether learning correlated with probability of recovery. We classified our subjects in two groups in relation to their clinical outcome, with no recovery comprising those subjects who showed no change in CRS scores (after 6 months to 2 years) and recovery consisting of those subjects who changed from the vegetative state to MCS or from the vegetative state or MCS to SED and those who increased their behavioral portfolio (CRS scores) without changing conscious state (**Fig. 4b**). We performed a logistic regression to evaluate whether conditioned stimulus late anticipatory-baseline could differentiate between recovery and no recovery. Learning (conditioned stimulus late anticipatory-baseline, $\chi^2 = 5.02$, $P = 0.025$) indicated, with an accuracy of 86%, whether a subject had shown signs of recovery or not.

The change of conscious state subgroup from the recovery group was composed of subjects VS3 and VS8, who changed to MCS, VS1 and VS4, who moved to SED, and MCS1, MCS2 and MCS5, who showed signs of improvement after the learning evaluation (**Supplementary Table 4**). These last three individuals could communicate (fulfilling the criteria for SED) at the time this manuscript was accepted for publication. The other three individuals from the recovery group showed greater CRS scores a few months after the full assessment; VS5 showed 3 to 6 in his CRS score, VS7 changed from 3 to 7 and MCS4 changed from 11 to 13. All patients in the recovery group suffered from TBI (and they all showed learning), the patients in the no recovery group did not suffer TBI (except for MCS6 and MCS7), with only 2 out of 10 showing learning.

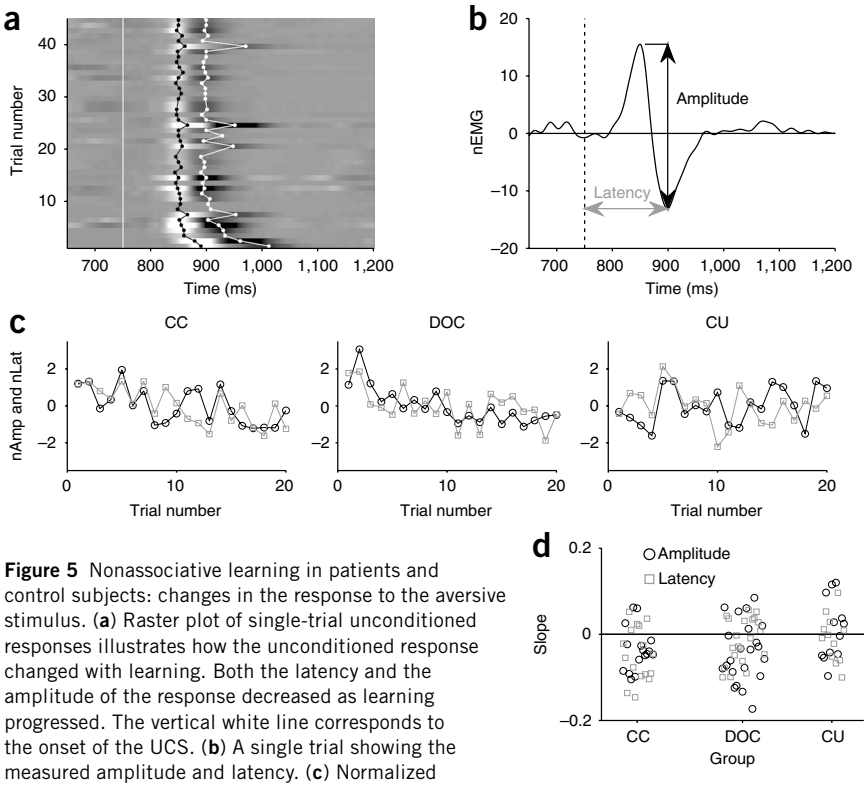

Figure 5 Nonassociative learning in patients and control subjects: changes in the response to the aversive stimulus. (**a**) Raster plot of single-trial unconditioned responses illustrates how the unconditioned response changed with learning. Both the latency and the amplitude of the response decreased as learning progressed. The vertical white line corresponds to the onset of the UCS. (**b**) A single trial showing the measured amplitude and latency. (**c**) Normalized amplitude (nAmp) and latency (nLat) of the response decreased as training proceed during the first 20 trials for the controlled consciousness and DOC groups. (**d**) Individual contributions to the decrease in latency and amplitude (measured by the slope).

Nonassociative learning

To further understand the global aspects of learning in individuals with DOCs, we carried out an analysis of the change in the eye blink response

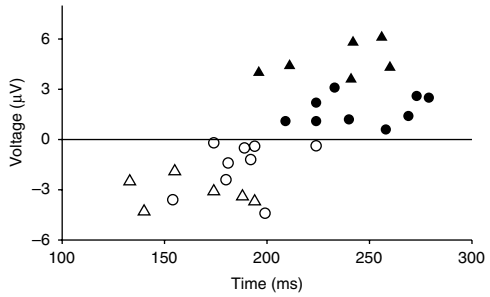

Figure 6 Intact latencies, but smaller amplitudes, in event-related auditory potentials in DOCs. Mean MMN negative (open symbols) and positive (filled symbols) related peaks' latencies and intensities of the difference between the responses to frequent and infrequent tones. Latencies and amplitudes were estimated for each individual subject for healthy volunteers (triangles) and individuals with DOC (circles). The positive peak amplitude was significantly smaller ($P < 0.001$) in DOC subjects than in normal volunteers.

(unconditioned response) to the air puff, which constitutes a nonassociative (not related to the conditioned stimulus) form of learning. Both the latency and amplitude of the response change during the course of learning (**Fig. 5a**,**b**). To quantify this observation, we estimated, for each individual trial, the latency and amplitude of the response.

Amplitude and latency decreased for the DOC and the controlled consciousness groups during the course of learning (**Fig. 5c**,**d**). This decrease was confined to the first 20 trials. When we considered the first 20 trials, the slope of the regression was significant for the control group (amplitude: $t = -3.1$, $P < 0.001$, degrees of freedom = 18; latency: $t = -4.4$, $P < 0.001$, degrees of freedom = 18) and for the DOC group (amplitude: $t = -4.8$, $P < 0.001$, degrees of freedom = 18; latency: $t = -2.5$, $P = 0.02$, degrees of freedom = 18) and was not significant for the anesthesia group (amplitude: $t = 1.3$, $P > 0.1$, degrees of freedom = 18; latency: $t = -0.9$, $P > 0.1$, degrees of freedom = 18). When we examined trials 20–40 (or beyond), none of these regressions were significant ($P > 0.05$). For the controlled consciousness and DOC groups, most subjects showed negative slopes, although there was a broad dispersion (**Fig. 5d**).

Auditory processing in individuals with DOCs

A critical aspect of trace conditioning is that it establishes a temporal gap between the conditioned stimulus and UCS. Because there is an overall attenuation of neural activity in individuals with DOCs[17,18,20], it is conceivable that it also presents long latencies and that some processing of the first stimulus may still be going on by the time the UCS occurs. To rule out this possibility, we measured the latency of evoked potentials in an electroencephalography experiment.

We tested nine individuals with DOCs in an oddball procedure, using the same stimuli as in the trace-learning experiment. This experiment served two purposes. First, we could measure the latency and the amplitude of the response. Second, we were able to determine whether the subjects reallocate attention to a different stimulus by measuring the mismatch negativity (MMN). Eight out of nine subjects (all except SED1) elicited an early electroencephalography response to the auditory stimulus, referred as a N1 component. There were significant scalp electrophysiological differences ($P < 0.05$) between tones, indicating a significant MMN in seven out of nine individuals with DOCs (SED1, VS12, MCS3, VS5, MCS4, MCS7 and VS4, but not VS7 and MCS6). In particular, this indicates that sensory responses in these subjects distinguished the two tones used in the trace-learning experiment. The latencies in the response components of the DOC subjects were not significantly different from the latencies of normal volunteers (negative peak $z = -1.653$, $P = 0.113$; positive peak $z = -0.59$, $P = 0.607$). However, the amplitude of the response in DOC subjects was significantly smaller than in control subjects (negative peak $z = -1.886$, $P = 0.066$; positive peak $z = -3.185$, $P < 0.001$; **Fig. 6**).

DISCUSSION

The maintenance of information in time is one of the main psychological attributes of conscious processing[15,30]. In fact, when there is no temporal interval between a conditioned stimulus and a UCS (delay conditioning), learning the conditioned stimulus–UCS relationship becomes automatic and reflexive and no longer requires declarative knowledge[1,31]. In this context, our finding that clinically defined vegetative state patients can acquire trace conditioning is surprising. As with other studies involving individuals with DOCs, this result has two interpretations. Individuals with DOCs may have partially preserved conscious processing, which cannot be exhibited overtly via intentional movement or verbal responses, or, alternatively, trace conditioning can indeed be acquired in the absence of consciousness.

Some considerations suggest that the first interpretation is more likely. First, subjects in a pharmacologically controlled unconscious state were incapable of eliciting trace-conditioning learning. However, as mentioned previously, this comparison must be made cautiously because of the many factors affected by anesthesia, including amnesia[31,32]. Another argument suggesting preserved conscious processing in individuals with DOCs comes from single-subject analysis. First, learning was a good predictor of recovery. Second, subjects that showed significant learning also showed other indications of partially preserved conscious processing in complementary neuroimaging studies. Three of the subjects fulfilling the clinical criteria for vegetative state that acquired trace conditioning had brain activity that may indicate partial preservation of awareness. Subject VS4 (**Fig. 3b**) showed specific brain activations when asked to imagine playing tennis or to imagine moving around the rooms of their house that were consistent with those of volunteers performing the same task[28,33]. Activation of the lateral premotor cortex was seen in subjects VS3 and VS8 when asked to move the opposite hand, suggesting motor intention, even when no muscle activity could be detected (unpublished data, T.A.B., F.F.M., M. Villarreal, A.M. Owen and V. Della Maggiore). The remaining vegetative state subjects who showed significant learning had activity in the lateral temporal cortex in response to speech stimuli[34]. Despite the previous arguments, we cannot exclude the possibility that trace conditioning is acquired, to a lesser extent, in the absence of awareness. Lower-order organisms, who most probably have nothing akin to human consciousness, can show this type of learning[35], suggesting that different learning mechanisms might be engaged when human conscious awareness is offline. In humans, other manners of acquiring trace conditioning in the absence of awareness have been demonstrated using emotionally salient stimuli as conditioned stimulus. Fear trace conditioning can be learned even when the conditioned stimulus is masked[36], but is not learned when the masked conditioned stimulus does not have a strong emotional content[37].

The functional neuroanatomy involved in trace learning seems to be consistent with its psychological attributes; bridging the cognitive gap generated by the silence between the tone and the air puff entails a functional neural network involving the cerebellum, the hippocampus and certain prefrontal cortex areas[3–5,38]. Because individuals with DOCs are a very heterogeneous group, classified mainly by clinical measures and resulting from a wide range of brain lesion patterns, it is important to understand which neuroanatomical and physiological indicators may be predictive of learning. In this study, we found that the degree of brain atrophy was a good predictor of learning capabilities. In addition, we observed a clear relationship between learning

and cause of injury: there was a very high probability of learning in TBI patients and a very low probability in hypoxic patients.

One of the most ambitious goals in DOC investigations is to establish measures that may work as indicators of recovery. We found that learning was a predictor of future recovery, with an accuracy of 86%. Moreover, individuals who showed learning and subsequently changed their state of consciousness (recovered) were reassessed behaviorally at least 2 years after the learning assessment; individuals who only showed improvement on the behavioral scale, but not to the point of a change in conscious state, were assessed less than a year after learning. These findings suggest there is room for late recovery in DOC patients[39] and that trace conditioning could predict this type of recovery in post acute DOC patients. These results suggest that an adequate classification of individuals with DOCs requires anatomical, functional and behavioral measures. Trace learning may be an important indicator in this integrative diagnostic battery.

Although it requires further investigation, our finding that individuals with chronic pathologies of awareness can acquire trace conditioning (and may recover) suggests that there is a window for cognitive neuro-rehabilitation. The underlying idea of clinical rehabilitation is to train the networks involved in the specific pathology. Although this has a straightforward implementation in muscular, sensory or mnemonic disorders, this becomes less clear when awareness is the process to be trained. Although extremely speculative, it is important to investigate whether training the circuits involved in awareness may help the recovery of consciousness.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

ACKNOWLEDGMENTS

We thank the care homes and rehabilitation centers in the UK and Argentina, the Cambridge Impaired Consciousness Research Group, the staff of the Wellcome Trust Research Facility for their contribution, and all the study's participants. We especially thank F. Klein and the Anesthesia Favaloro Team. We also thank L. Naccache and C. Koch for comments on an early version of the manuscript. This study was funded by an Antorchas Foundation grant (T.A.B.), a Marie Curie IIF grant (T.A.B.), a StartUp grant (F.F.M.), the Human Frontiers Science Program (M.S.) and a Medical Research Council Acute Brain Injury Collaborative grant (G0600986).

AUTHOR CONTRIBUTIONS

T.A.B. and C.F. designed the study. T.A.B. and F.F.M. conducted the behavioral and neurological assessments. T.A.B., C.F., M.R.C., M.H. and D.E.S. conducted the eyeblink conditioning task in the normal volunteers group and T.A.B., M.R.C. and C.F. conducted the task in the patient group. T.A.B., D.E.S., C.F., M.H., M.R.C., F.F.M. and M.S. analyzed and interpreted the data. T.A.B., D.E.S. and M.S. performed the statistical analysis. T.A.B., D.E.S. and M.S. drafted the manuscript. All of the authors revised the manuscript for important intellectual content.

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ONLINE METHODS

Patients. We chose 22 individuals with DOCs were chosen from a pool of 62 vegetative state or MCS patients using several inclusion criteria. The subjects were between 18 and 60 years of age, had relatively preserved auditory evoked potentials (normal or mildly delayed wave V) on at least one side, were three months from ictus or more, and had a preserved eye-blink reflex. We conducted comprehensive evaluations during 5 d of hospitalization in Buenos Aires or Cambridge. All of the DOC subjects underwent a full clinical assessment that included neurological examination, CRS[40] and Wessex head injury matrix[41] to establish each patient's behavioral profile. Subjects were given structural MRI, short latency auditory and somatosensory evoked potentials assessments. Additional evaluations were conducted in some patients: fMRI speech task, fMRI movement intention task, fMRI volition task, visual and motor evoked potentials, novelty evoked related potentials and a movement intention electromyographic task. We assessed the degree of cortical and subcortical atrophy using a visual rating scale[42] inspired by a previously developed scale[43]. Briefly, we first defined, using T1 three-dimensional anatomical images, atrophy levels from 0 to 4 (0 = no atrophy, 1 = very low, 2 = mild, 3 = severe and 4 = highly severe atrophy) in a group of 12 patients with neurodegenerative disorders and applied the scale to the DOC subjects (**Supplementary Table 4**). The subjects' T1-weighted images were assessed by two experienced raters (T.A.B. and F.F.M.) using a blind procedure. Signed assent from subjects' next of kin was acquired before investigation. The Cambridge Local Research Ethics Committee, the Raul Carrea Ethics Committee and the Fundacion Favaloro Ethics Committee approved this study.

Healthy volunteers (controlled consciousness group). Normal volunteers were free of physical and psychiatric illnesses, including head trauma. We carried out the trace-conditioning experiment on 16 subjects. None of them had a history of alcohol dependence or substance abuse and refrained from smoking and having caffeine intake for at least 4 h before the test. All control subjects included in the analysis matched DOC subjects in age and gender. Control subjects had normal hearing for their age, were naive to eye-blink conditioning procedures and were instructed to watch a silent video (Classic Chaplin movie) during the presentation of the stimuli. They were also told that they would receive a questionnaire about their experience during the task. Every participant gave written informed consent. No payment was received for taking part in the study.

Anesthetized subjects (controlled unconsciousness group). We carried out the test on 12 patients under the effect of intravenous propofol during standard endoscopic procedures (video-gastroscopy and colonoscopy), no muscle relaxants were given during the procedure and all of the subjects showed preserved eye-blink reflexes. The depth of anesthesia was controlled using clinical markers (heart rate, respiratory rate, muscular activity and bispectral index) by a specialized anesthesiologist. The learning procedure was performed during programmed endoscopic procedures of at least 30-min duration and it started no less than 3 min after anesthetic induction or when bispectral index levels reached 60 or less[44]. Every participant gave written informed consent. No payment was received for taking part in the study.

Experimental procedure. The stimulation apparatus was designed on the basis of the classic human eye-blink conditioner and consisted of an electronic device that controlled the delivery of the air puff and the auditory stimuli and sent a synchronization signal to the EMG recorder. Eye blinks were recorded with surface electrodes placed around the orbicularis oculi muscle of the right eye. One electrode was placed 1 cm lateral to the outer canthus and a second was placed 1 cm below the right eye. The ground electrode was aligned at the center of the subject's forehead. Electromyographic activity was acquired with a Keypoint machine (Medtronic) at 2,000 Hz in continuous recording mode. The conditioning parameters were similar to those used in a previous study[3]. The conditioned stimulus was a 75-dB, 250-ms, 1- or 2-kHz tone with a 5-ms rise-fall time; delivered binaurally (when appropriate) through earphones. It was followed by a silent period of 500 ms after which a 100-ms, 3-psi corneal air puff UCS was delivered to the right eye. The unpaired tones was either a white (static) noise or a 1- or 2-kHz tone that was not paired to an air puff. All DOC subjects' reflex responses to the air puff were tested at least 2 d before the conditioning experiment. The procedure consisted of a total of 140 conditioning trials (70 conditioned stimulus and 70 unpaired tones), with an intertrial interval ranging from 10–15 s. The order of conditioning trials was pseudorandom with the constraint that neither trial type occurred more than twice consecutively. No unpaired pseudo-conditioning trials were used before paired conditioning trials. The data was stored and later preprocessed with MATLAB (Mathworks).

The continuous EMG data was segmented in epochs starting 1,000 ms before and ending 2,000 ms after the conditioned stimulus onset. Data was low-pass filtered with a cutoff frequency of 50 Hz. We implemented an automatic artifact-detection algorithm. For each subject, we estimated the mean values and the s.d. of the baseline interval. Trials for which these values differed in more than two s.d. from the distribution were discarded. For further analysis, the EMG signal was normalized on a trial by trial basis, subtracting the mean and dividing by the s.d. of a window of 290 ms, starting 928 ms before the beginning of the trial. We then rectified the resulting signal. We refer to this as the nEMG, which has units of s.d. We then parsed this nEMG response in four critical temporal intervals (**Fig. 1**): the period before tone onset (baseline, −400 to 0 ms), a short period of 300 ms following the presentation of the tone (α response), a subsequent period (anticipatory interval) following this transient response and before the presentation of the puff (conditioned response), and the period following the onset of the air puff (unconditioned response). The anticipatory interval was divided into two intervals of the same duration: early (first half of the anticipatory interval) and late (second half of the anticipatory interval). We then averaged the nEMG activity to get a single value for each trial and interval. These values were used in all data analysis of anticipatory response with the exception of **Figures 2a** and **3a**, in which nEMG activity is averaged across trials.

The averaged nEMG activity for each trial and interval was submitted to a by subject ANOVA with 2 × 2 factorial design, in which the main factors were interval (baseline or late anticipatory), and stimulus (conditioned stimulus or unpaired tones). The significance level was set to $P = 0.05$. This value was used even when we presented multiple comparisons. Multiple comparison corrections would decrease the number of false positives at the expense of an increase in the number of false negatives; it was critical for our analysis to avoid false negatives. The significance of our results at the group level was very high; the probability of having as many as 9 out of 16 (as in control group) or 10 out of 22 positive results (as in DOC group) just by chance is $P < 10^{-6}$. As *post hoc* tests, we performed one-tailed paired *t* tests to evaluate the significance level of the difference between baseline and late anticipatory interval for both stimulus types (conditioned stimulus and unpaired tones) and for each subject (**Table 1**). Individual *t* values of these tests were plotted in **Figure 3b**. To compare the anticipatory interval between both stimulus types and for baseline interval between conditioned stimulus and unpaired tones, we performed unpaired *t* tests.

To quantify the change in the unconditioned response, as a measure of nonassociative learning, we estimated the latency and amplitude of each individual trial. To estimate it, we searched the amplitude and latency of the maximum and minimum peaks in the normalized, but not rectified, EMG data. The amplitude was calculated as the difference between both peaks' amplitudes and the latency was taken from the later of both. Because both parameters can vary broadly between subjects, we normalized them by subtracting the mean and dividing by s.d. for each subject separately. Finally, we plotted the first 20 trials of each subject (the average for the three groups is shown in **Fig. 5e**) and performed a linear regression to trial number.

Statistical analysis at the group level. We used SPSS v16.0 for nonparametric statistics at the group level and for regressions. Because of the small sample size and nonGaussian distribution, we used Kruskal-Wallis one-way ANOVA for main group comparison in the core measure of learning (conditioned stimulus late anticipatory period/baseline) and its control condition (unpaired tones late anticipatory period/baseline). For each measure, the differences between groups were investigated using a nonparametric Mann-Whitney U with exact (that is, nonasymptotic) significance assessment method. This method has been suggested to increase the reliability of results in small sample sets by dispensing with the assumptions required for the asymptotic method[45]. Two backwards regressions (on conditioned stimulus late anticipatory period/baseline and unpaired tones late anticipatory period/baseline) were performed to evaluate the explanatory power of the clinical measures (age, CRS, CAS and TFI) on learning for the DOC group. Two binary logistic regression were employed to evaluate the predictive power of learning measures (conditioned stimulus Late anticipatory period/baseline and unpaired tones Late anticipatory period/baseline) to discriminate between groups, one for vegetative state or nonvegetative state (clinical diagnosis)

doi:10.1038/nn.2391

and the other for TBI or nonTBI (cause of injury). Another binary logistic regression was undertaken to assess the power of conditioned stimulus Late anticipatory period/baseline to classify individuals in two groups. Inclusion of only one factor in the binary logistic regression allows us to maintain an approximate 1 to 20 ratio between regressors and observations[46].

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