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Behind an eye blink:

a new empirical perspective on intentional action

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Non chiedermi parole oggi non bastano.
Stanno nei dizionari: sia pure imprevedibili
nei loro incastri, sono consunte voci.
È sempre un prevedibile dejà vu.
Vorrei parlare con te - è lo stesso con Dio -
tramite segni umbratili di nervi,
elettrici messaggi che la psiche
trae dal cuore dell'universo.
Un fremere d'antenne, un disegno di danza,
un infinitesimo battere di ciglia,
la musica-ultrasuono che nemmeno
immaginava Bach.

Non chiedermi parole, oggi non bastano, Maria Luisa Spaziani.

Sommario

Il “blink” o “batter d’occhi” è un movimento di rapida chiusura e riapertura delle palpebre. Il “blink” può essere un movimento spontaneo, riflesso o volontario. “Blink” con identiche caratteristiche cinematiche possono avere differenti origini e significati. Per esempio, un blink può essere spontaneo quando ha la funzione fisiologica di creare un film lacrimale evitando la seccazione della cornea, può essere riflesso in riposta a stimoli esterni ed infine può essere volontario per comunicare un messaggio attraverso un canale comunicativo preservato, per esempio quando un paziente *locked in* cerca di comunicare gioia, accordo o disaccordo, frustrazione attraverso gli occhi (Laureys *et al*., 2005).

Lo scopo principale di questo studio è stato quello di trovare una misura oggettiva relativa alla distinzione tra un blink spontaneo e uno volontario: il “potenziale di preparazione” (*Readiness Potential*).

Il presente studio è quindi rilevante per due ragioni:

1. Nei soggetti sani, i blink spontanei appaiono con una frequenza di circa 1 ogni 5 secondi. Allo stesso tempo, i soggetti sani possono “controllare” il movimento spontaneo e riprodurlo in maniera intenzionale se opportunamente istruiti. In questo modo, il “blink” o “ammiccamento oculare” offre un contrasto ideale tra atto conscio e inconscio, a parità di cinematica. In questa prospettiva, l’analisi dell’attività cerebrale che precede un atto spontaneo o automatico messa a confronto con l’attività che precedere un “blink” volontario può offrire uno sguardo unico sui correlati neurali di un atto cosciente.
2. Nei pazienti con gravi cerebrolesioni, il “blink” è spesso l’unico atto motorio che può essere individuato. È infatti impossibile per molti pazienti effettuare movimenti più complessi. Per questo motivo, attraverso un condizionamento operante in cui ad uno specifico comportamento viene associato un rinforzo positivo, il nostro scopo è quello di indirizzare i pazienti ad associare un determinato tipo di “blink”, opportunamente selezionato, con un rinforzo positivo rappresentato da voci familiari/amiche che si suppone possano avere una valenza emotiva positiva per il paziente. Nella prima parte della tesi verranno introdotte le premesse teorico/sperimentali alla base dello studio e verranno presentati i materiali e metodi e i risultati relativi alla popolazione di controllo

(soggetti sani). Nella seconda parte, verrà introdotto il "disturbo di coscienza" dal punto di vista clinico, il nuovo protocollo sperimentale applicato ai pazienti con disturbo di coscienza e i risultati preliminari. In conclusione, verranno valutate le potenzialità dello studio da un punto di vista teorico, da un punto di vista clinico/riabilitativo ed infine da un punto di vista etico.

Abstract

Blinking is a rapid closing and opening of the eyelid. Eye blinks with identical kinematical features can have different origins and meanings. For example, one can blink automatically, due to a simple reflex arc – such as when moistening the cornea – or one can blink voluntarily to communicate a fundamental message – such as when a locked-in patient communicates that he/she is happy or frustrated (Laureys, *et al*., 2005) The main aim of the present project is to find a brain-based objective way to know whether a given blink is a meaningless automatic neural event or the endpoint of a complex conscious process. The proposal builds up on the empirical work by Kornhuber & Deecke and Benjamin Libet, who showed that the awareness of intention to move is preceded by a recordable cerebral activity called "Readiness Potential".

The present proposal is relevant for two reasons:

1. In healthy subjects, automatic blinking occurs spontaneously every 5 seconds, or so. At the same time, healthy subjects can be instructed to blink voluntarily in a controlled fashion. In this way, blinking offers the ideal contrast between unconscious and conscious acts – the physical, kinematic aspects of the movement being equal. In this perspective, analyzing brain activity prior to automatic and voluntary blinks may offer a unique insight on the neural correlates of a conscious act.
2. In patients with severe brain injuries blinking is often the only motor act that can be reliably detected. By employing operant conditioning, we aim at training patients on the association between a specific eyelid closure and a positive reinforcement.

   Specifically, Readiness Potential like activity will be computed on the cortical activity preceding eye blinking as a measure of "volition," first in healthy controls and then in vegetative and minimally conscious state patients undergoing operant conditioning.

In healthy controls, we will contrast spontaneous blinks against voluntary blinks. The results of this experiment are meant to explore the dynamic range of the changes in brain activity that underlies voluntary vs. spontaneous blinks in controlled conditions.

In patients, detecting a progressive increase in the strength or complexity of brain activity (up to the levels obtained in healthy subjects during voluntary blinks) during the

course of the conditioning sessions will indicate that their blinking might reflect a voluntary act.

Ultimately, this project, if successful, will link operant conditioning to the long-standing topic of the neural substrates of a wilful decision to act, bearing important scientific/ethical implications.

The novelty of this project rests on:

a. Exploring, empirically, the relationships between brain activity and the will. The underlying hypothesis guiding this project is that a wilful act should be reflected, to some measurable degree, in high levels of anticipatory brain dynamics.

b. Taking Libet's work one-step forward, by using slow cortical potentials such as the "Readiness Potential" as a neural marker of volition.

c. Using the "Readiness Potential" to distinguish between spontaneous and voluntary blinks.

d. Answering the critical question of whether the blinks produced by vegetative patients after a conditioning protocol are voluntary or not.

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List of abbreviations

BA: Brodmann Area

BP: Bereitschaftspotentiale

CMA: Cingulate Motor Area

CNC-S: Coma Near-Coma Scale

CNV: Contingent Negative Variation

CRS-R: Coma Recovery Scale – Revised

DC: Direct Current

DOC: Disorder of consciousness

EEG: Electroencephalography

EMCS: Emergence from Minimally Conscious State

EMG: Electromyogram

EOG: Electrooculogram

ERP: Event-Related Potential

FEF: Frontal Eye Fields

fMRI: functional Magnetic Resonance Imaging

FPN: Fronto-parietal network

GCS: Glasgow Coma Scale

LIS: Locked-in Syndrome

LPS: Levator Palpebrae Superioris

LRP: Lateralized Readiness Potential

MCS: Minimally conscious state

MP: Motor Potential

MRCP: Movement-related Cortical Potentials

NS: Negative Slope

OOm: Orbicularis Oculi muscle

PLV: Phase Locking Value

RAP: Reafferent-Potential

RAS: Reticular Activating System

RP: Readiness Potential

SCP: Slow Cortical Potentials

SEF: Supplementary Eye Fields

SMA: Supplementary Motor Area

SMC: Suppementary Motor Cortex

SNW: Slow Negative Wave

SPN: Stimulus-Preceding Negativity

UWS: Unresponsive wakefullness syndrome (also referred as VS)

VS: Vegetative state (also referred as UWS)

Introduction

"She raised one hand and flexed its fingers and wondered, as he had sometimes before, how this thing this machine for gripping, this fleshy spider on the end of her arm, came to be hers, entirely at her command. Or did it have some little life of its own? She bent her finger and straightened it. The mystery was in the instance before it moved, the dividing movement between not moving and moving, when her intention took effect. It was like a wave breaking. If she could only find herself at the crest, she thought, she might find the secret of herself, that part that was really in charge. She brought her forefinger closer to her face and stared at it, urging it to move. It remained still because she was pretending, she was not entirely serious, and because willing it to move, or being about to move it, was not the same as actually moving it. And when she did crook it finally, the action seemed to start in the finger itself, not in some part of her mind. When did it know to move, when did she know to move it?"

Ian McEwan, *Atonement*

The study of volition is tightly linked with the notion of consciousness. In this thesis, I will approach the notion of volition from a neuroscientific perspective. I will use the term "volition" and "intentionality" as synonymous, in a naïve way, simply to refer to conscious acts performed and thus intentioned by the subject him/herself both as self-initiated or self-paced. This thesis is based on the idea that there is a difference, in terms of neural activity, between intentional movements and non-intentional movements. The experience of an intentional action makes a difference in the brain.
Chapter 1 is based on the theoretical investigation of "Slow Cortical Potentials," the class of brain potentials usually connected with brain preparatory processes. In particular, I focused on a specific slow cortical potential, the "Readiness Potential," that starting from the pioneering work of Kornhuber & Deecke is associated with intentional/self-paced movements. The Readiness Potential received greater attention since the seminal work of Libet, who demonstrated that the specific time course of this

potential is not compatible with the concept of "free will."[1] Basically the Readiness Potential, as "neural signature of volition" appeared well before the subject became conscious of the intention to move. To what extent conscious activity is causally relevant for the action remains an open question of scientific and philosophical relevance, but this will not be directly addressed in this thesis. Rather, I will discuss how the different interpretations of the Readiness Potential could provide information about different aspects of volitional movements.

Chapter 2 presents the description of spontaneous, voluntarily, and reflex eye blinks in human physiology.

In Chapter 3, I will present a new empirical setup aiming at distinguishing between a spontaneous and intentional movement, namely "eye blink" (Chapter 2), in healthy control subjects. The importance of the "eye blink", as one of the few motor acts that can be executed both spontaneously and voluntarily, has increased in the light of the results obtained from healthy subjects. Intentional blinks are differentiated from spontaneous blinks, in that the Readiness Potential activity emerges just before intentional eye blinks, and not for spontaneous eye blinks.

Results from the experiment and their relevance in the context of the state of art will be discussed in Chapters 4 and 5.

In Chapter 6, I will introduce the peculiar medical condition defined as "Disorder of Consciousness" (DOC). After a theoretical characterization of this condition, I will explain why such condition requires a specific and tailored examination both from the behavioural and from the neural perspectives.

Starting from healthy subjects' results, I will then describe how the "eye-blink protocol" is applied to one DOC patient and one Locked-in Syndrome patient (LIS).

This study has clinical, theoretical and ethical implications. I will discuss the importance of our study in light of the existent field of research on patients with disorder of consciousness.

Finally, in Chapter 7 I will present some results derived from the study of the Readiness Potential for voluntary eye blink in epileptic patients stereotactically

[1] This is a theoretical inference derived from Libet's experiments; it is not directly showed by Libet's empirical results. On the contrary, Libet claimed that Readiness Potential is compatible with free will.

implanted with depth multi-lead electrodes (SEEG). The study of the Readiness Potential from a different perspective—that is, intracranial activity—can in fact shed light on the actual origin of such slow cortical potentials.

Part 1. Introduction to slow cortical potentials and Eye-Blink

Chapter 1

1.1 Slow cortical potentials

Slow cortical potentials (SCP) are described as electrical potentials recorded from the scalp with a frequency below 1Hz. They are also referred to as DC-potentials (amplifier no high-pass filter –low cut off). They do not represent rhythmic activity, in fact they may change within a period of a few hundred millisecond up to several seconds (fluctuations not oscillations). (Caton, 1875)

Cortical generators of slow cortical potential are identified in excitatory postsynaptic potentials (EPSPs) at the apical dendrites of vertical arranged pyramidal neurons in the cortex. They have a large distribution since these neurons have large distance connections.

While SCPs are always present, they show specific characteristics if they are recorded time-locked to a stimulus as event related potential (ERP) (Birbaumer et al. 1990)

Principal "Slow Cortical Potentials" are:

1. SNW (slow negative wave) – it follows an unexpected stimulus, resulting in an orienting response

2. SPN (stimulus preceding negativity) – sustained negativity over parietal and frontal cortex during the waiting period for a feedback stimulus after a time estimation task (Brunia, 1988)

3. CNV (contingent negative variation) – when the first stimulus indicates the occurrence of the second stimulus. CNV can be recorded in the inter-stimulus interval. (Walter, 1964)[2] (see Figure 1)

[2] In 1964 slow cortical potentials received a great attention. In fact, while Kornhuber and Deecke theorized for the first time the existence of a recordable brain activity preceding voluntary movement ("Readiness Potential"), Walter, a British neurophysiologist, created protocol to elicit a different potential called "Contingent

4. BP/RP (readiness potential) – it precedes a voluntary movement of approximately 1/1.5sec.

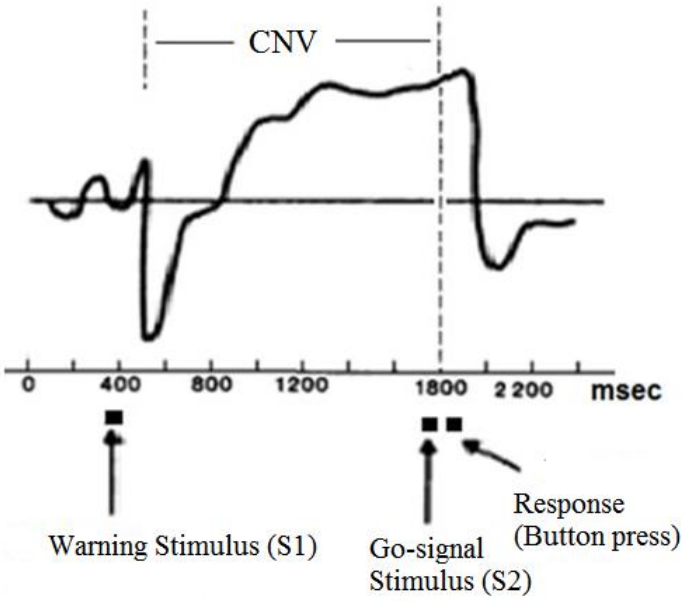

Figure 1. *Contingent Negative Variation (CNV) elicited by a Warning Stimulus (S1) and a Go-signal Stimulus (S2)*

1.1.1 Readiness potential, or Bereitschaftspotential: a slow negative potential before self-paced movements

Since Hans Helmut Kornhuber and Luder Deecke's seminal work a huge amount of empirical evidence was collected in order to demonstrate that well before an intentional action occurs, a significant activity – a negative slow voltage in EEG signal – appears in specific brain regions, namely motor cortex and supplementary motor area.

Negative Variation (CNV)". The idea is to present two stimuli, a conditional stimulus indicating that after 1 or 2 sec an imperative stimulus is going to be presented. "Contingent Negative Variation" is described as the Event Related Potential (ERP) that appears between these two stimuli. In particular, Walter described a negative potential that appears immediately after the conditional response and endures until the imperative response, after which it decreases immediately or becomes positive.

The context of the discovery, as usually happens for some lucky findings in science, was pretty casual. In the spring of 1964, while discussing about brain research over a lunch break in "Gasthaus zum Schwanen," at the foot of the Schlossberg hill in Freiburg, Kornhuber and Deecke –a Tenured Professor and a Ph.D. student, respectively– found themselves agreeing on a very simple fact. At that time brain investigations were mainly focused on the analysis of passive states of the brain or the sensory evoked brain potentials. What was lacking was a specific examination of active states, in particular self-initiated actions. Starting from that moment, their work was focused on the creation of an experimental setup aiming at characterizing the peculiar aspects of intentional action in terms of brain activity. The simple but powerful idea was to collect a series of intentional movements (each movement corresponding to a trial), for example finger flexions, and to average out brain activity time-locked to the onset of each movement.

From the average of at least 40 trials (e.g. finger or wrist flexions) they found a specific surface negative slow potential appearing 1/1.5 sec before movements. This specific activity was then called "Bereitschaftspotential," the German word for "Readiness Potential."

The "Readiness Potential" is a slow cortical negative voltage that appears with a frequency below 1 Hz. This means that compared to other well known potentials, such as "alpha rhythm," the Readiness Potential is ten to a hundred times or slower. The direct implication is that the Readiness Potential is not clearly visible from a single trial, the average of different trials is necessary in order for this potential to appear. Moreover, it is usually best visible only when the EEG recording is acquired in DC (*direct current*). A DC amplifier does not omit any low frequencies (no high-pass filter/low cut-off) and the DC component of the signal is captured along with all the rest.

For this reason, such slow changes in the EEG had been disregarded for a long time. Moreover, while slow cortical potentials are always present, they show a specific characteristic if they are recorded time-locked to a stimulus such as an event related potential. The clever idea from Kornhuber and Deecke was to use the self-paced or

intentional movement as the event, and then average out brain activity prior to that movement.

There are small potentials hidden in the rhythmic activity of the human EEG that can only be recorded if they are reproducible and time-locked to a certain event capable of triggering an averaging computer.

(Kornhuber & Deecke, 1976)

In Kornhuber and Deecke's experimental protocol, the subjects are instructed to perform their movements at irregular intervals based on their own will (i.e. they had to move out of free will). In this protocol movements are internally driven without any external cues. This protocol is usually referred as "self-paced" protocol because the subject has to decide "when to move" of his/her own volition. It is worth noting that subjects are explicitly asked to avoid rhythmic activity and they are asked to make movements at irregular intervals in order to preserve the "volitional" aspect of action, otherwise there is a risk to create an automatism. In self-paced protocols, as the one developed by Kornhuber and Deecke the action does not have to be pre-planned but it has to emerge as the actual product of free will or intentionality. In this respect, this is the first scientific protocol that tries to operationalize volition based on the intuition of following the development of intentional action not only from the response, but also by looking at what happens in the brain well before the response itself occurs.

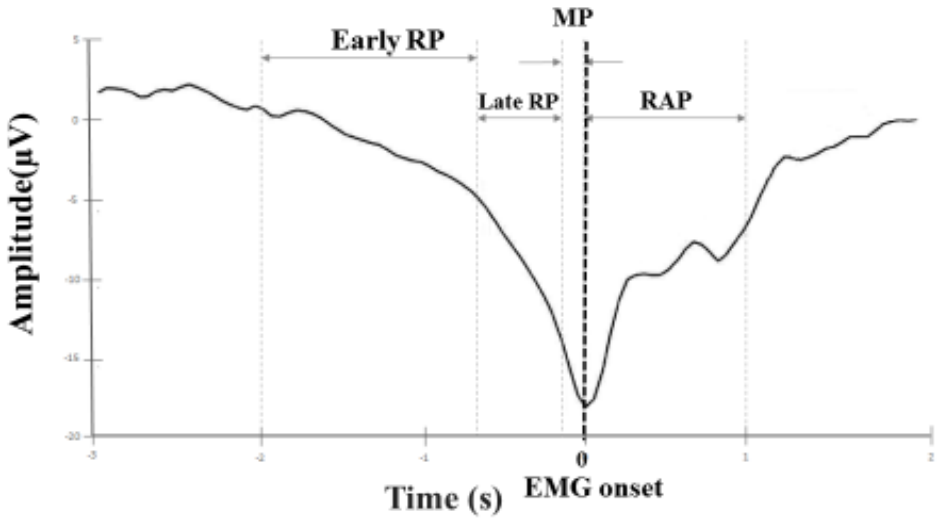

Figure 2. *Different parts of Readiness Potential: Early RP, Late RP, Motor Potential (MP), Reafferent Potential (RAP). All these potentials are also commonly named "movement-related cortical potentials" (MRCPs)*

As shown in figure 2, the Readiness Potential seems to be composed by two different parts. The early part of the RP is a slowly rising phase of the waveform, while the late RP is an abrupt change in the slope of the wave. These two different parts of the wave are usually denominated "BP, early BP, BP1 and negative slope 1 (NS1)," and "late BP, BP2, NS', NS2," respectively. After movement, there is a specific pattern that is usually referred to as the "Reafferent Potential" (RAP), as shown in Figure 2.

The early, late, and motor potential differ in terms of distribution over the scalp. The early part of the RP is bilaterally symmetrical, while the late and motor potential are asymmetrically distributed and maximal over the contralateral precentral areas.

Different parts of the Readiness Potential are meant to reflect different levels of intentionality at the brain level. Since Benjamin Libet's groundbreaking investigations (see Section 1.1.1) the early part of the RP is usually considered to start unconsciously and it might represent a general mechanism of arousal that indicates that the brain is starting to prepare for the future movement. The late part of the RP is associated with

the action itself, and it seems to reflect the volitional aspect of the movement, as it is absent for automatic or non-intentional movements. The "Motor Potential" (MP) is totally related to movement, regardless of whether it is pre-planned or not. It can be present both if the movement appears as the endpoint of a complex cascade of neuronal and cognitive events, and if the movement seems to be performed without any specific pre-planning, out of the blue.

The Reafferent potential (RAP), might account for the somatosensory feedback deriving from the match between the intended movement and the actual movement performed.

To estimate the precise localization of generator sources of the RP various studies have been conducted with several dipole source localization techniques.[3] The main hypothesis about likely generators of the RP is that the early RP starts first in the supplementary motor area (SMA), shortly after in the lateral premotor cortices bilaterally, until the activation of M1 and the premotor cortex for the generation of the late RP (NS') at about 400 ms before the movement onset. Then, just before the movement onset, the late BP culminates in MP in M1.

The early RP is mostly modulated by cognitive functions, such as the level of intention, movement selection, and preparatory state, while the late RP is sensitive to the movement's features, such as discreteness, precision and complexity.

In general, the Readiness Potential can be influenced by several factors, such as:

- Preparatory state (see Libet's distinction between type I and II of the RP; see Figure 4)
- Movement selection (freely selected vs. fixed)
- Learning and skill acquisition
- Pace of movement repetition
- Praxis movement

[3] Nagamine et al. 1996; Cui and Deecke, 1999; Toma et al., 2002; Kornhuber and Deecke 2003

- Perceived effort
- Force exerted
- Speed and precision of movement
- Discreteness and complexity of movement
- Pathological lesions of various brain structures

The Readiness Potential should be regarded as a complex and dynamic phenomenon. It is very difficult to isolate a single component or the specific processes involved in the movement. For example, as suggested by Jahanashi,[4] it is possible that different frontal and prefrontal areas differentially contribute to the various cognitive, motivational, and motor processes involved in the RP. It might be that while the prefrontal area is engaged in the decision-making process for response selection, timing or initiation of action, the pre-SMA/cingulate motor area and lateral premotor cortex may be involved in the preparatory processes. This can be in contrast to the activation of the SMA proper and motor cortex, which may be most likely associated with the actual initiation, and execution of movement. To this purpose, it could prove pivotal to develop specific tasks, with the aim to disentangle different concurrent processes in volitional action.

1.1.2 Benjamin Libet and the unconscious preparation of self-initiated voluntary movement

In 1983 the psychologist Benjamin Libet replicated Kornhuber & Deecke's experiment, while adding a modification of the protocol. He asked subjects to indicate on a rotating cathode ray oscilloscope (CRO) (Figure 3) the position of a moving bright dot when he/she was aware of the conscious decision to move a finger (W-time). The quadrangle of the oscilloscope was divided into 60 parts, such as those of a clock, but each lap lasted 2.56 seconds, so every second corresponded actually to 43 milliseconds. The experiment is conducted in this way: for each subject the EMG activity is recorded to detect the starting point of the movement (finger or wrist

[4] Jahanshahi et al., 2001.

flexion) trough specific EMG electrodes. Simultaneously also recorded EEG activity trough EEG cap. After each movement – that corresponds to a trial[5] – the subject has also to report after the execution of the movement when he/she became consciously aware of the decision to move the finger. In particular, he/she has to bear in mind and then report after each trial the dot position on oscilloscope when he/she felt the urge to move. Subjects are specifically instructed not to target a particular time interval as the time of their movement, but rather to "let the urge to act appear on its own at any time without any preplanning or concentration on when to act" (Libet, 1983). For this reason, these kinds of movements are classified as "self-initiated" to distinguish them from "self-paced" ones, in order to stress the fact the self-initiated movements seem to be carried out by the subject's free will, i.e., without any concern about when to act.[6]

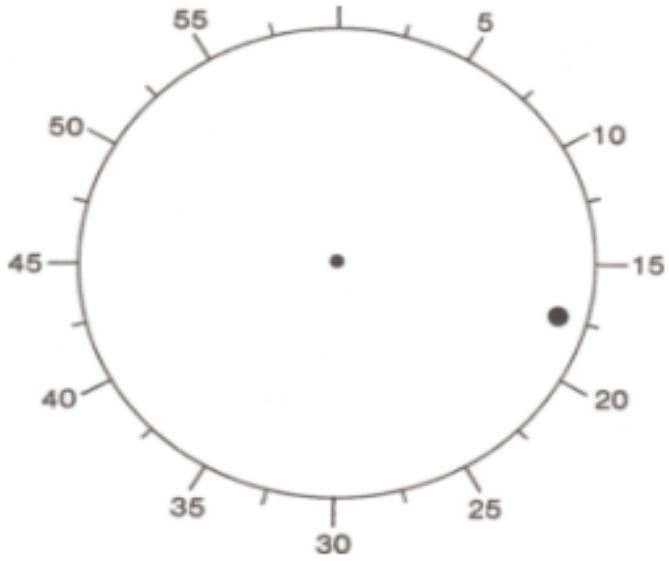

Figure 3, *Cathode ray oscilloscope*

[5] For each subject blocks of 40 trials (on average) are recorded.

[6] In Kornhuber & Deecke's protocol subjects produce self-paced movements at irregular intervals; they are also required to vary the intervals between movements and to keep intervals between two consecutive movements within certain limits. In contrast, in Libet's protocol movements have to be performed just by following the "urge to move".

If we assume that the Readiness Potential reflects the neural preparation for voluntary movement, then what Libet found is a gap between neural preparation to act and the conscious will to act. Specifically, the subject's reported a W-time (the time of will or the decision to move) that is/was systematically shifted about 500 ms after the rise of Readiness Potential and before the purely motor preparation (M-time). Libet also created a different set of tasks to systematically assess how the "preplanning" component might affect the duration of the RP. As showed in figure 4, pre-planned movements seem to be characterized by a longer RP (around 1000 ms before of action itself) in comparison with not pre-planned movements, where the RP appears around 500 ms before the movement.

What do these results mean? For the first time in modern neuroscience, an experimental protocol tried to demonstrate that a conscious decision does not cause the movement, but rather that the brain activity (the Readiness Potential) bringing about the movement starts before the individual wills anything to happen.

Starting from these findings, a huge amount of literature was produced in order to discuss how Libet's experiments might create a serious problem for free will. The existence of a neural indicator (RP) that appears before the conscious will to act seems in fact to challenge the common sense's intuition about our free deliberation, or the folk-psychological notion of intention.[7]

More recently, in 2011, Fried[8] replicated Libet's findings at the scale of the single neuron. This experiment represents an important step towards a deeper understanding of volition. Intracranial data provide a unique contribution too, in that they can help explore the actual origin of Readiness Potential not only from the cortical areas but also from the subcortical structures, thereby providing reliable information about the Readiness Potential timing. On those specific patients, intracranial electrodes are used for evaluation prior to neurosurgery. Through depth electrodes it is possible to detect

[7] Folk-psychology's intuition about intention and motor acts is that intention is the cause of future action. In a hierarchical structure, intentions trigger a cascade of more concrete processes: from deliberation through intention, from the planning to the actual execution of the action. (Schurger & Uithol 2015)

[8] Fried et al. (2011)

the firing patterns of single neurons. Subject were asked to perform the same task as the one proposed by Libet, in order to reproduce the same experimental conditions.

Results showed a gradual build-up of activity of a cluster of single neurons in the medial frontal lobe. Interestingly, Fried's study suggested that a preconscious activity in the SMA at the level of single neurons was present prior to subject's actual intention to move (W-time). As Fried wrote: "taken together, these findings lend support to the view that the experience of will emerges as the culmination of premotor activity – probably in combination with networks in parietal cortex– starting several hundreds of ms before awareness." However, "the scientific, philosophical and societal implications of these findings remain open for debate."[9]

What Libet's and other similar experiments suggest (*e.g.* Soon *et al*., 2008, 2013) is in fact that our brain, rather than our subjectivity, is in charge when we decide to act. But is this the case?

Libet himself did not want to push that way further, as he also made room for a veto (free won't) over the alleged brain decision, before the action's taking place. In this respect, the subject would keep control over the temporal dynamics of his/her voluntary action, and has therefore free will to decide whether to do it or not. The following figure from Banks and Pockett, 2007 summarizes well Libet's findings:

[9] Fried et al. (2011)

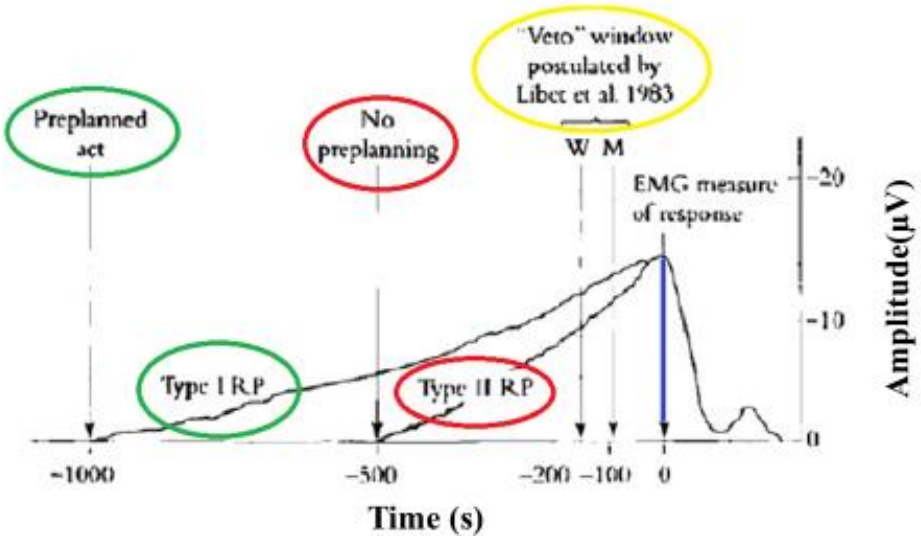

Figure 4. *Preplanned vs no preplanned acts: Type I RP, Type II RP*

1.1.3 Volition and self-paced protocols

This is not the place to discuss in depth the issue of free will and all its possible theoretical implications. However, it is important to mention a few possible criticisms to Libet's findings:

1. These experiments do not probe directly the decision to move or not to move, but rather the decision about when to follow an instruction.
2. Simple motor acts, such as finger flexion, are not the right paradigm for the investigation of free will. These acts cannot catch the complexity and the dynamical aspects of actions from which free will emerges.
3. Much longer timescales or real dynamic environment are the significant context where to test assumptions about free will.

In general, Libet-like experiments can be seen as an attempt to operationalize free will. The experimental environment is restricted and deals with very few variables in comparison to the complex and multi-faceted aspects of every day life's decision-making.

In addition, in Libet-like protocols the understanding of voluntary action is restricted to self-paced or self-initiated movements, which represent a specific class of actions. As showed in figure 5 below, a plethora of different movements can be grouped in different types. Along a hypothetical continuum that goes from reflex movements to goal directed movements, self-paced movements partially reflect heterogeneous aspects of volition.

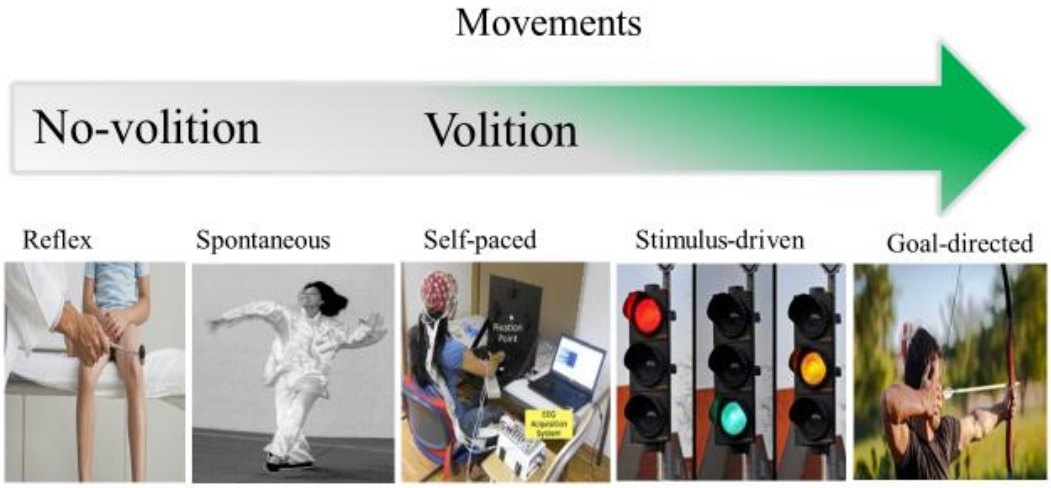

Figure 5. *An hypothetical continuum of volitional movements*

Voluntary movements can be sub-grouped in three class: cued or stimulus-driven actions, self-initiated/self-paced, and goal directed. As showed in table 6, in self-paced or self-initiated protocol the subject has to decide "when" to move. The given instruction "move your finger when you feel the urge to move" can be in fact interpreted as "decide when to move." The task is restricted to the fact that:

1. The subject has to move sooner or later; the decision about whether to move is therefore not taken in account by the subject.
2. She/he knows which movement has to do or what to do; the choice is not related to the type of movement to execute.
3. The only free choice seems to be the one about when to move.

A more comprehensive account of voluntary movements needs to take in account different aspects of volition or decision. It can be useful to build a better theoretical

schema of what are the characteristics of voluntary action. For example, a hypothetical continuum of voluntary action whose extremes are represented by the self-paced protocol on one side, and cued-actions on the other side as shown in table 6, can be constructed in order to best understand which processes are needed for the manifestation of voluntary action.

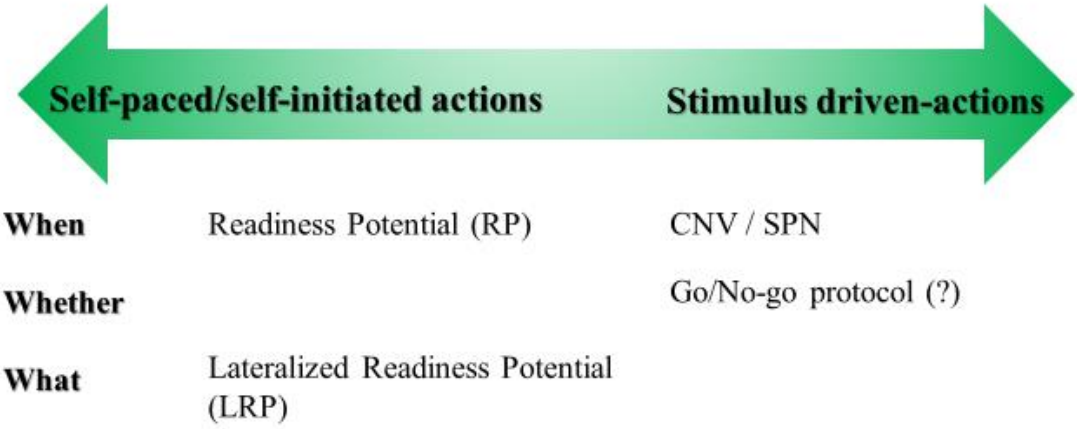

Figure 6. *Self-paced vs Stimulus Driven Actions*

As shown in the table 6, manipulating the "when" variable results in two different potentials: Readiness Potential and Contingent Negative Variation (CNV).

Traditionally, self-initiated actions are taken to differ from cued-actions in that while in self-initiated actions the decision about when to move is internally driven by the subject, in the cued protocol the cue is externally driven. In the stimulus-driven protocol, the trigger of movement is outside the subject.
Externally driven actions are in fact forced with respect to the time (a cue can signal when to move as in the CNV protocol), or with respect to the "whether"-choice (for example in the Go/No-go protocol, a test that requires a participant to perform an action given certain stimuli e.g., press a button – Go— and to inhibit that action under a different set of stimuli —e.g., not press that same button - No-Go.

The "what" dimension can affect the decision-making process, but it does not seem crucial in volition. The choice of what to move seems in fact to represent a further dimension in the volitional process, one that appears before the "when"- and the

"whether"- decision. The Lateralized Readiness Potential (LRP), which is associated with unilateral hand movement, appears as a negative slope more prominent over the motor cortex contralateral to the chosen hand. The LRP usually appears after the early RP in the late part of the RP as a final choice relative to the side of movement to perform.

Regarding the CNV and the RP, they appear as potentials with a similar morphology. Conceivably, both the RP and the CNV deal with "volition" in different manners. The RP is presumably elicited by self-paced/self-initiated protocols, while the CNV emerged in cued protocols.

Self-paced and stimulus-driven action can be described as two species of the same genus: volitional acts.

Goal-oriented actions are instead a more complex behaviour to test experimentally; they represent in fact dynamic aspects of action, usually requiring long-term decision making, whose composite temporal-dimension is difficult to replicate in the experimental environment.

Summarizing, in the experimental protocols regarding volitional actions, manipulated variables are the "when," "whether," and "what" variables. In particular, the "when" variable seems to be crucial both in self-paced protocols and in stimulus-driven protocols.

1.1.4 Different routes for the same potential?

Several neuroimaging studies[10] comparing the neural activity for self-paced actions and stimulus driven actions found that the activation of the pre-SMA is stronger for self-paced actions than for stimulus-driven ones. As Haggard pointed out,[11] the starting point of the Readiness Potential is seen as the initiation "of a cascade of neural activity" that flows from the pre-SMA back to the SMA proper and the M1 (see Figure 7, left side). However, the pre-SMA itself has to be triggered in order to produce the neural activity cascade. It has been suggested that the basal ganglia can convey inputs

[10] Jenkins I.H. et al., (2000); Deiber M. P et al., (1999)
[11] Haggard P., (2008)

to the pre-SMA. Intracranial studies[12] showed that the RP could be recordable also from subcortical sites; in particular, the RP in the basal ganglia preceded the onset of movement by 1500 ms. As shown in figure 7 (right side) information from early sensory cortices (S1) is linked to a level-representation in the parietal cortex in order to orientate goal-directed movements, such as grasping. This has been suggested as a plausible network for stimulus-driven actions.

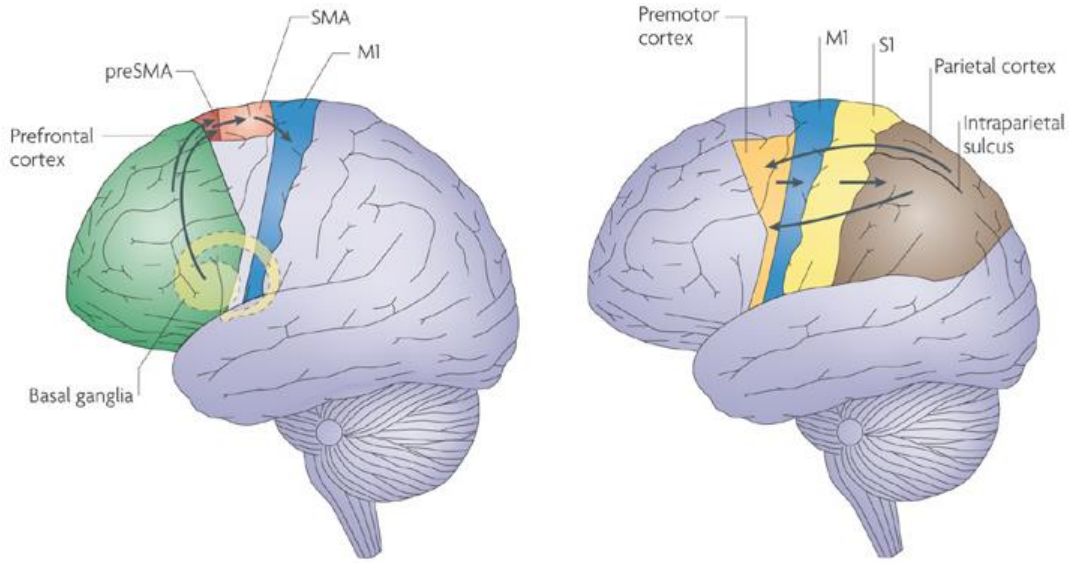

Figure 7. *Brain pathways for voluntary action: on the left side the brain circuit for self-paced action, on the right side the brain circuit for stimulus-driven action (Haggard, 2008)*

In conclusion, the specific shape of the Readiness Potential can be explained, as showed in Figure 7 (right side), by an activation of the prefrontal cortex, in which deliberation takes place, followed by the pre-SMA, where the action is planned together with other pre-motor areas. Immediately before the action takes place, there is an activation of the M1 (that is specific in respect of the side of the movement).(Figure 8)

[12] Rektor I., (2008)

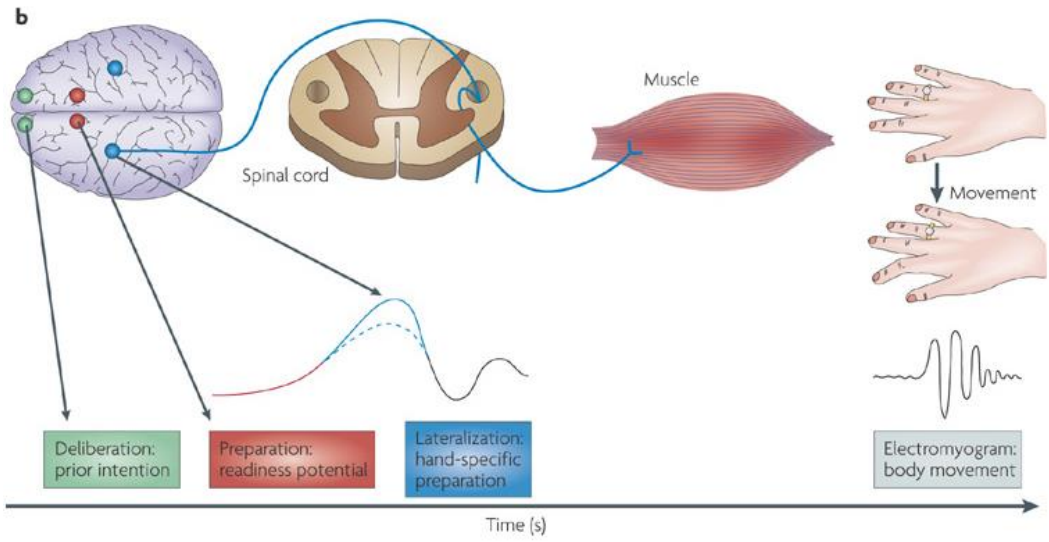

Figure 8. *Lateralized Readiness Potential (LRP)*

1.1.5 The Readiness Potential's slippery slope[13]

In the free-will debate there are at least two categories of interpretation regarding the nature of the "Readiness Potential:"

1. The Classical view: the RP is the neural signature of volition – or the RP causes the action (Kornhuber & Deecke, Libet)[14] (Figure 9)
2. The Alternative view: the RP is not causally related to volitional act – the RP might correlate with the voluntary action (Schurger)[15] (Figure 9)

[13] The *Slippery slope argument***,** in logic, is the fallacy of arguing that a certain course of action is undesirable or that a certain proposition is implausible because it leads to an undesirable or implausible conclusion via a series of tenuously connected premises, each of which is understood to lead, causally or logically, to the premise (or conclusion) that follows it. Sometimes the same fallacy is also called "domino fallacy" precisely because we can never know if a whole series of events and/or a certain result is determined to follow one event or action in particular.
Here, we use "slippery slope" as a word pun where the slope is represented by the characteristic shape of the Readiness Potential and the adjective "slippery" refers to the "standard" interpretation of the negative rising slope preceding a voluntary movement that, starting from Libet, was interpreted as the causally deterministic process taking place before the occurrence of a voluntary act.
[14] Kornhuber & Deecke (1965)
[15] Schurger, A., (2012)

2.1 The RP appears also for invertebrates before spontaneous (non-intentional) initiation of movement (Kagaya &Takahata)[16]

The interpretation of Readiness Potential in Libet's framework has been already discussed in section 1.1.1; here the alternative interpretation to the "classical" view of the Readiness Potential will be described.

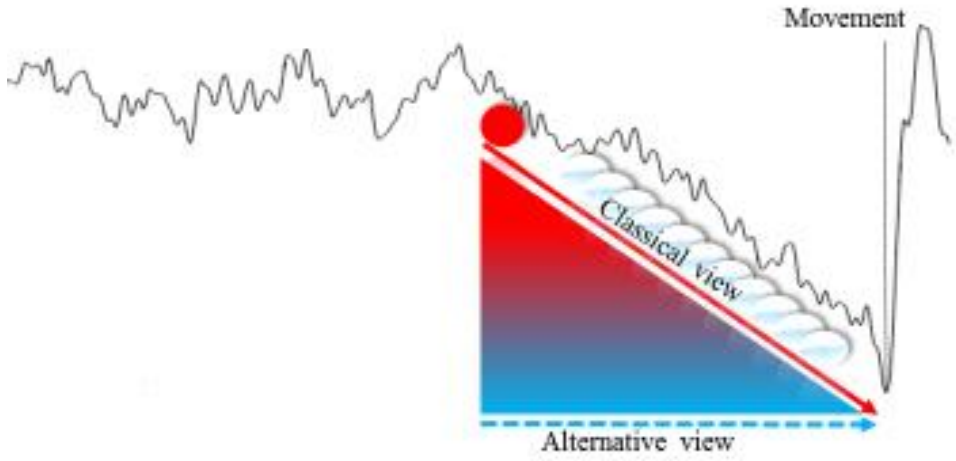

Figure 9. *The Classical and the Alternative interpretation of the Readiness Potential*

The "alternative" view generates from the intuition that:

> A gradual increase in neural activity preceding spontaneous[17]movements appears to be a very general phenomenon, common to both vertebrates and invertebrates alike.[18]

The actual nature of the Readiness Potential can be understood, according to Schurger, only if we discard our common intuitions about free will and causation. The Readiness Potential is not the neural sign, nor the neural mechanism of volition, but it should be interpreted as a general (not specific) fluctuation of the EEG activity.[19] The RP appears

[16] Kagaya K., Takahata M., (2010)

[17] The term "spontaneous" in this specific framework refers to a non-intentional movement.

[18] Schurger, A., (2012)

[19] This idea is already present in some other authors as Eccles (1985): "there is a tendency for the initiation of movement to occur during the excitatory phase of the

in fact only if we average out different trials, as it is very unlikely to detect the RP at a single trial. Fluctuations of the EEG activity are present both during the rest-activity (no task-condition) and during the voluntary movement. Even though these fluctuations can contribute to voluntary movement, they are not the cause of it.

Taking in mind the assumption that neural activity might undergo spontaneous fluctuations,[20] and borrowing from "decision-making" theories the notion of "accumulator models,"[21] Schurger postulated that voluntary movements occur whenever there is enough accumulation of evidence in order for the neural activity to reach the threshold, in lack of any external cue. But what is the origin of the evidence? In Libet's task, the subject is instructed not to pre-plan the action but to move whenever he/she feels the urge to move, so the evidence is based on a "weak imperative to move." In other words, there is an implicit imperative during all task: subjects are free to move whenever they want but they have to move at some point. Moreover, if the imperative to move is weak, the precise moment at which the decision (threshold) is crossed is determined by spontaneous sub-thresholds fluctuations in neural activity. In order to test empirically this model, Schurger created the "Libetus interruptus" task. The task is identical to the classical Libet's task, with the addition of a few random interruptions: an audio tone (click) that "forces" the subject to move as fast as possible after the sound. The model predicts that faster reaction times were preceded by a negative-going voltage deflection before the movement onset. In other words, faster reaction times are obtained if the neural activity is closer to the postulated threshold.

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random spontaneous activity." Schurger's theoretical framework is based on Eccles' suggestion regarding the existence of spontaneous fluctuations in global activity of large populations of cortical neurons; these fluctuations can be present either without any intention or movement, or to facilitate and allow a movement.

[20] Spontaneous fluctuations in the brain can be understood as $1/f$ noise or auto-correlated noise (pink noise). This means that faster fluctuations are smaller in amplitude or that there is a diminishing power for higher frequency. In some studies, "channel noise" in neurons, which is thought to arise from the random opening and closing of ion channels in the cell membrane, is seen to be $1/f$.

[21] In accumulator models a response is triggered when a signal that represents the decision process (activation function) grows over time to reach a threshold level. (See Hanes & Schall, 1996)

This build-up even preceded random unpredictable cues; in this respect, the RP cannot reflect the preparation of movement.[22]

In this new framework, the Readiness Potential is not a unique phenomenon directly linked with voluntary action, but it is best characterized as composed by two parts. The first part is primarily based on stochastic fluctuations, while the last part –around 150 ms before of the movement that coincides approximately with W-time— is purely motoric, in the sense that it is linked with the movement and it can be seen as pure "motor preparation."

As Gomes noticed well before Schurger's argument:

> […] the hypothesis that spontaneous fluctuations in the global activity of large populations of cortical neurons, such as those reflected in the earlier part of the RP, should regularly occur, independently of any intention or movement, so as to allow the production of voluntary acts […] would be very difficult to test, due to the absence of a common reference time from which to average different EEG tracings of subject at rest. […].
>
> In fact, in a single EEG tracing the RP itself is not visible. […] One needs to average many EEG tracings to obtain the RP, and a common reference time is necessary for this (in the case of voluntary acts, it is the movement onset time). At present, there seems to be no way of testing this hypothesis, but in principle it should be testable.[23]

In addition to, and in line with, Schurger's perspective, different authors stressed the idea that the Readiness Potential might not reflect just pure motor preparation.[24] Mixing trials where the subject decided to choose a letter in a stream of letter (rapid serial visual presentation (RSVP)) with no movement, and trials where decision is matched with a movement, these authors found that the Readiness Potential occurred

[22] A critique to this conclusion derives from the fact that subject – even if the cue is unpredictable – is always waiting for a cue to happen. Slow cortical potentials (described in Chapter 1) and in particular SPN can be found in the waiting time for a pending cue. The RP like shape found before an unpredictable cue can be also explained in this way. Unpredictable does not mean unexpected.

[23] Gomes, G., (1999)

[24] Alexander P., (2015)

also in the absence of movement, and that motor-based processes did not significantly modulate the Readiness Potential.[25] In this empirical framework the RP is not seen as the reflection of purely motor preparation, but it may represent a wider phenomenon of anticipation.

1.1.6 A biological mechanisms?

Another possible interpretation of the Readiness Potential is provided by several authors, in particular by the work of Kagaya & Takahata,[26] who showed that also in invertebrates, in particular in crayfish, spontaneous initiation of movement (walking) is preceded by readiness discharge neurons that become active more than 1 sec before walking, while they remain inactive at the onset of the mechanical stimulus-evoked walking. The assumption is that animals can initiate behaviour not only in response to an external stimulus, but also "voluntarily", depending on their internal state. Brain neurons active before of spontaneous initiation of movement in crayfish follow a similar temporal dynamic of the Readiness Potential in human animal. The authors in fact called this activity "readiness discharge". The Readiness Discharge is not associated with any specific direction of walking. Other specific neurons are active after the Readiness Discharge. This suggests a hierarchical control for the spontaneous activations of walking. Moreover, Kagaya & Takahata found brain neurons whose activities increase during walking and others that are active at the termination of walking. This also suggested a hierarchical organization of spontaneous walking in crayfish, from behavioural initiation to continuation and termination. In addition, intracellular studies showed that Readiness Discharges are based on sequential excitatory and inhibitory synaptic inputs rather than on endogenous excitability changes.

Another different perspective might highlight the classical view of the RP, which is based on cognitive and cortical phenomena. Since the crayfish is an invertebrate, and as such has no cortex and very few characteristics similar to the mammalian motor system, even though it seems to share the same mechanism of neuronal readiness

[25] This is also true for other slow cortical potentials such as CNV and SPN (See Brunia, 1988 – 1991)

[26] Kagaya K., Takahata M., (2010)

before a self-initiated movement, a comprehensive account of self-initiated actions needs to comprise also this aspect: the RP can be a biological mechanism that does not entail consciousness or volition

It is hard to understand what "spontaneous initiation" amounts to in the case of the crayfish, but it might be possible that the analogous neuronal mechanisms of the "readiness discharge" and the "readiness potential" can be described as a general neuronal mechanism or neuronal triggers for motor initiation. It might be a biological mechanism independent from any conscious control. This could explain Libet's gap between neural decision and conscious awareness of decision. What differentiates the Readiness Discharge from the Readiness Potential, then, might be the emergence of agency or the feeling of awareness of intention, which makes a difference between invertebrates and human animals. At the same time, the specific localizations, in different cortical areas of the human brain, that are active before the intentional action, cannot be negligible. Voluntary action seems to be encoded in the brain with a specific temporal and hierarchical dynamic.

What is interesting, with regard to the Readiness Discharge in the crayfish, is the absence of this neuronal activity for externally-triggered movements, suggesting that the Readiness Discharge process might be specific to spontaneous/freely deliberated movements. This suggestion could be of potential interest to best characterize the differences between humans' self-paced and stimulus driven actions.

Chapter 2

2.1 Eyeblink types

In healthy subjects, automatic blinking occurs spontaneously every 5 seconds, or so. The eyelids constitute a protective barrier between the cornea and anything that might inflict damage from the outside world. If the cornea loses its transparency, eyesight will be compromised or altogether destroyed. In order to prevent such a thing from happening we are equipped with several types of blinks. Blinks can occur either voluntarily, spontaneously or as a reflex in response to external stimulation. In addition, blinks can also be a learned response, like in eyeblink conditioning. Different pathways control the different types of blinks, although some overlaps occur. Regarding the kinematic properties, voluntary, spontaneous and reflex blinks have very similar features (Evinger, Manning and Sibony, 1991).

Reflex blinks are the fastest types of blinks and can be evoked in numerous ways. All reflex blinks are elicited by external stimuli; the three primary sensory modalities activated in reflex blinking include tactile, optic and acoustic sensations. Several reflex blinks can be distinguished. Reflex blinks can be divided into two categories, the trigeminal and non-trigeminal blinks. Tactile threats to the eye are likely to be perceived in the facial area which is innervated by the trigeminal nerve. The trigeminal reflexes are the corneal, ciliary (eyelash) reflex and supra-orbital reflex, evoked by electrical stimulation or glabellar tapping, are trigeminal reflexes. Corneal and ciliary reflexes can also be induced by tactile stimulation or air puffs. Tactile stimuli outside the face and non-tactile stimuli can also threaten the eye, which is then protected by non-trigeminal reflex blinks. Blink reflexes evoked by non-trigeminal inputs are the somatosensory, acoustic, photic and optic blink reflexes. A somatosensory reflex can be induced by an electrical stimulus to peripheral nerves, for example the median nerve at the wrist (Miwa et al. 1995). An acoustic blink reflex can be evoked by sudden loud sounds. Photic blinks are elicited after exposure to light stimuli. In clinical practice blink reflexes are usually evoked by mechanical stimulation of the cornea or eye lashes

or with glabellar tapping or electrical stimulation of the supra-orbital branch of the trigeminal nerve (Majeurs et al., 2005; Thomas, 1994).

Spontaneous blinks spread a protective tear film over the cornea in order to prevent the ocular surface from drying (Bour et al., 2000). The rate with which spontaneous blinks occur is thought to be determined by a generator. FMRI studies show activation in the right medial frontal gyrus possibly corresponding to the supplementary motor area when compared to blink inhibition activation and in Brodmann areas 7, 17 and 19 compared to keeping the eyes closed during spontaneous blinking (Bardouille, Picton and Ross, 2006). This implicates a role for these areas in the generation of spontaneous blinks. Independent of the exact location, the generator controlling the spontaneous blink rate appears to be influenced by several internal and external factors.
Voluntary blinks are blinks initiated by conscious thought. Blinks are used to communicate, for instance to emphasize innocence or indicate that what you said is not very serious. Voluntary blinks are even used to replace speech by patients with severe motor paralysis. Several MRI studies have been conducted investigating blink-related neuronal activation. Subjects showed bilaterally increased activation in the primary visual cortex, central thalamus, posterior putamen, and supplementary and primary motor cortex and cerebellum during voluntary blinking (Dimitrova et al. 2002).

2.2 Anatomical substrates

In all types of eyelid movements, two skeletal eyelid muscles, the levator palpebrae superioris (LPS) and the orbicularis oculi (OO) muscles, and two smooth muscles, the superior tarsal and inferior tarsal muscles (Mu¨ller's), are involved. In blinking, LPS and OO muscles act antagonistically (Bour, Aramideh, and De Visser, 2000; Evinger et al. 1991). Motoneurons in the central caudal subnucleus of the oculomotor complex innervate the LPS muscle, while OO muscle fibers receive their innervation from the intermediate zone of the facial nucleus (VanderWerf et al. 2003). During a blink, LPS motoneurons stop to fire briefly, while OO motoneurones produce a short burst, that in turns activates OO muscle fibers triggering a rapid lowering of the upper eyelid.

Stereotyped eye movements during blinking accompany eyelid movements. At the onset of a blink, the eye rotates normally from the initial position nasally downward and the extent of eye rotation depends on the initial eye position (Bour, Aramideh and De Visser, 2000)

2.3 Blink kinematics

2.3.1 Eyelid kinematics

For all kind of blinks, muscle activity onset precedes eyelid movement onset with about 12 ms and the maximum velocity of the closing eyelid (down phase) is generally twice as high as the maximum velocity during eyelid opening (up phase) (Evinger et al. 1991). Reflex, voluntary and spontaneous blinks also have their own characteristics. For the reflex blink the largest burst of muscle activity is generated by very powerful simultaneous motorunit activation resulting in fast and strength eyelid closure and somewhat slower eyelid opening. The voluntary blink has a less significative but longer contraction of the OO muscle which creates a slower eyelid movement with an amplitude similar to a reflex blink. The spontaneous blink has the smallest muscle activity and therefore the slowest and smallest eyelid movement (Evinger et al. 1991, VanderWerf et al. 2003).

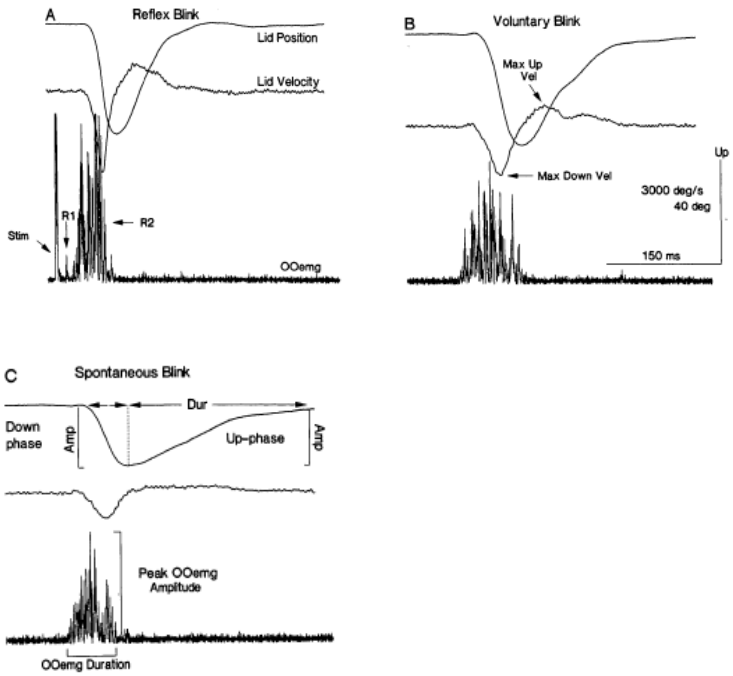


Fig. 10. *Eyelid kinematics and Orbicularis Oculi muscle EMG activity associated with: (A) reflex, (B) voluntary and (C) spontaneous eyeblinks. Stim=stimulus artefact from electrical stimulation of the supra-orbital nerve; Amp=amplitude; Dur=duration; Vel=velocity* (adapted from Evinger et al., 1991).

2.3.2 Eye kinematics

During blinking the eye rotates down towards the nose and back up again (Evinger et al., 1991). The blink appears to be a synchronic movement of both eyes and eyelids. Although when measured by means of electrophysiology, eye movement during voluntary blinks as well as reflex blinks is slightly asymmetric. Besides the rotation the eye is also displaced 1-2 mm back into the orbit during blinking (Evinger et al., 1984). Eye movement during blinking is achieved through co-contraction of all extraocular muscles except the superior oblique. The exact shape of the rotation of the eye is dependent on the start position (Bour, Aramideh and De Visser 2000, Evinger et al. 1991). The eye usually comes back to the initial gaze position after a blink. Bour and co-workers (2000) found that for reflex blinks this was independent of the intensity of the stimulus that evoked a blink. In rabbits, for example, the latency for extraocular muscle activation is longer than for OO muscle activation and varies depending on the muscle and blink-evoking stimulus (Evinger and Manning 1993).

2.4 Neural correlates and pathways

Most of the influence exerted by the higher order or supranuclear areas modulates the excitability of the blink, through for instance, descending cortical projections via the thalamus and superior colliculus (SC) via tecto-reticular projections (Basso, Powers and Evinger, 1996). Supranuclear projections involved in the act of blinking are distributed over several regions. FMRI studies revealed that the primary motor cortex, supplementary motor cortex, cingulated motor cortex and the central thalamus are active during spontaneous and voluntary blinking (Evinger and Perlmutter 2003). Direct projections have been described from the motor cortex to the facial nucleus and lateral medullary reticular formation (Jenny and Saper 1987). The basal ganglia can also influence reflex blinking through different pathways (Esteban 1999). For voluntary blinking, the neuronal circuit of eyelid movements is partly controlled by the neuronal blinking circuit, but for reflex blinking, the neuronal circuit of eyelid movements is more or less operating independently from the eye blink's circuit.

The upper face movements are controlled by multiple cortical motor regions, with specific involvement of the cingulate medial areas (CMAs) in the medial part of the frontal lobe, the primary motor areas (M1) and the lateral premotor areas (PMAs) (Hanakawa, Dimyan and Hallett, 2008). These areas contain also premotor neurons of the OOC (Orbicularis Oculi muscle), and from this stimulation might be obtained muscle activity from OOC (Sohn et al., 2004). The lateral precentral areas project predominantly to the contralateral lower facial muscles and relatively weakly, but consistently, to the upper facial musculature via the facial nucleus (Morecraft et al. 2001). This projection accounts for the movement patterns elicited by cortical stimulation to the lateral cerebral hemisphere (Cohen and Hallett 1988; Paradiso et al. 2005). Recent fMRI studies (Hanakawa, Dimyan and Hallett 2008; Hanakawa and Fukuyama, 2005) suggests that the motor representations of intentional bilateral blinking (oculomotor representation) and the upper face movements are adjacent but segregated in the cingulate medial frontal areas, hence supporting the somatotopically organized multiple CMA scheme proposed by Pickard and Strick in 1996.

Part 2. Experimental Protocol on healthy subjects

Chapter 3

Blinking is a rapid closing and opening of the eyelid. Blinks with identical kinematical features can have different origins and meanings. In patients with severe brain injuries blinking is often the only motor act that can be reliably detected. The main aim of the present study is to find a brain-based objective way to know whether a given blink is a meaningless neural event or the endpoint of a complex conscious process. We build up mainly on Benjamin Libet empirical work who showed that the awareness of intention to move is preceded by a recordable cerebral activity called "Readiness Potential" starting about 700 ms before the movement.

3.1 Participants

Six participants (mean age 25.7 years; range: 25 to 27 years, 5 females) were tested at the Department of Biomedical and Clinical Sciences L.Sacco of the University of Milan. All participants gave their written informed consent to take part in the experiment, according to the Declaration of Helsinki. All had normal or corrected to normal visual acuity and did not wear glasses or contact lenses during the experiment. The experimental procedure was approved by the Ethics Commettee of the Ospedale Luigi Sacco Milano.

3.2 Experimental protocol

The subjects were seated in a comfortable chair in the experimental room and were just requested to look at a dark screen set 1,5 m away at eye level. The subject had to maintain a central gaze position and should avoid body and head movements as well as facial muscle contractions. The experiment consists of three phases:

1. A "Spontaneous blink" phase to obtain the spontaneous blinks, used as control condition;

   This phase was always the first in order to maintain the participants unaware of the objective of the study and avoid attention focusing on the execution of spontaneous

eye blinks. In this phase no further instructions were given. The EEG recording starts when the subject considers himself/herself comfortably ready. Since we expect that each subject will blink spontaneously 10-15 times per minute on average (Evinger et al., 1991) a total of 120 minutes of recording was collected in order to obtain a total amount of at least 1000 spontaneous blinks. The whole recording was subdivided in 8 sessions (15 minutes each one, to prevent fatigue).

2. A voluntary "brisk" phase, during which the subject was required to blink as fast as possible.
3. A voluntary "slow" session to obtain eyeblinks whose velocity should be as natural as possible with the only request to perform them intentionally.

   These last two phases were alternated. In the voluntary phases, the subject was required to intentionally blink at any time they feel like doing so and to try to avoid spontaneous blinks. Eye blinks had to be performed not too closely without implementing any strategy (e.g. counting, pacing). Both phases were preceded by a training session in which the participant learned to properly perform the required movement. The aim of the training session was also to minimize task-related cognitive efforts. The duration of each phase was at least 120 minutes (8 sessions, 15 minutes each) to obtain a number of eye blinks similarly to the spontaneous phase.

3.3 Electrophysiology recording

We used a 64-channel EEG amplifier (BrainAmp DC, Brain Products, Germany). Sixty surface electrodes were placed in a cap according to the International 10/20 System. All electrodes were referenced to the linked earlobes and a ground electrode was placed on the forehead. Impedances were kept below 5 kΩ and the signal was acquired at a sampling rate of 5000 Hz (DC acquisition). Four out of 64 electrodes were used for EOG and EMG recording and positioned according to the most suitable placement for quantitative measurement eye blink detection according to Kaneko & Sakamoto (Kaneko & Sakamoto, 1999). To detect eye movements and identify the blink artifacts a vertical electrooculogram (EOG) was recorded by two electrodes placed above and below the right eye (in line with the pupil when the gaze was in central position). The EMG electrodes were located on the inferior portion of the Orbicularis oculi muscle to specifically detect the blink movement.

3.4 Data analysis

Data analysis was performed with MatLab (Math Works Inc., R2015b) and the SSP BioMedical Data Analysis Package (SiSyPhus Project; Version 2.0e, 2.1e), developed by members and collaborators of TMS-EEG LAB of the Department of Biomedical and Clinical Sciences "Luigi Sacco", University of Milan.

All signals were filtered using a high-pass, 1st order finite impulse response (FIR) filter (corresponding to a detrend).

3.4.1 Trigger positioning

As a first step of the signal pre-processing, we used the onset of EMG activity related to the eye-blink as the trigger to identify each trial.

To detect the eye blink onset, the EMG signal was processed as follows:

- 40-2000 Hz band-pass Butterworth filtering;
- EMG rectification;
- Peak interpolation using Hilbert transform function;
- Smoothing using a Gaussian filter;

Amplitude and standard deviations of the EMG signal were then computed. The first standard deviation was chosen as threshold value to position the trigger, which identify the eye blink onset.

All signals (EEG, EMG, EOG) were segmented from -2000 ms to 1000 ms around the trigger to define each epoch of interest (trial). The time-window was chosen to possibly include both a Readiness potential, which is supposed to begin at least 1 second before a movement (Burr, 2005) and the whole eye blink movement dynamics.

3.4.2 Kinematic analysis

Before segmentation, EOG signal was filtered (band-pass 0,2-8 Hz). Both EMG and EOG were baseline corrected (time-interval -1900 -1400).

To characterize the kinematic features of the three types of eye blinks, five parameters were selected.

Regarding the EMG signal we computed:

1. Peak to peak amplitude (on the non rectified signal; A max $_{EMG}$ (µV));
2. Duration (Δ(t) EMG $_{i\text{-}f}$ (ms)).

Regarding the EOG signal we computed:

3. Maximal amplitude of the maximum positive peak (A max $_{EOG}$ (µV));
4. The time-difference between the EMG onset and the EOG onset (Δ(t) $_{EOG\text{-}EMG}$ (ms), defined as the time at which the amplitude reaches 5% of the maximum);
5. The time-difference between the EMG onset and the time point at which the EOG reaches the maximal amplitude (A(t)max $_{EOG}$ (ms)).

Then in order to assess if the features of the eye blink kinematic significantly varies between the three different conditions (blink type), a one-way ANOVA was performed for each parameter. Post-hoc comparisons were finally applied to assess significant differences between conditions.

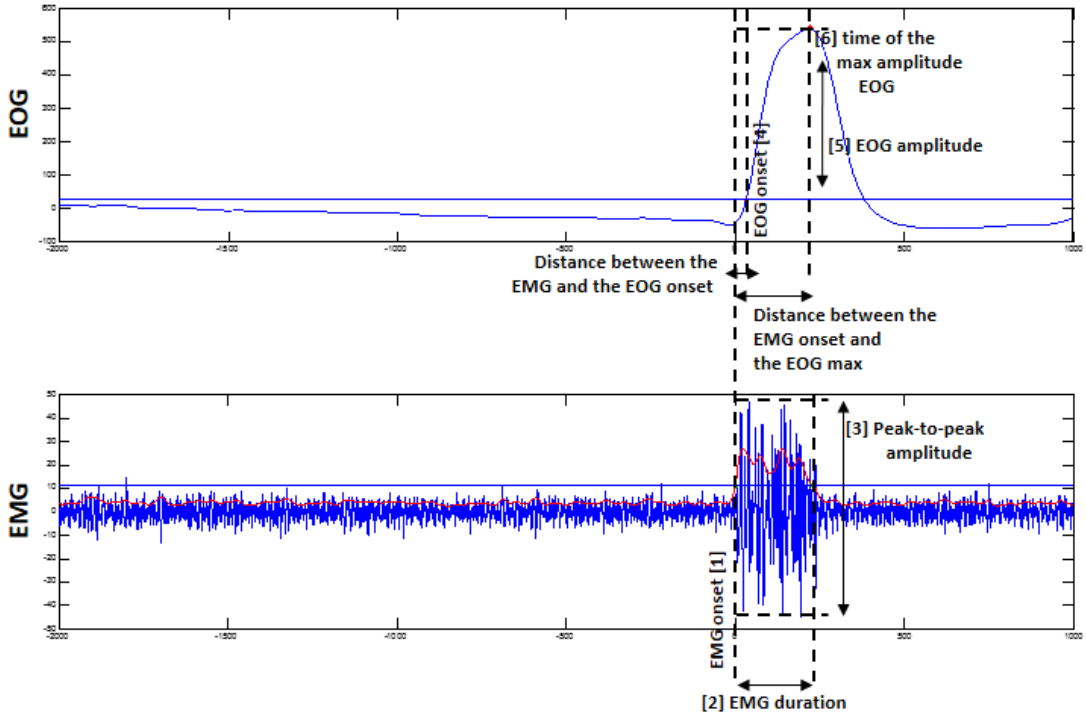


Fig. 11.Kinematic characteristics of eyeblinks as described by EOG and EMG

3.4.3 EEG analysis

On each EEG trial, the epoch was visualized within a time-window from -2000 ms to 2000 ms around the onset of the eye blink and then manually inspected to reject bad trials (those in which muscle artefacts, line drifts and double blinks). Artifactual channels were removed and then interpolated.

The continuous EEG signal filtered (band-pass 0.1-45 Hz and notch filter 49-51Hz), segmented (according to the previously described time-window) and then down-sampled at 500 Hz. Sessions belonging to the same condition were concatenated and the time interval from -1900 ms to -1400ms was used for baseline correction. The Independent Component Analysis (ICA; Makeig, Debener, Onton, & Delorme, 2004) was applied in order to remove EMG artifacts and main ocular movements (i.e. saccades).

Subsequent EEG analyses were performed exclusively on Cz (channel 29) since, according to the literature, the RP effect is supposed to be maximal on this site. A Bootstrap statistics was applied to the baseline in order to obtain two statistical thresholds, detecting the significance of negative and positive amplitude values, respectively.

Then, in order to quantify and describe the Readiness Potential, we evaluated four parameters:

1. The *cumulative amplitude* along the interval time of statistical significance (A cumulative $_{Cz}$ (μV));
2. The *onset time* of the significant EEG activity with respect to the negative statistical threshold (I $_{Cz}$ (ms));
3. The *maximum amplitude* reached in the interval time between -200 and -100 ms (A max $_{Cz}$ (ms));
4. The *slope* of the negative drift (Slope RP; assessed only when present, i.e. in the two voluntary conditions).

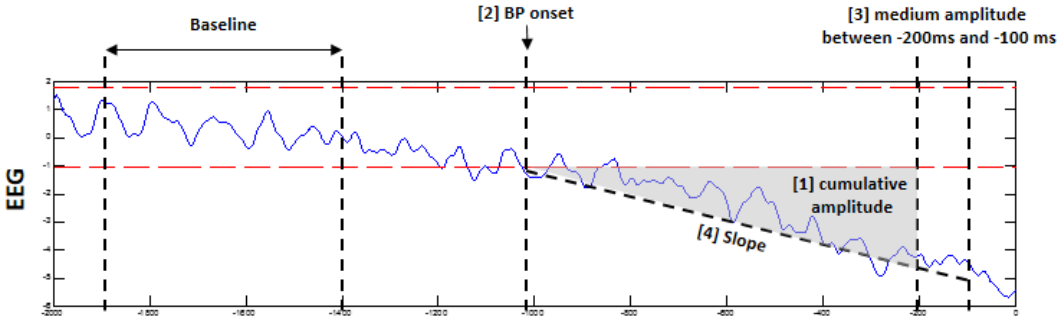


Fig 12. RP parameters

Then in order to assess if the features of the RP significantly varies between the three different conditions (blink type), a one-way ANOVA was performed for each parameter. Post-hoc comparisons (paired t-tests corrected with Bonferroni) were finally applied to assess significant differences between conditions.

3.4.4 Source modeling and statistics

We then localized the primary electromagnetic sources of scalp EEG activity by performing a source modeling. This modeling was applied to EEG data in order to detect the significant cortical activations which generated the scalp EEG signal and to estimate the time-space distribution of the cortical current density.

The conductive head volume was modeled according to the 3-spheres BERG method (Berg & Scherg, 1994). According to this method, the inner and outer surfaces of the skull and the scalp were represented by three ideal concentric spheres with specific and homogeneous conductance.

The cortical surface is modelled according to a tridimensional grid composed of 5120 fixed dipoles which are perpendicularly oriented to the cortical surface, adapted to the real anatomy of a single subject using the Statistical Parametric Mapping software (SPM12).

It is known that an infinite number of different configurations of sources may explain a given electric field measurement; this problematic localization of cortical generators is known as inverse problem. In this case, the inverse problem was solved by the Weighted Minimum Norm (WMN) constraint applied to an “empirical” Bayesian approach (56-58).

With this WMN approach, the weights are estimated in such a way to produce the source distribution with the minimum power that fits the measurements in a least-square-error sense. The source- modeling results in a matrix which represents the current cortical distribution as a function of time (samples) for each trials belonging to the same EEG session. After source-modeling, the significant cortical activations were evaluated by applying a non-parametric bootstrap-based statistical procedure to the obtained cortical currents.

First, each source's activity was centralized and normalized on the mean and standard deviation of its baseline level (from -1900 to -1400 ms). Then, surrogate average baseline activity was obtained for each source by randomly shuffling pre-stimulus samples at the single-trial level (Lv, Simpson & Bell, 2007). At this point, the maximum absolute value of the surrogates across all sources was calculated (Pantazis, Nichols, Baillet, Leahy, 2005). This entire procedure was repeated 500 times to obtain a distribution of 500 bootstraps, corrected for multiple comparisons. The one-tail (1-α) 100th percentile of the distribution of the maximum absolute values was used to estimate a significance threshold T(α) for all normalized sources and samples.

The spatio-temporal patterns of the significant cortical activations were identified according to a well-known anatomical classification (Brodmann Areas) and then compared between conditions (within-subject) and between subjects (for each condition).

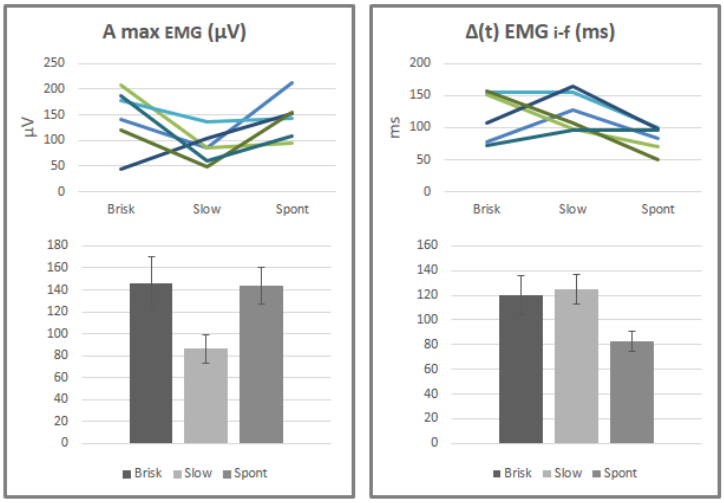
Chapter 4

After bad-trial rejection, an average number of 420 spontaneous, 660 voluntary brisk and 540 voluntary slow eye blinks per subject was obtained. These "good" eye-blink trials were then considered for the analysis of the kinematic characteristics and the RP parameters of the three eye blink conditions.

4.1 Results: kinematic parameters

In Fig.1, the within-subject variations and the between-subject mean value of the two EMG parameters (the peak-to-peak amplitude and the duration of the O.Oculi muscle activation related to the blink) for the three conditions are represented. A one-way ANOVA was applied in order to assess if the parameters variations between the tree conditions were significant. The statistical comparison revealed no effect of the condition for both parameter variations ($A\ max_{EMG}$ (μV): F=3,37, p=0,062; $\Delta(t)_{EMG}$ i-f (ms): F=3,3, p=0,064).

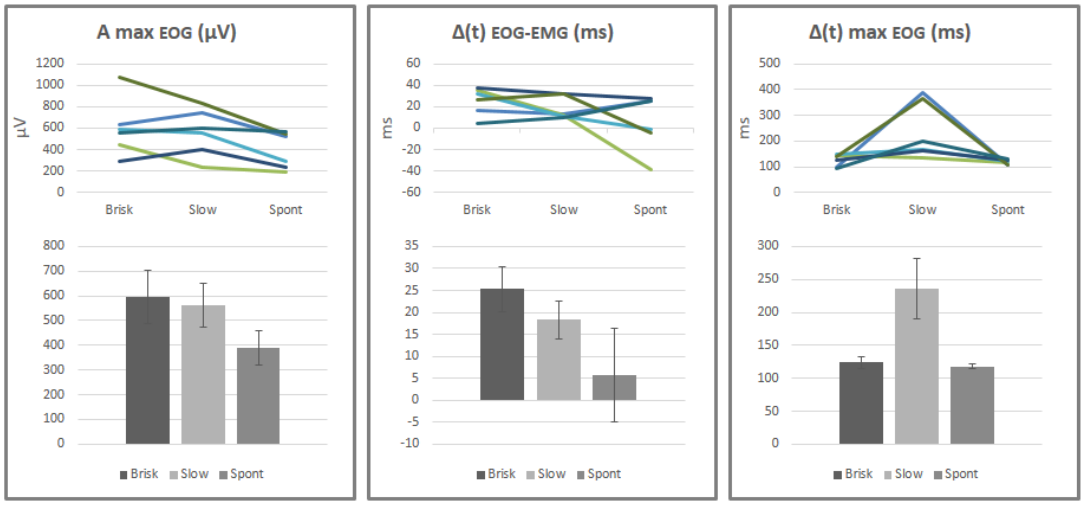
In Fig.2 the within-subject variations and the inter-subject mean value of the three EOG parameters for the three conditions are represented. No significant differences were found for both the maximum amplitude ($A\ max_{EOG}$ (μV): F=1,51; p=0,253) and the time of the EOG onset with respect to the EMG ($\Delta(t)_{EOG-EMG}$ (ms): F=1,84; p=0,192). Conversely, the third parameter (time at which EOG reaches its maximum amplitude) significantly varied among conditions ($A\ max_{EOG}$ (μV): F=1,51; p=0,014). As expected, the maximum peak of amplitude occurred later in the slow condition with respect to the brisk and the spontaneous ones. However, a post-hoc analysis (paired t-test corrected with Bonferroni) revealed no significant differences among comparisons; the effect was indeed most likely driven by two subjects.



	Brisk	Slow	Spont
m	146,3604	86,45172	144,0308
se	23,97604	12,8648	16,91462

	Brisk	Slow	Spont
m	119,9892	124,6591	82,87187
se	16,16363	12,14057	8,179018

Fig. 13. EMG parameters in three eyeblink conditions. Left panel shows the values of the maximum EMG amplitude while right panel shows the the time-point value at which the EMG activity reaches its maximum amplitude, for the three conditions. In the upper graph of each panel, mean values for each subject were plotted and, in the lower graphs, cumulated values with standard error bars are showed. Mean and standard errors related to the above mentioned parameters for each condition are also reported.



	Brisk	Slow	Spont
m	597,3969	560,4121	389,9649
se	107,7883	89,14113	69,32681

	Brisk	Slow	Spont
m	25,35005	18,32524	5,823115
se	5,113174	4,360231	10,67565

	Brisk	Slow	Spont
m	123,9734	236,5609	118,1085
se	9,464172	45,59449	4,146166

Fig 14. EOG parameters in the three eyeblink conditions. Left panel shows the values of the maximum EOG amplitude, Central panel shows the values of the EOG onset time with respect to the EMG onset time (corresponding to the zero point). The right panel shows the time values at which the EOG reached it maximum amplitude. In the upper graph of each panel, mean values for each subject were plotted and, in the lower graphs, cumulated values with standard error bars are showed. Mean and standard errors related to the above mentioned parameters for each condition are also reported.

Although the differences did not reach the statistical significance, some trends in the group-level data can be observed; in particular, considering the *maximum EMG amplitude* (A max EMG (µV)) brisk and spontaneous eye blinks values were comparable and higher than the slow condition (in 5 out of 6 subjects). The EOG amplitude, instead, revealed a gradient between the three conditions, with the highest values for the brisk and the lowest for the spontaneous condition. Regarding the *mean eye blink duration*, the EMG of the two voluntary conditions showed very similar values and were slightly longer than the spontaneous ones (in 4 out of 6 subjects). Conversely, spontaneous and brisk eye blinks showed a comparable EOG duration, which looked longer than the corresponding slow condition. Furthermore, the

difference between the EMG and the EOG onset times showed that in the voluntary conditions the eye blink EMG onset always precedes the EOG onset.

In summary, the kinematic parameters related to the EMG and the EOG did not reveal any peculiar pattern associated to a specific eye-blink condition and no statistically significant differences between kinematic parameters could be found at the group level.

Table 1: Values associated to the kinematic parameters for the EMG and the EOG

Subjects		EMG parameters						EOG parameters								
		$A\max_{EMG}(\mu V)$			$\Delta(t)\,EMG_{i\text{-}f}(ms)$			$A\max_{EOG}(\mu V)$			$\Delta(t)_{EOG\text{-}EMG}(ms)$			$\Delta(t)\max_{EOG}(ms)$		
		Brisk	Slow	Spont	**Brisk**	Slow	**Spont**	Brisk	Slow	**Spont**	Brisk	Slow	Spont	Brisk	Slow	Spont
S1		140,9262	86,37217	213,1346	77,23721	127,5466	84,02457	635,0798	739,6373	517,5913	16,73866	13,07279	25,3405	96,31307	389,4672	107,0196
S2		206,5069	84,64261	95,10301	151,7636	97,65853	69,88025	446,0436	237,6951	190,6558	35,00586	12,47453	-39,1516	141,9586	134,6282	118,2936
S3		178,5825	135,7398	142,0176	154,4866	154,9196	100,25	583,9667	553,2224	288,9588	31,63501	10,55417	-0,66389	147,5691	167,2104	119,5676
S4		45,1302	104,4264	151,7564	107,2622	164,9653	98,33391	291,862	399,3331	234,9903	37,31709	32,30495	27,61391	125,9507	163,0667	127,12
S5		120,2638	47,74299	153,6147	156,9616	107,1407	49,18526	1075,538	833,5971	542,1193	26,83872	31,74359	-4,04947	137,3729	366,6513	105,8895
S6		186,7527	59,78632	108,5586	72,22374	95,72384	95,5572	551,8913	598,9876	565,4736	4,564985	9,801423	25,84923	94,67596	198,3416	130,7608
Mean		146,3604	86,45172	144,0308	119,9892	124,6591	82,87187	597,3969	560,4121	389,9649	25,35005	18,32524	5,823115	123,9734	236,5609	118,1085
Standard error		23,97604	12,8648	16,91462	16,16363	12,14057	8,179018	107,7883	89,14113	69,32681	5,113174	4,360231	10,67565	9,464172	45,59449	4,146166
ANOVA	F	3,37			3,31			1,51			1,84			6,12		
	p	0,062			0,064			0,253			0,192			0,011		

4.2 Results: EEG parameters

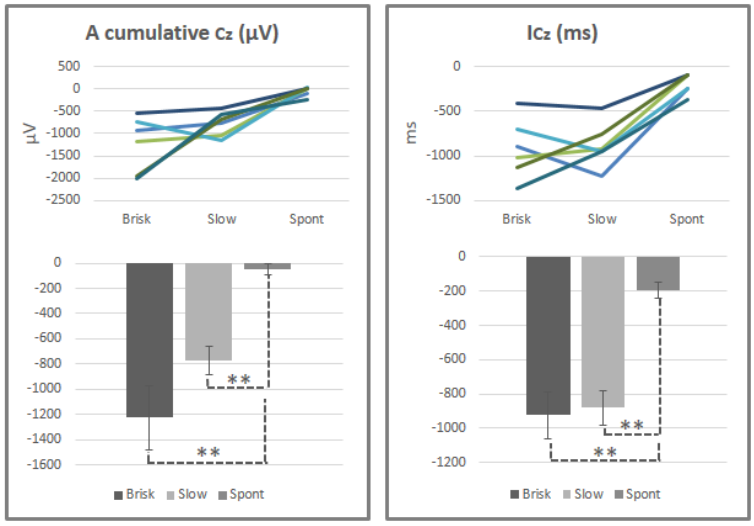
In Fig. 15 and 16 the within-subject variations and the inter-subject mean values of the four parameters associated to the Readiness Potential for the three conditions are represented. A one-way ANOVA with subsequent post-hoc analyses were applied and showed that the parameters varied significantly as an effect of condition.

Specifically, the *cumulative amplitude* did not significantly differ between the two voluntary conditions, but both the brisk and the slow eye blink types showed significantly higher values with respect to the spontaneous one (A cumulative $_{Cz}$ (μV): F=13,37; p=0,0005; T-test Brisk vs Slow: p=0,1892; T-test Brisk vs Spont: p=0,0038; T-test Slow vs Spont: p=0,0034).

The *RP onset time over Cz* did not significantly differ between the two voluntary conditions. Both the brisk and the slow eye blink types showed a significantly different (earlier) onset with respect to the spontaneous one (I $_{Cz}$ (ms): F=15,88; p=0,0002; T-test Brisk vs Slow: p=0,7553; T-test Brisk vs Spont: p=0,002; T-test Slow vs Spont: p=0,0005).

The *maximum amplitude over Cz* did not significantly differ between the two voluntary conditions, but both the brisk and the slow eye blink types showed significantly higher values with respect to the spontaneous one (A max $_{Cz}$ (ms): F=25,95; p=0,0000135; T-test Brisk vs Slow: p=0,0332; T-test Brisk vs Spont: p=0,0009; T-test Slow vs Spont: p=0,0125).

Since in the spontaneous condition a consistent negative deflection cannot be detected, only the two voluntary conditions were considered for comparison of the *slope* parameter. A statistically significant difference of this parameter was found between the two voluntary conditions (Slope $_{RP}$; T-test Brisk vs Slow: p=0,0084).



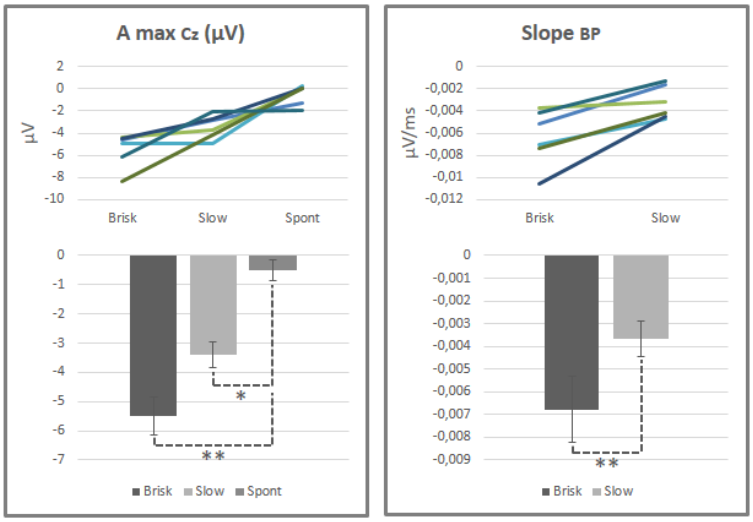
	Brisk	Slow	Spont
m	-1224,67	-772,078	-50,2137
se	253,0472	112,8663	43,95789

	Brisk	Slow	Spont
m	-923,667	-881	-193,667
se	137,3438	103,5835	45,97511

*Fig. 15. EEG parameters in three eye blink conditions. Left panel shows the cumulative EEG amplitude while right panel shows the time-point value at which the EEG activity become significant, for the three conditions. In the upper graph of each panel, mean values for each subject were plotted and, in the lower graphs, cumulated values with standard error bars are showed (** corresponds to p<0.003). Mean and standard errors related to the above-mentioned parameters for each condition are also reported.*

The *maximum amplitude over Cz* did not differ significantly between the two voluntary conditions, but was significantly higher with respect to the maximum amplitude of the spontaneous condition (A max $_{Cz}$ (ms): F=25,95; p=0,0000135; T-test Brisk vs Slow: p=0,0332; T-test Brisk vs Spont: p=0,0009; T-test Slow vs Spont: p=0,0125).

Regarding the *Slope of the RP*, only the two voluntary conditions were considered for comparisons, since no presence of a consistent negative deflection was revealed by the analyses made on the EEG data for the spontaneous condition. A statistically significant difference in the slope was found between the two voluntary conditions (Slope $_{RP}$; T-test Brisk vs Slow: p=0,0084).



	Brisk	Slow	Spont
m	-5,49327	-3,4073	-0,50311
se	0,632571	0,433257	0,371996

	Brisk	Slow
m	-0,00678	-0,00366
se	0,001466	0,000771

*Fig. 16: EEG parameters in three eye blink conditions. Left panel shows the maximal EEG amplitude for the three conditions while right panel shows the slope of the two voluntary conditions. In the upper graph of each panel, individual mean values of each subject were plotted and, in the lower ones, cumulated values with standard error bars are showed (** corresponds to p<0.009; * corresponds to p<0.01). Mean and standard errors related to the above-mentioned parameters for each condition are also reported.*

In figure 17a, for each subject, the averaged EEG activity preceding the blink onset (from -2000ms to 0) is displayed. The three traces corresponding to different eye blink conditions are superimposed (black trace corresponding to the spontaneous, red trace corresponding to the slow and blue trace corresponding to the brisk) for each channel on the basis of the topographical arrangement of the 60-electrodes cap. In each subject, the EEG activity, identified in the voluntary conditions (brisk and slow) as the RP, is maximal represented over the fronto-central electrodes and in particular at the level of the Cz channel (circled in dark gray). For each condition (spontaneous on the left side, slow in the middle, brisk on the right side), two color-coded maps showing the instantaneous voltage distributions at two selected latencies (-500ms and -200ms respectively, voltage color-scaled between +4 and -4 µV, red and blue respectively) were also presented.

Interestingly, only two subjects (i.e. subject 1 and 6) showed a slight negative voltage in the spontaneous condition at both selected latencies (of note, only in subject 6 this negative voltage increases at -200ms compared to -500ms).

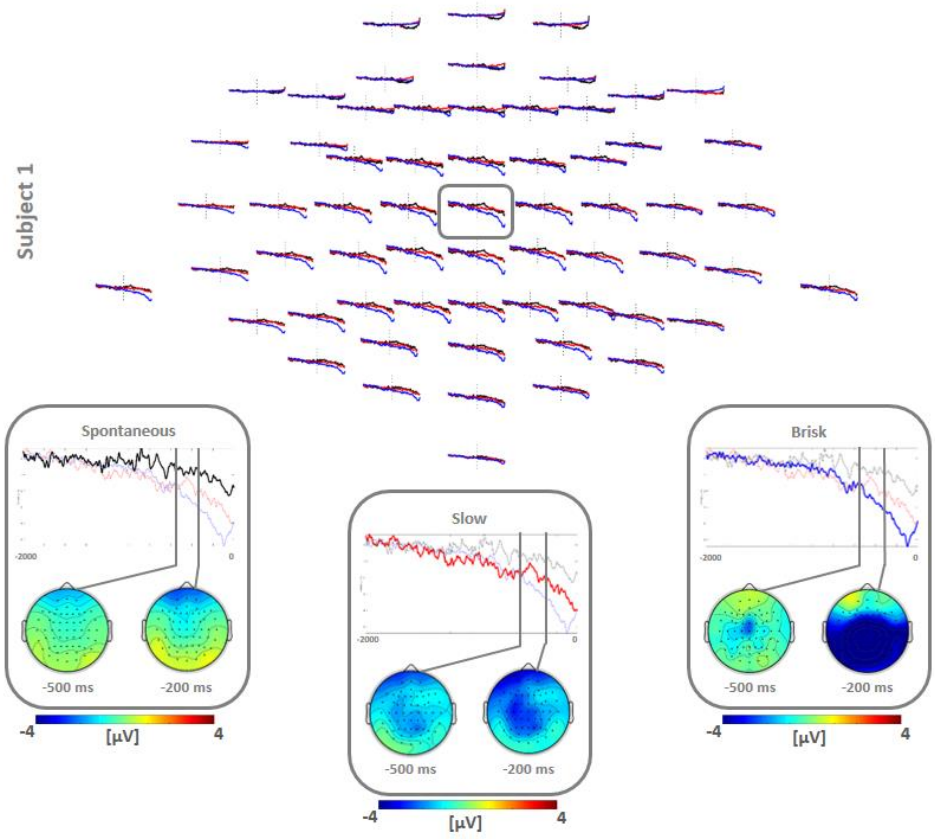
As regards to the voluntary conditions, the instantaneous voltage maps always showed a voltage increase between the two latencies (i.e. more negative values when approaching the blink onset); this negative voltage increase was consistent between subjects and well depicted the slowly progressive negative RP course.

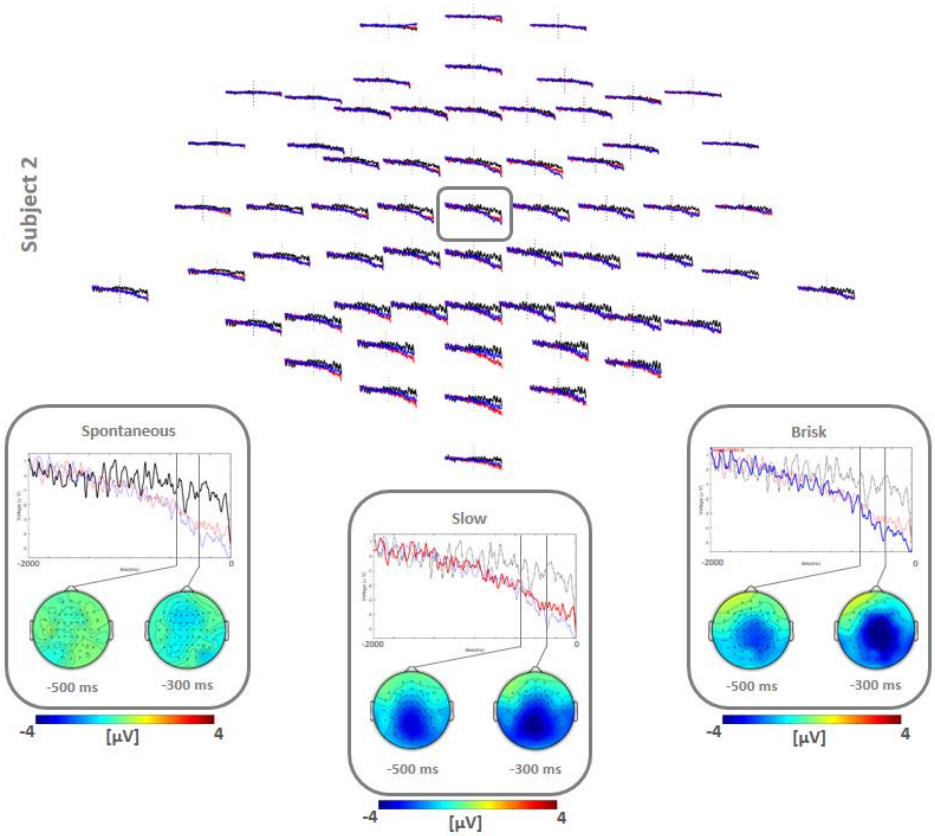
In conclusion, we found a significant difference of the EEG activity preceding the onset of the eye blink if the spontaneous and voluntary conditions were compared. Specifically, a substantial and consistent slow negative potential (mean voltage -4 μV across all subjects) which precedes the intentional eye blink was detected in all subjects for both voluntary conditions (brisk and slow). This component was maximally represented over fronto-central electrodes. A not-negligible between-subject variability in latency and maximum amplitude of this negative potential was however present.

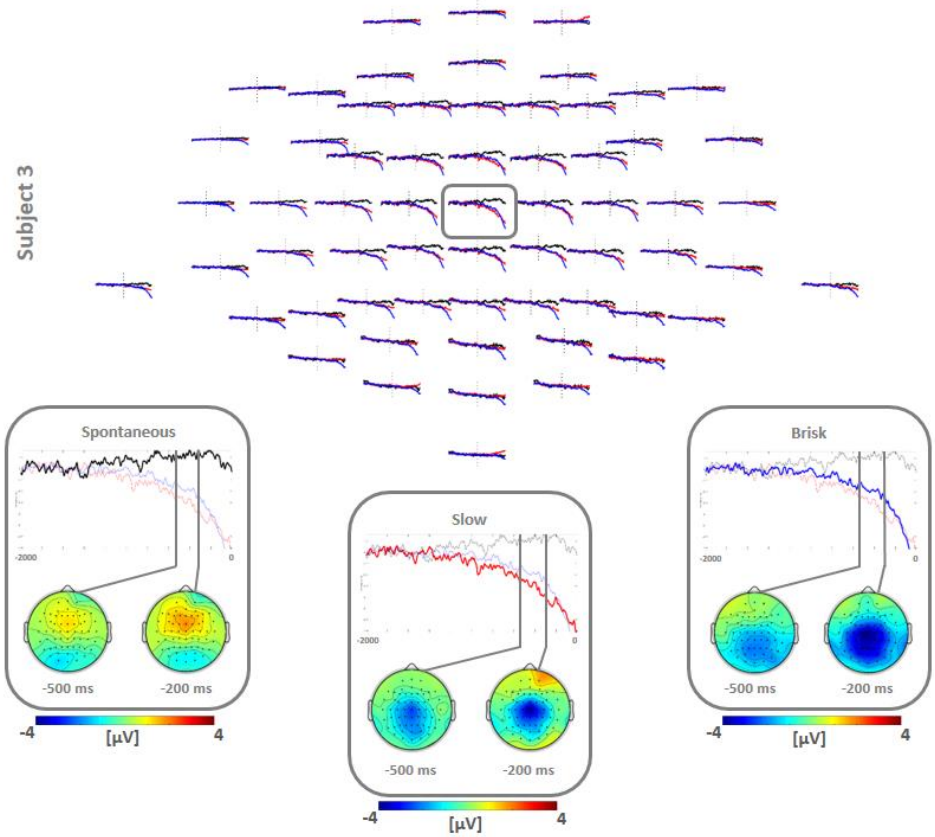
Of note, only two subjects showed, also in the spontaneous condition, a negative value of the cumulative amplitude (although not significant at the group level). Specifically, this negative drift may represent a different phenomenon, since it showed a smaller amplitude (as expressed by both amplitude parameters) and a delayed onset if compared to the two voluntary conditions.

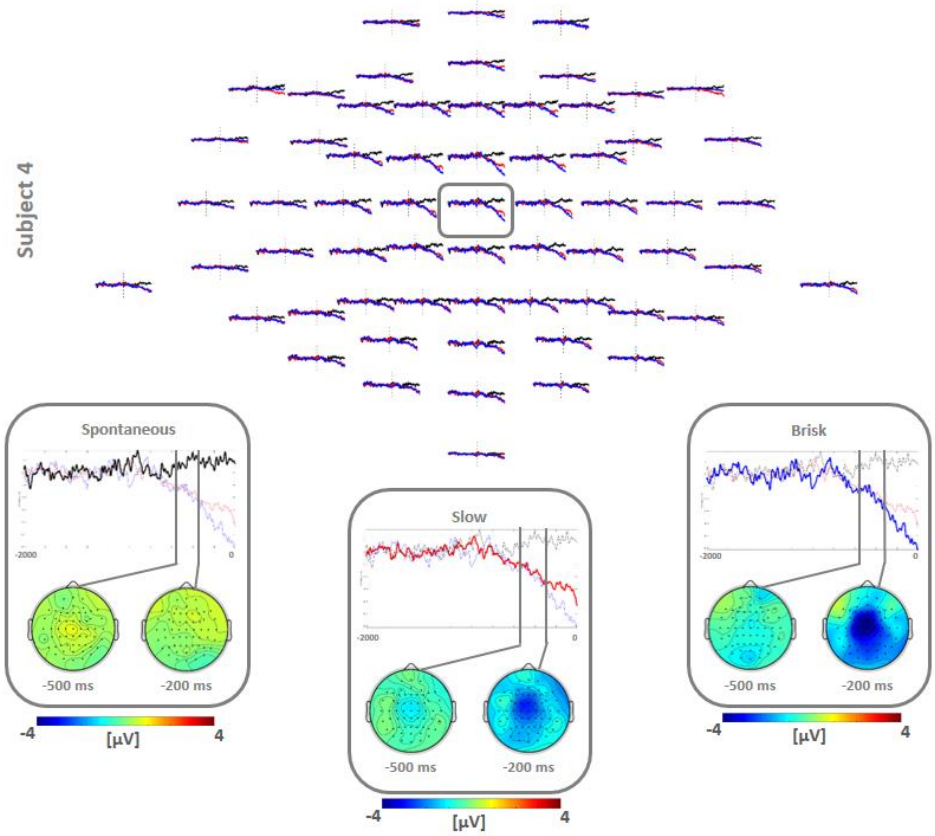
***Panel A (Figure 17a)**: displays of the averaged activity over channel 29 (Cz) in the two seconds interval preceding the eye blink for the three conditions;*

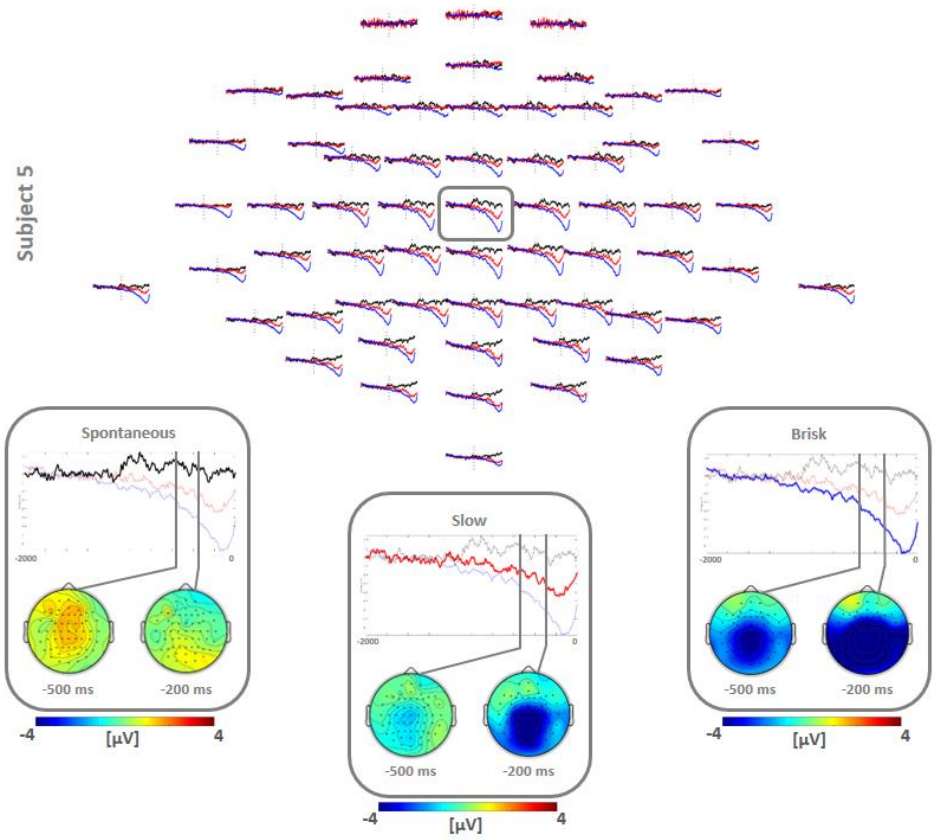
***Panel B (Figure 17b)**: voltage scalp topographies at time point -300 ms (300 ms preceding the eye blink EMG onset) in the three conditions (those belonging to the spontaneous condition in the left column, the ones belonging to the voluntary slow in the central column and the topographies relative to the averaged voluntary brisk trials on the right).*


Subject 1
Spontaneous
-2000
0
-500 ms
-200 ms
-4
[µV]
4
Slow
-2000
0
-500 ms
-200 ms
-4
[µV]
4
Brisk
-2000
0
-500 ms
-200 ms
-4
[µV]
4


Subject 2
Spontaneous
-2000
0
-500 ms
-300 ms
-4
[µV]
4
Slow
-2000
0
-500 ms
-300 ms
-4
[µV]
4
Brisk
-2000
0
-500 ms
-300 ms
-4
[µV]
4


Subject 3
Spontaneous
-2000
0
-500 ms
-200 ms
-4
[µV]
4
Slow
-2000
0
-500 ms
-200 ms
-4
[µV]
4
Brisk
-2000
0
-500 ms
-200 ms
-4
[µV]
4


Subject 4
Spontaneous
-2000
0
-500 ms
-200 ms
-4
[μV]
4
Slow
-2000
0
-500 ms
-200 ms
-4
[μV]
4
Brisk
-2000
0
-500 ms
-200 ms
-4
[μV]
4


Subject 5
Spontaneous
-2000
0
-500 ms
-200 ms
-4
[µV]
4
Slow
-2000
0
-500 ms
-200 ms
-4
[µV]
4
Brisk
-2000
0
-500 ms
-200 ms
-4
[µV]
4

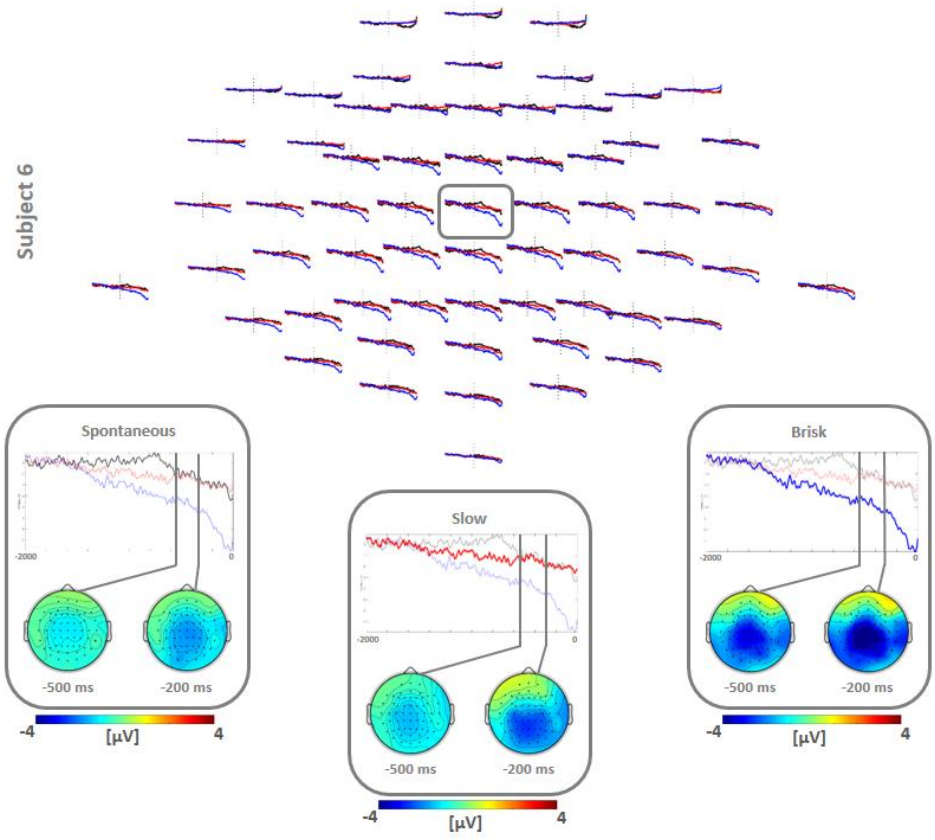

Subject 6
Spontaneous
-2000
0
-500 ms
-200 ms
-4
[μV]
4
Slow
-2000
0
-500 ms
-200 ms
-4
[μV]
4
Brisk
-2000
0
-500 ms
-200 ms
-4
[μV]
4

Table 2: Values associated to the RP parameters for the EEG

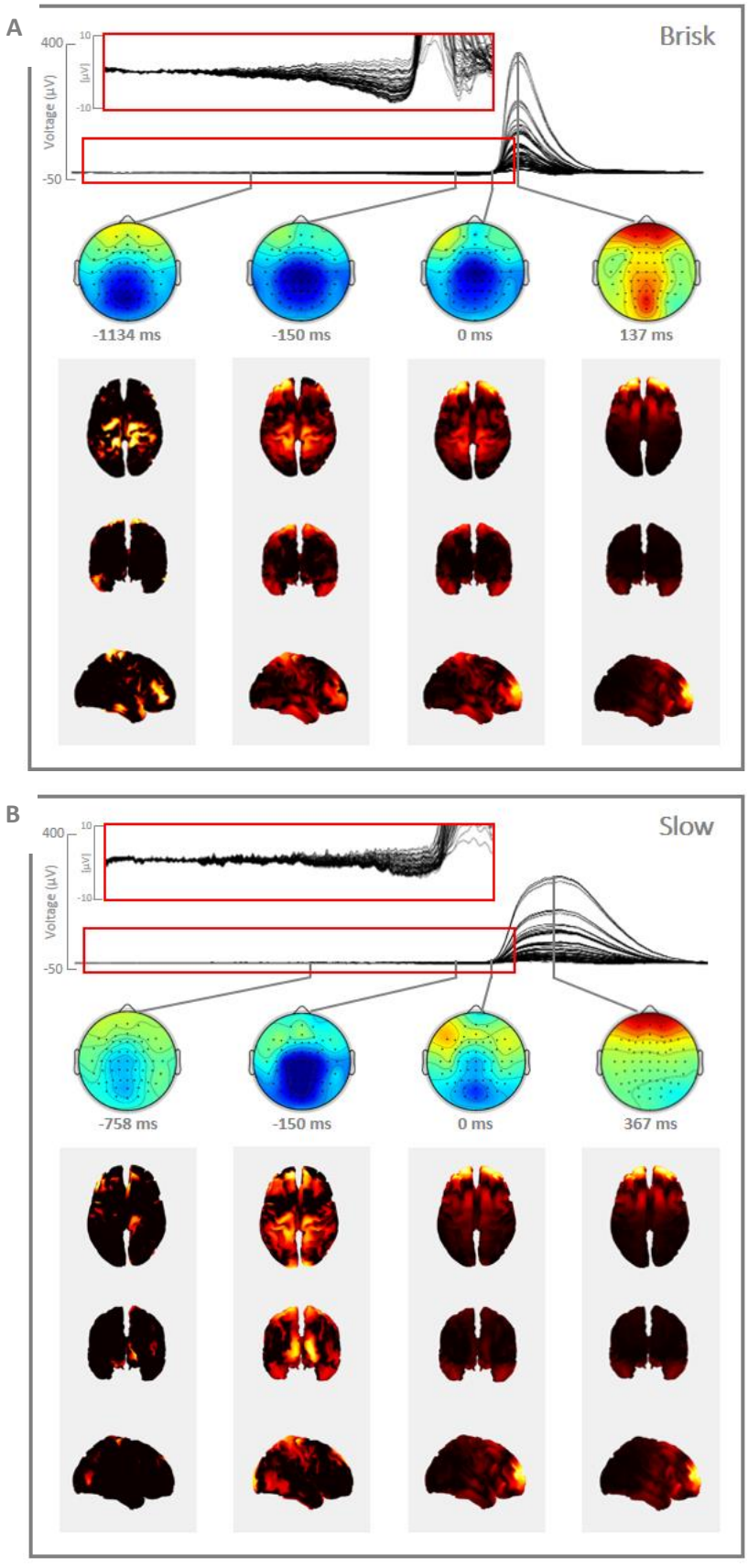
Subjects	EEG parameters										
	A cumulative $_{Cz}$ (µV)			I $_{Cz}$ (ms)			A max $_{Cz}$ (µV)			**Slope $_{RP}$ (µV/ms)**	
	Brisk	Slow	Spont	**Brisk**	Slow	**Spont**	Brisk	Slow	**Spont**	Brisk	Slow
S1	-938,27	-763,209	-97,7724	-892	-1234	-248	-4,62614	-2,80812	-1,27222	-0,00518	-0,00163
S2	-1176,52	-1030,39	0	-1026	-926	-100	-4,36693	-3,73549	0	-0,00378	-0,00317
S3	-744,322	-1158,35	44,58325	-706	-954	-242	-4,9338	-4,9569	0,25073	-0,00702	-0,00477
S4	-536,262	-427,355	0	-412	-468	-100	-4,49673	-2,72985	0	-0,01055	-0,00451
S5	-1944,64	-676,473	0	-1134	-758	-100	-8,3585	-4,1372	0	-0,00738	-0,00423
S6	-2008,03	-576,689	-248,093	-1372	-946	-372	-6,17753	-2,07625	-1,99719	-0,00413	-0,00134
Mean	-1224,67	-772,078	-50,2137	-923,667	-881	-193,667	-5,49327	-3,4073	-0,50311	-0,00678	-0,00366
Standard error	253,0472	112,8663	43,95789	137,3438	103,5835	45,97511	0,632571	0,433257	0,371996	0,001466	0,000771
ANOVA	F	13,37		15,88			25,95			/	
	p	0,0005		0,0002			0,00001			/	
t-Test (p)	**Brisk vs Slow**	0,1892		0,7553			0,0332			0,0084	
	Brisk vs Spont	0,0038		0,002			0,0009			/	
	Slow vs Spont	0,0034		0,0005			0,0125			/	

4.3 Source analysis, preliminary results

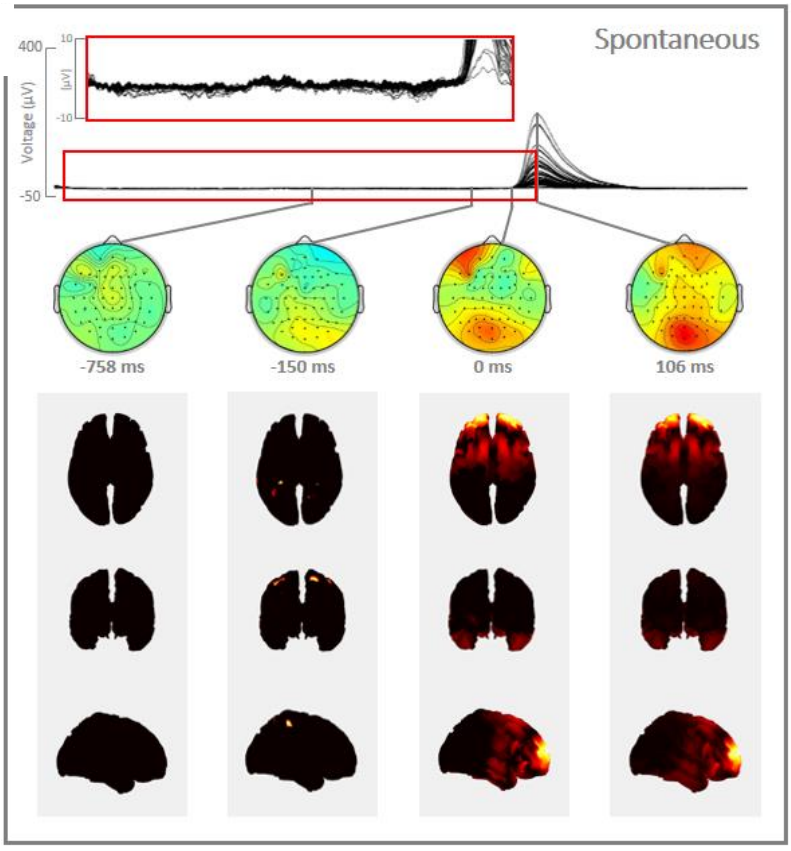
In panels A, B and C the source modeling preliminary results referring to a representative subject (Subject 5) for the three eye blink types (Brisk, Slow and Spontaneous, respectively) ae shown. In each panel, are illustrated (from the top to the bottom of the figure):

- the butterfly plot (the average potential of each channels superimposed) corresponding to one of the three types;
- the corresponding topographical representations of the scalp voltage at four different latencies, specifically: the onset time of the RP (calculated as onset of the cumulative amplitude over Cz), the time at which the maximum amplitude occurred (150 ms before eye blink EMG onset), the EMG eye blink onset time (0 ms) and the time of the EOG maximum peak (eye blink maximum amplitude);
- the relative current density distributions represented in dorsal, posterior and lateral views at the aforementioned time points.

  In both the voluntary conditions (brisk and slow) there is an early bilateral activation of the precentral frontal area (Brodmann area 4) and the postcentral parietal area (Brodmann area 6), with a later activation of bilateral medial and inferior frontal lobes (corresponding to the Supplementary eye fields –SEF- and the Frontal eye fields –FEF-, respectively). The sensorymotor areas (BA 4 and BA 6) remain active until eyeblink onset. At this point, a huge current activity become clearly visible over the frontal pole (BA 10) and remain active for all the movement duration. A slighter activation of the frontal pole starts from 150 to 100 ms before the eyeblink onset can also be identified; however, the subsequent more evident activation of the same area during the blink movement suggests an artifactual origin rather than a genuine cortical generation.


A
Brisk
400
10
[µV]
-10
Voltage (µV)
-50
-1134 ms
-150 ms
0 ms
137 ms
B
Slow
400
10
[µV]
-10
Voltage (µV)
-50
-758 ms
-150 ms
0 ms
367 ms

C



Chapter 5

5.1 Comparison with previous studies

5.1.1 Blink kinematics

Healthy subject's study provides the kinematic characteristics of three different types of blink (spontaneous blink, an unintentional movement, and two types of voluntary blink, brisk and slow) and the related electro-cortical potentials elicited by these movements.

Kaneko and Sakamoto (1999) studied a quantitative EMG-EOG method to identify spontaneous, reflex and voluntary blinks. Their results showed that the three types of blink could be identified on the basis of the mean amplitude and duration of EOG and EMG signals, and that the voluntary blink significantly differs from the other blink types. Specifically, the EMG signal seemed to be more appropriate to make this distinction. The spontaneous blink had significantly smaller EMG values than the other types of blink for both duration and amplitude parameters. The amplitude and the duration of EMG for voluntary blinks were greater than for the other types of blink. In disagreement with this previous study, our results did not show statistically significant differences in the kinematic characteristics of the three types of blink at the group level.

Similar to Kaneko and Sakamoto (1999), our experiment involved the use of EMG and EOG for recording and evaluation of the blink characteristics and also shared the specific electrode placement, the selected kinematic parameters and their measurement. However, our study mainly differs for the sample size (6 vs 11 subjects) and for the specific instructions provided to perform voluntary movements; Kaneko and Sakamoto (1999) asked to perform a voluntary blink as fast and weak as possible. Conversely, in our study we tested two intentional blinks which differs for the execution modality: the first movement (i.e. brisk) should be executed as fast as possible, but without any instruction on how intense/strong the movement should be, while the second movement (i.e. slow) should be performed as natural as possible. In this perspective, our results are only partially comparable with those of Kaneko and Sakamoto (1999). In fact, while these authors found a statistically significant

difference between the different types of blink, we did not find a significant difference in strictly statistical terms. Looking at the measurements, however, some trends could be observed; in particular, voluntary blinks roughly seem to show greater duration and amplitude values compared the spontaneous blinks. The lack of statistical significance could be due to the training session which may cause the brisk blinks to resemble the spontaneous ones and could be also due to the large amount of voluntary trials performed by each subject (more than 400 per type) which may include some spontaneous blink. The small sample size (6 subjects) should also be taken into account to explain the high variability between subjects. Increasing the number of subjects could allow to reliably define whether these parameters (i.e. amplitude and duration) are able to discriminate between the eyeblink types.

5.1.2 Blink-related cortical potential

The role of the cerebral cortex for the preparation and the generation of voluntary blink was previously studied by Montagna & Zucconi (1984) and Kaneko and colleagues (2004) and recently by Mota & Lins (2017) who evaluate the cortical potential related to the voluntary blink movement. They found that voluntary performed eyeblinks were preceded by a negative cortical potential that begins approximately 600 ms before the onset of EMG activity of the orbicularis oculi muscle.

The maximum amplitude of this potential was found to be localized over central electrodes, in particular at the level of the Cz channel, in line with previous results focused on more commonly studied voluntary movements (hand or foot movements). This result showed that a voluntary blink did require cortical processes of preparation and execution similar to other voluntary movements. While Kaneko and colleagues (2004) found that the cortical potential related to the spontaneous movement was similar to the one preceding the reflex but strongly different from the one preceding the voluntary movement, Montagna & Zucconi (1984) did not find any change in the brain activity preceding spontaneous eyeblinks. Hence, these authors concluded that the neural processes related to a spontaneous movement take place at a subcortical level rather than at the level of primary and supplementary motor areas as it happens for a voluntary movement.

Our study confirmed the main findings of previous studies. In fact, we observed that a negative potential starting on average 900 ms before the execution of a voluntary blink was present in all subjects. Since this potential was mainly expressed at the midline channels, we decided to analyze this activity at the Cz site also according to the RP literature. The selected parameters of amplitude, time onset and slope of the EEG potential allowed a good characterization of the Readiness Potential preceding a voluntary blink. These parameters, in fact, significantly differed from the corresponding ones that described the EEG activity preceding a spontaneous blink.

Previous studies (for a review see Shibasaki and Hallett, 2006; Verbaarschot, Farquhar, Haselager, 2015) have shown that the RP is influenced by several factors, as, for example, the attentional level and the "volitional" level involved in the performance a specific movement; in this perspective, higher grades of volition turned into an increased RP amplitude.

In our experiment, we assumed that the "brisk condition" in which the blink has to be performed rapidly should require more attention and intention than the "slow condition", in which the blink has to be performed as naturally as possible. The comparison of the mean amplitude values, computed both as the cumulate both as medium amplitude in the time interval between 100 and 200 ms (before the blink), did not show any statistically significant differences between the brisk and the slow blink. Conversely, a statistically significant difference was found by evaluating the slope of the curve of the RP which resulted higher in the brisk compared to the slow brisk. Thus, we can infer that the execution of these two blinks, roughly comparable for the voluntary purpose but slightly different for the execution modality, critically affects the RP characteristics with a significant effect on the slope and a less marked effect on the amplitude parameters (where the difference does not reach statistical significance).

To sum up, besides replicating previous findings, we found that the RP 1) specifically preceded intentional movements, 2) was consistent among subjects and 3) was partially modulated by the execution modality, as showed by the change of the slope parameter.

Furthermore, in full agreement with Libet's perspective, what clearly differentiated the voluntary from the spontaneous motor acts was the subjective decision to perform them. In our experiment, such decision was unconstrained and occurred only when the

subject felt to do so. Given that the action execution assumed a self-paced rhythm, we always monitored that the subject did not use strategies (e.g. counting) or give an explicit rhythmicity to the motor acts.

Compared to classical Libet's-like experiments, we decided not to specifically test the temporal occurrence of the awareness of the urge to act because this issue can be assessed only through a subjective report. However, requiring such report can add an additional cognitive dimension, which may be reflected and partially overlap with brain activity preceding movement execution (the RP). Moreover, this subjective report cannot be interpreted as an objective and reliable measure of the time course of the intention generation (Haggard, Clark and Kalogeras, 2002) mainly because this estimated temporal onset seems to be dependent on an attentional bias. Therefore, we decided not to investigate the time of awareness of intentionality but to focus only on the intentionality itself. We assumed the RP as the electrophysiological marker of voluntary movements, irrespectively of its specific causal or consequential role, and without stepping into the philosophical debate about intentionality and free will.

Part 3. Experimental Protocol on DOC and LIS patients

Chapter 6

6.1 Disorders of Consciousness

"The words "conscious" and "consciousness" *in philosophical perspective* are umbrella terms that cover a wide variety of mental phenomena. Both are used with a diversity of meanings: the adjective "conscious" is heterogeneous in its range, being applied both to whole organisms (creature consciousness) and to particular mental states and processes (state consciousness)."[27]

An animal, person or other cognitive system may be considered as conscious in different ways:[28]

1) *Sentience.* It may be conscious in the generic sense of simply being a *sentient* creature, one capable of sensing and responding to its world. (Armstrong 1981)

2) *Wakefulness.* A subject counts as conscious only if he/she is *awake and normally alert.*

3) *Self-consciousness.* A third and yet more demanding sense might define conscious creatures as those that are not only aware, but also aware that they are aware, thus treating creature consciousness as a form of *self-consciousness*. (Carruthers 2000)

4) *What it is like.* Thomas Nagel's (1974) pivotal *"what it is like"* criterion captures the most private and subjective notion of being a conscious organism. According to Nagel, a creature is conscious just if there is "something that it is like" to be that creature, i.e., some subjective way the world seems or appears from the creature's mental or

[27]Van Gulick, R., (2017) "Consciousness", *The Stanford Encyclopedia of Philosophy* (Summer 2017 Edition)
[28]See, "Consciousness", *The Stanford Encyclopedia of Philosophy* (Summer 2017 Edition)

experiential point of view. In this respect, being conscious is just a matter of being an experiential creature, of any kind.

Regarding "state of consciousness", there are at least three major options:

1) *States one is aware of*: states in which some information is available for control of behaviour and for guiding verbal reports. (Chalmers, 1996)

2) *Qualitative states:* states that involve qualitative or experiential properties of the sort often referred to as *qualia*, or raw sensory feels.

3) *Access consciousness:* a state's being conscious is a matter of its being available for interaction with other states, and of the access that one has to its content. (Nagel, 1995)

From a clinical perspective, the notion of consciousness is reduced into two major components: the *level* of consciousness (i.e., wakefulness or vigilance and arousal) and the *content* of consciousness (i.e., awareness of the self and of the environment) (Plum and Posner, 1983). Numerous brainstem neuronal populations that directly project to both thalamic and cortical neurons support arousal. Depression of either brainstem or global hemispherical function may therefore cause reduced wakefulness. Awareness is thought to be dependent upon the functional integrity of the cerebral cortex and its reciprocal subcortical connections; each of its many aspects resides to some extent in anatomically defined regions of the brain (Majeurs et al. 2005).

Clinically speaking, the term Disorders of Consciousness (DOCs) refers to a heterogeneous group of individuals who have survived severe brain damage (Bekinschtein et al., 2009). In cases of severe cerebrovascular accident (GCA) of different aetiology (traumatic, anoxic, haemorrhagic), patients may lose consciousness and fall into a comatose state. Coma is a state of non-responsiveness in which patients lie with eyes closed and cannot be awakened even when intensively stimulated (Plum and Posner 1983). Comatose patients are characterized by a lack of sleep–wake cycles (Teasdale and Jennett 1974) and they have neither verbal production nor response to command, but can present reflexive responses to painful stimulation. In these patients, there is no awareness of self or of the environment. The autonomous functions such as breathing and thermoregulation are reduced and patients require respiratory assistance.

Global brain metabolism (i.e. energy use) is also diminished by 50–70% of normal (Laureys 2005). Coma results from a diffuse cortical or white matter damage, or from a brainstem lesion (Vanhaudenhuyse et al. 2010). Coma must last at least 1 h to be distinguished from syncope, concussion, or other states of transient unconsciousness. The prognosis is often made within 3 days. If the aetiology is traumatic, half of the patients who have no chance to recover will die during this short period (Schnakers, Majeurs and Laureys, 2004). During the evolving state of consciousness following coma, two clinical conditions may be present: vegetative state (VS) or minimally conscious state (MCS +/-).[29] The Vegetative State (VS) has been recently redefined as a syndrome (UWS) (Laureys et al., 2010), and is characterized by the re-opening of the eyes (after a coma state) and a partial recovery of the sleep/wake cycle and reflexive movements (but with no voluntary/intentional response). On the other hand, Minimally Conscious State (MCS) is characterized by fluctuating behavioural signs characteristic of a certain degree of awareness of the surrounding environment and, sometimes, of residual communicative abilities. (Figure 18)

[29] MCS was recently subcategorized based on the complexity of patients' behaviours: MCS+ describes high-level behavioural responses (i.e., command following, intelligible verbalizations or non-functional communication) and MCS- describes low-level behavioural responses (i.e., visual pursuit, localization of noxious stimulation or contingent behaviour such as appropriate smiling or crying to emotional stimuli). (MA Bruno, 2011)

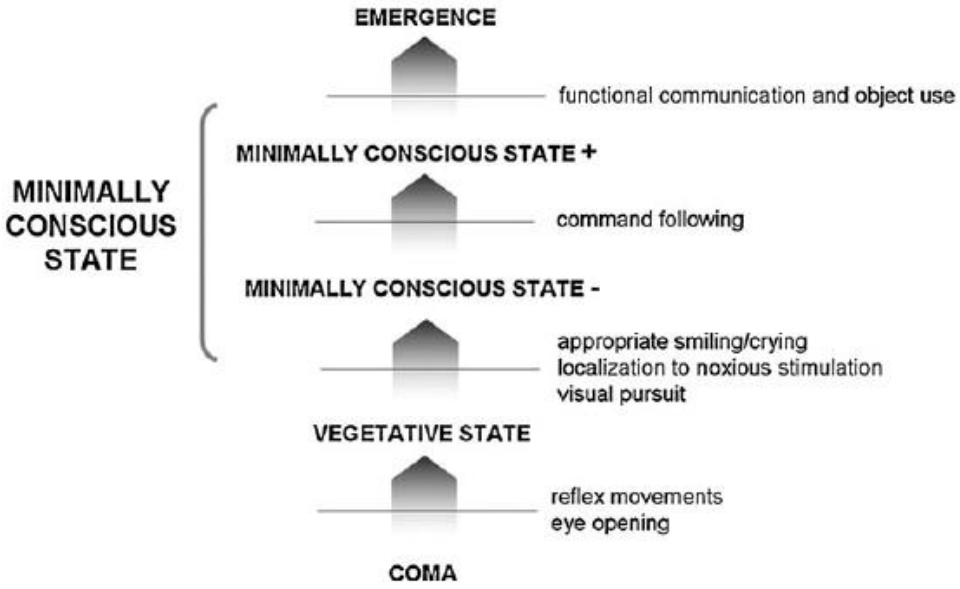


Figure 18. Schemes of stages after coma

6.2 The Diagnostic Problem

The clinical assessment of patients with DOCs critically depends on the patients' residual ability to create a connection with the outside world by conveying their subjective experience through motor behaviour.

However, frequently, the severity of the brain injury is such that the patient, although conscious, is unable to perform any kind of movement or adequately understand the required command. Therefore, there is a gap between the behaviour and the residual cerebral complexity. Voluntary movements may be wrongly interpreted as reflex movements and motor responses may be very limited due to a paralysis of all limbs (quadriplegia) (Gosseries et al., 2011). Motor responses can also be quickly exhaustible and therefore not reproducible (Schnakers et al. 2004). The level of arousal can also fluctuate and patients may become drowsy or even fall asleep while evaluating them. All these constraints lead to diagnostic errors. Studies have shown that 20–40% of patients diagnosed as vegetative showed signs of consciousness when assessed with sensitive and reliable standardized tools (consciousness scales) (Childs and Mercer 1996, Schnakers et al. 2009). Indeed, the distinction between movement or involuntary reflex and intentional movement is often difficult to be performed at the bedside and

typically requires repeated and careful evaluation. Even when intentional, the behavioural manifestations of this population may be very little appreciable. Together with the frequent fluctuations in the level of consciousness characteristic of these patients, this gap makes the differential diagnosis between VS and MCS subjects very challenging. Moreover, patients with DOCs might have deficits in the domain of language comprehension and production, such as aphasia.[30] This makes even more complicated the assessment of consciousness, because the patient might be unable to understand the given commands.

In summary, problems of misdiagnosis could be due to at least three reasons:

1) The discrimination between VS and MCS hinges upon the (in)ability of any given patient to signal their awareness by sustained, reproducible, purposeful or voluntary (motor) response.

2) Arousal and motivation fluctuation

3) Aphasia

6.2.1 Behavioural scales

In order to help clinicians involved in the care of patients with DOCs in the challenging task of distinguishing the vegetative (VS) from the minimally conscious state (MCS), specialized neurobehavioural rating scales have been developed, providing a reliable and valid means of detecting signs of consciousness. However, there are significant differences among these scales with respect to diagnostic sensitivity (Majerus, Thwaites, Andrews, and Laureys, 2005). The most used ones are the Glasgow Coma Scale (GCS), the Coma Near-Coma Scale (CNC-S) (Rappaport et al., 1992), the Sensory Modality and Assessment Rehabilitation Technique (SMART; Chatelle et al., 2010), and the Coma Recovery Scale-Revised (CRS-r) (Kalmar & Giacino, 2005).

The Glasgow Coma Scale (GCS) remains the most widely and internationally used scale of reference nowadays, probably due to its simple and short administration (Gosseries et al., 2011). However, it is commonly considered more suitable for the

[30] Aphasia is an inability to comprehend and formulate linguistic behaviour because of damage to specific brain regions.

assessment of the acute state after brain injury than for the monitoring of consciousness in the chronic states after coma.

Glasgow Coma Scale		
Response	Scale	Score
Eye Opening Response	Eyes open spontaneously	4 Points
	Eyes open to verbal command, speech, or shout	3 Points
	Eyes open to pain (not applied to face)	2 Points
	No eye opening	1 Point
Verbal Response	Oriented	5 Points
	Confused conversation, but able to answer questions	4 Points
	Inappropriate responses, words discernible	3 Points
	Incomprehensible sounds or speech	2 Points
	No verbal response	1 Point
Motor Response	Obeys commands for movement	6 Points
	Purposeful movement to painful stimulus	5 Points
	Withdraws from pain	4 Points
	Abnormal (spastic) flexion, decorticate posture	3 Points
	Extensor (rigid) response, decerebrate posture	2 Points
	No motor response	1 Point
Minor Brain Injury = 13-15 points; **Moderate Brain Injury** = 9-12 points; **Severe Brain Injury** = 3-8 points		

Figure 19. *Glasgow Coma Scale*

More detailed and specific assessments of patients' level of consciousness are provided mainly by the Coma Near-Coma Scale (CNC-S) (Rappaport et al., 1992), the Sensory Modality and Assessment Rehabilitation Technique (SMART) (Chatelle et al., 2010), and the Coma Recovery Scale-Revised (CRS-r) (Kalmar & Giacino, 2005). These scales provide a standardized evaluation of the arousal level, of the auditory, visual, gustatory, and motor functions, and of the verbalization and communication abilities. For each explored sensory/motor modality, scores are provided according to the presence/absence of the response (both intentional and reflexive), and to its consistency and functional complexity.

The Coma Recovery Scale-Revised (CRS-R) by Giacino et al. (2004) is considered the most suitable scale to assess changes in the level of consciousness. It also provides a more fine-grained assessment of the recovery of consciousness. The basic structure is similar to the GCS. It includes similar visual, motor and verbal subscales as the GCS, but there are three additional sub-scales: an auditory function scale, a communication scale, and an arousal scale. Furthermore, the visual, motor, and verbal

subscales are much more detailed than they are in the case of the GCS. For example, the visual subscale assesses visual startle responses, eye fixation, eye movement, visual object localization, and object recognition. These items are critical for identifying subtle signs of recovery of consciousness. Furthermore, for each item, fully operational definitions are provided and special importance is given to the consistency of behaviours assessed via the establishment of baseline observations and repeated administration of the item. This two-step procedure (baseline observation followed by repeated administration of a given item) allows for a greater certainty that a given behaviour is contingent upon a given stimulus and not simply random or reflex; in other words, the behaviour has to be reproducible in the same context.

The CRS-R has been designed to be particularly helpful for discriminating between vegetative and minimally conscious state (Gaicino et al., 2004; Kalmar & Giacino, 2005). A number of specific items are proposed that should permit discrimination between vegetative and minimally conscious state (e.g., the observation of item 2 '*fixation for more than 2 seconds'* on the visual function scale is supposed to incompatible with a diagnosis of a vegetative state, but supports a diagnosis of a minimally conscious state).

6.2.2 Active Paradigms

For the reasons described above, behavioural assessment is not sufficient to describe the complexity of the disorder. Such conditions in fact require a more tailored assessment of the patient's state of consciousness in order to overcome the limits of clinical-behavioural evaluation.

The logic of the consciousness *conundrum* can be summarized as follows:

- If a patient exhibits purposeful and reproducible behaviour, then he/she must be aware and thus (at the very least) minimally conscious.
- But what if he/she does not exhibit a purposeful behaviour? Is he/she not conscious? This is a fallacious conclusion.
- The patient could be aware, but unable to produce a motor output.

- The absence of evidence is not the evidence of absence.

Patients with DOC need therefore a careful examination of their state of consciousness; one that cannot rely only on behavioural performance. The clinical evaluation of patients with DOCs must also involve a quantitative assessment based on the principles of single-subject experimental design.

Several studies provide empirical evidence that functional magnetic resonance imaging (fMRI) and electroencephalography (EEG) offer additional and crucial information ancillary to standard clinical assessment of severe brain-injured patients. A specific subset of protocol called "active paradigms" (Laureys and Schiff, 2012) employs "active tasks" such as mental imagery or selective attention, as an alternative to behavioural response to commands. In particular, in a seminal work (Owen *et al*., 2009) researchers showed how mental imagery can modulate patients' brain activity in a manner similar to healthy subjects. They conducted a fMRI study during which patients are given verbal instructions to perform two mental imagery tasks at specific points during the scan. In the first task, patients are asked to imagine playing tennis, while in the second task they are asked to imagine visiting the rooms of their home. During the periods that they are asked to imagine playing tennis, significant activity was observed in the supplementary motor area. In contrast, when they are asked to imagine walking through their home, significant activity is observed in the para-hippocampal gyrus, the posterior parietal cortex, and the lateral premotor cortex. Their neural responses are very similar to those observed in healthy subjects performing the same imagery tasks during different scan session (Figure 20).[31]

These results confirm that, despite fulfilling the clinical criteria for a diagnosis of vegetative state, this patient retains the ability to understand

[31] In another study from Monti et *al*., (2010) *Wilful Modulation of Brain Activity in Disorders of Consciousness,* different mental imagery (motor imager/spatial imagery) was used as a channel of communication. In this study, the patients were required to give a YES/NO answer to autobiographical questions. Specifically, the patients were instructed to perform either motor imagery or spatial imagery (depending on the trial) in order to answer YES or NO. Patients produced activations indicative of a correct answer in response to 5 of the 6 questions.

spoken commands and to respond to them through her brain activity, rather than through speech or movement.[32]

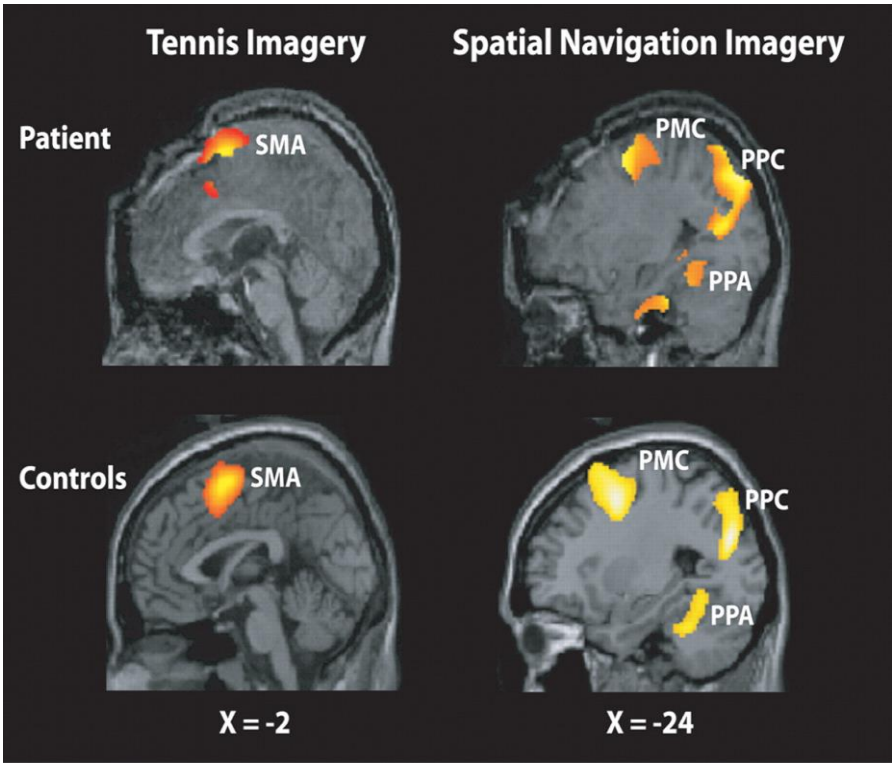


Figure 20. *Brain activity in two different tasks. Controls Subject vs VS patient*

6.2.3 Perturbational Complexity Index (PCI)

Starting from this ground-breaking work on the comprehension of the disorders of consciousness, a lot of empirical and clinical work was done in order to fill the gap between behavioural and neurophysiological evaluation of the level and the content of consciousness (Monti & Vanhaudenhuyse, Coleman, Boly, Pickard, Tshibanda, 2010, Stender J *et al*, 2014).

In line with this effort, some recent studies (Casali, 2013, Casarotto 2016) have developed an objective (brain-based) measurement, independent of behavioural feedback, based on brain response (EEG) to transcranial magnetic stimulation (TMS). The quantification of the electroencephalographic response of TMS results in an index, the "perturbational complexity index" (PCI), that directly gauges the ability of many

[32] Owen et al., (2008) *Detecting Awareness in the Vegetative State*, Science 1402.

functionally specialized modules of the thalamocortical system (differentiation) to interact rapidly and effectively (integration), thus producing complex patterns of activity. PCI has thus allowed to investigate DOCs in an innovative way and has also allowed a stratified classification of clinically-diagnosed non-responsive patients such as VS (or UWS) in three subcategories, based on the level of cerebral complexity: "no response," "low complexity," and "high complexity." Different types of EEG patterns induced by transcranial magnetic stimulation (TMS) add a fundamental dimension to the understanding of the notion of consciousness. The PCI as a measure of complexity can be interpreted as an index of brain potential. In particular, "high complexity" sub-population of patients identified by the PCI would be the category where a dissociation between brain-based measures and clinical assessment is most visible. "High-complexity" group of patients are, in fact, clinically non-responsive, even if they exhibit brain potential of consciousness.

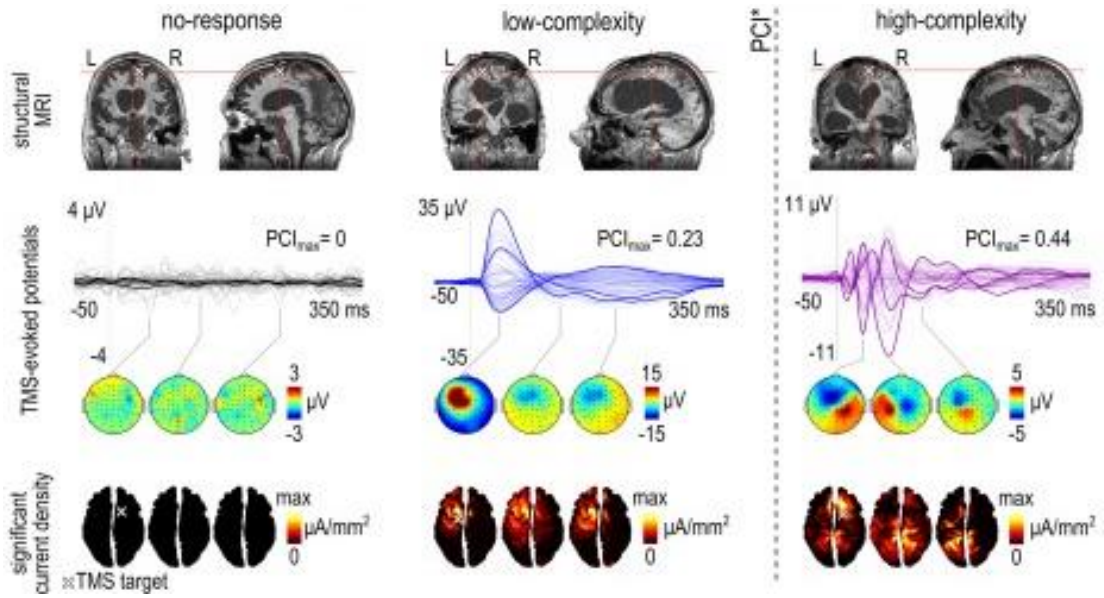


Figure 21. *Representative VS patients resulting from PCI based stratification*

6.2.4 Intentional Action and Consciousness

Nowadays, the assessment of consciousness in patients with severe brain injuries remains a highly demanding challenge from both the clinical and the theoretical perspectives.

The attribution of consciousness to patients still relies on interpreting bodily movements as intentional action, but as a different number of works showed, the absence of intentional movements does not imply the absence of consciousness.

A paradigmatic case is represented by the Locked-In Syndrome (LIS).[33] The LIS is a complete paralysis of the body resulting from a lesion in the brainstem (American Congress of Rehabilitation Medicine, 1995). Gestural and oral communication is impossible, but these patients are often able to blink and move their eyes. Even though the patients cannot move, their sensations are still intact and they are fully aware of both themselves and the surrounding environment (Laureys et al. 2005). The only way for these patients to communicate with their environment is through eye movement and sometimes also with the tip of a finger. Indeed, some control of the fingers, toes or head is generally regained. The patient with LIS is able to answer questions by a simple code such as looking down for "no" and up for "yes", or blinking once for "yes" and twice for "no" (Laureys et al., 2005).

However, in some extreme cases, defined as Total Locked-In Syndrome (CLIS),[34] also eye movements are impaired. The patient is fully conscious but unable to perform any kind of intentional movements.

Is he/she no longer conscious? The LIS and CLIS syndromes, although rare, are well-known syndromes that do not entail lack of consciousness.[35] In absence of manifested movements, only the examination of the patient's brain activity[36] during so-called "active paradigm" might provide information on the patient's level of consciousness.

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[33] The aetiology of patients with LIS is of vascular origin nearly 90% of the cases, but it can also be traumatic. When the lesion is restricted to the brainstem, cognitive functions are fully preserved. If additional cortical lesions are present, the cognitive functions associated with these cortical areas may be affected (Schnakers et al. 2008).

[34] Plum and Posner (1966).

[35] Schnakers, C., (2009).

[36] Principal techniques for such investigation are the EEG, fMRI and PET.

From a philosophical perspective (see Drayson 2013), the assessment of "consciousness" in patients with DOCs where movements are not possible anymore is based on the equation between "intentional bodily actions" and "intentional mental actions." In this respect, cognitive functions such as "mental imagery" are used as vicarious motor functions.

One possible critique to this approach is that the equation between mental and motor function is misleading. It might be the case that "mental imagery" does not require volition. As Owen himself points out, "it is theoretically possible that the mere instruction to imagine such actions triggers specific and automatic changes in brain activity," but, he concludes, "the complexity of the commands used here and the richness of the imagery that is likely to be required to produce a response that is indistinguishable from that of healthy individuals, make this possibility extremely unlikely."[37]

This might mean that even if "mental imagery" is not an optimal candidate to test volition, it definitely requires conscious processing of the stimulus.

6.3 Application of Eye-Blink Protocol on DOC Patients

6.3.1 Introduction

Our study aims to investigate consciousness capability in patients with DOC from a behavioural point of view, by focusing on one of the few preserved movements, the eye blink, and from a brain perspective, by assessing whether the spatiotemporal cortical dynamics that precede a "reinforced" movement, measured with the EEG, exhibit similarities to those found in healthy subjects (i.e. Readiness Potential).

The strengths of this approach are manifold:

1) It is focused on movement – eye blink, as the only behavioural channel preserved.
2) It evaluates cerebral dynamics preceding an intentional movement, based on tested data (healthy controls) and a well-known potential in the literature – the Readiness Potential.

[37] Owen, AM., (2006).

3) It probes volition in an "operant conditioning" protocol, where the learning process is accompanied by a supposed intention to move in order to obtain a pleasant stimulation. It has been reported that patients with disorders of consciousness (DOCs) can learn a complex behaviour (Lancioni *et al*. 2011, 2012), although the conscious and voluntary nature of these learning behaviours has never been quantified or verified objectively. The definition and implementation of brain activity's measure that reflects the ability of a patient with DOCs to perform a voluntary act has clinical, therapeutic and ethical implications.

   A fundamental notion in this study is the one of "operant conditioning."[38][39]

   Operant conditioning differs from classical conditioning for the fact that the stimulus-response association in the classical conditioning is based on temporal contiguity and frequency of repetition of association. In the classical conditioning framework, the learning process is automatic and physiological, without intermediate cognitive processes such as stimulus comprehension and stimuli's expectation. In this respect, in the classical conditioning the subject is seen as passive. In the operant conditioning framework, the establishment and the enhancement of the associative link between stimulus and response is not based just on temporal contiguity, but on the effects derived from the response. In the operant conditioning, a voluntary response is then followed by a reinforcing positive or negative stimulus. If the reinforcement is positive, the likelihood of the response will increase; otherwise, if the reinforcement is negative, the response will be extinguished.

   In the positive reinforcement paradigm, cognitive representations are the medium through which the subject learns how to obtain a positive stimulus. The term "operant" is referred to any active behaviour that operates upon the environment to generate consequences.

   Given that patients with DOCs are characterized by a heterogeneous spectrum of motor disability, we have identified the blinking as one of the few movements preserved in patients with DOCs, and as such, it represents a possible candidate for the exploration of the patients' learning ability.

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[38] Thorndike, E.L. (1901). "Animal intelligence: An experimental study of the associative processes in animals." *Psychological Review Monograph Supplement*. 2: 1–109.

[39] Skinner, B. F. "Science and Human Behaviour," 1953. New York: MacMillan

6.3.2 Experimental Procedure

To this end, a Matlab graphic user interface (GUI) was developed to detect the kinematic characteristic of the blink both *online*, during operant conditioning protocol, and *offline*, during baseline recording.

During the first phase of the study, 5 patients (VS (2), MCS (2), EMCS[40] (1)) with sufficiently preserved spontaneous eye blinking are selected, and recorded sessions are targeted to characterize spontaneous motor repertoire baseline. Within each patient-specific motor repertoire, a distinct kinematic pattern is isolated. This pattern has to be reproducible, but not frequent, and therefore usable as a target movement to be reinforced (Figure 2). The specific movement must be present in the spontaneous repertoire of the patient, but not very frequent, otherwise the conditioning would not take place. In fact, if every blink is positively reinforced, the patient would be unable to recognize the peculiar movement that generates the reinforcement.

In particular, those blinks that have deviating characteristics with respect to the normal distribution, in terms of amplitude and/or duration, are reinforced online.

Ideally, if the patient recognizes and learns the relationship between a certain type of blink and a positive reinforcement (familiar voices, pleasing music for the patient), the distribution of kinematic parameters will progressively move to the less frequent selected parameters.

The conditioning that is to be practiced is therefore "operant": if the subject recognizes the contingency between a specific "outlier"[41] blink and the reinforcing stimulus, the likelihood of occurrence of the same type of response in the presence of same context is expected to increase.

A further prediction based on the empirical evidence obtained in the control subjects is that the blink performed to obtain positive reinforcement might be preceded by a preparatory brain activity similar to the one preceding the voluntary blink in the control population.

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[40] Emerged from Minimally Conscious State (EMCS).

[41] An outlier is an observation that lies at an abnormal distance from other values in a random sample from a population.

6.3.3 Stages and description of the experimental protocol

Experimental procedure:

- Blink recording (periphery - only EOG)
- Offline analysis – GUI/ Definition of parameters (both visually and automatically)
- Creation of a template (average of outlier blinks)
- Check of template (correlation and amplitude)
- Stimuli for conditioning
- Online conditioning
- Online conditioning and simultaneous EEG recording
- Offline analysis (behavioural and EEG recording)

Description of each stage:

1. To detect eye movements and identify the blink artifact, a vertical electro-oculogram (EOG) was recorded by two electrodes placed above and below the eyelid (in line with the pupil when the gaze was in central position, to exclude saccadic movements (horizontal movements). The electrodes used are very light and disposable surface adhesive electrodes, not intrusive for the patient. Peripheral recording is necessary to collect a sufficient number of events (blinks) in order to create a distribution of events where to individuate outlier blinks.
2. An offline GUI on Matlab was created to detect and classify blink parameters. Specifically, the offline blink analysis follows these steps:

- Selection of the channels for EOG detection on BrainAmp System
- Offline trial rejections of non-blinks events (saccades, muscle artifact, etc.)
- Manually selection of the start and end points of blink events
- Calculation of different parameters (Figure 22): time to peaks EOG (ms), number of blinks (in case of double or triple blinks that can be present in DOC patients), EOG duration (ms), EOG area, EOG amplitude referred to the baseline, EOG amplitude referred to the onset. Starting from the parameters distribution, it is possible to individuate which parameters are outlier compared to the patient's normal distribution.

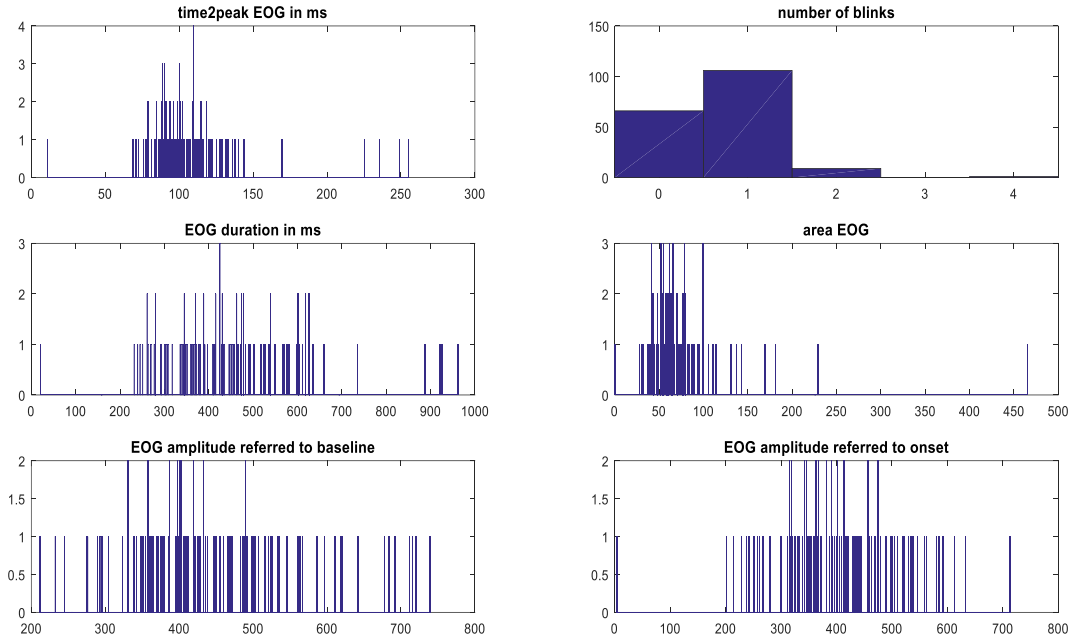


Figure 22. *EOG parameters calculated offline*

3. After the selection of the blinks'outlier parameters, a template – obtained as the average of different outlier blinks - is created to be used for the online reinforcement.
4. The creation of a template is necessary for the online reinforcement: the online GUI is built in such a way as to deliver a stimulus whenever eye blinks detected online match with the template. Specifically, blink events have to correlate (0.9 of correlation) with the template. Moreover, it is possible to set other parameters, such as amplitude, and set a threshold on it in order to create a more accurate match between the chosen outlier blink and the selected template.
5. The selection of stimuli for the online conditioning protocol is based on the anamnestic data about each patient. The involvement of relatives and friends in the production of stimuli is fundamental, because the aim is to create stimuli with an emotional valence for the patient. The requirement for the "audio-files" is that they have not to be too long (around 10 sec), otherwise the risk might be to not catch patient's attention; moreover, audio-files have to contain the patient's name. There are some evidences[42]

[42] Tamura *et al*., (2016) Hearing subject's own name induces the late positive component of event-related potential and beta power suppression, *Brain Research.*

that the patient's hearing of his/her own proper name might facilitate a bridge for communication.

6. The online conditioning is based on the GUI created in Matlab, aiming to detect all blink events and to reinforce only the ones that match (correlation > 0.9) with the template. Whenever the correlation requirement is satisfied, the system provides the stimulus. If during the stimulation another blink to reinforce occurs, this is recorded as "reinforced event", but it does not interrupt the stimulation. Each conditioning session can last from 10 to 15 minutes, to maximize the efficacy of the stimulation. Conditioning sessions are made each day, approximately 2 hours per day, for a period of at least 10 days. Every day's behavioural responses, and any increase of "reinforced events," are monitored.
7. If the conditioning protocol works as expected, behavioural conditioning sessions will be joined to the EEG recording. As for the healthy subjects, it is employed a 64-channel EEG amplifier (BrainAmp DC, *Brain Products*, Germany). Sixty surface electrodes are placed in a cap according to the International 10/20 System. All electrodes are referenced to the linked earlobes. The final purpose of this work is in fact to understand if blinks made to obtain a pleasant stimulation ("reinforced events") are preceded by a recordable cerebral activity similar to the Readiness Potential.
8. Two types of analysis are performed: the first is relative to behavioural results, the second is relative to the EEG recordings.

   Behavioural data are stored according to the following parameters for each day of conditioning:

- nr. of sessions
- type of session (baseline / conditioning in the case of the EEG recording)
- total events (tot of blinks)
- reinforced events (blinks reinforced)
- correlation setting
- template used
- duration of session

9. EEG recording

   As for the healthy subjects, a 64-channel EEG amplifier (BrainAmp DC, *Brain Products*, Germany) is employed. Sixty surface electrodes are placed in a cap

according to the International 10/20 System. All electrodes are referenced to the linked earlobes and a ground electrode was placed on the forehead. Impedances were kept below 5 kΩ and the signal was acquired at a sampling rate of 5000 Hz (DC acquisition). To detect eye movements and identify the blink artifact, a vertical electrooculogram (EOG) is recorded by two electrodes placed above and below the right eyelid (in line with the pupil when the gaze was in central position).

6.4 Case 1: DOC patient

6.4.1 Eye-blink protocol on DOC patient

The first patient (A.S.) involved in this study is diagnosed as Vegetative State patient (VS).[43]

(Figure 23)

Date	Total score	Auditory	Visual	Motor	Oromotor / Verbal	Communica-tion	Arousal
13/01/2014	6*	1	0	2	1	0	2
02/09/2016	11*	2	3	2	2	0	2
06/10/2017	7	2	0	2	1	0	2
09/10/2017	7	2	0	2	1	0	2
13/10/2017	8	2	1	2	1	0	2

Figure 23. *CRS'scores: description of behavioural variability during years*

The aetiology of the disorder is traumatic brain injuries.[44] Neuroimaging data show more post-traumatic bilateral fronto-basal lesions on left hemisphere.

The patient is peculiar because A.S. is characterized as "high-complexity" by TMS-EEG evaluation: PCI=0.44.

Blink-periphery is recorded on different days, during different parts of the day in a 3-month time period, in order to catch the variability of the behaviour. During the 3-month recording (for a total of 14 sessions), blink detection has also been refined and

[43] The CRS's score is respectively:
13/01/2014 – 6*best score (Vegetative State)
2/09/2016 – 11*best score (Minimally Conscious State) (score for each subscale: auditory_2 visual_3 motor_2 oromotor_2 communication_0 arousal_2).
6/10/2017 – 7 (Vegetative State)
9/10/2017 – 7 (Vegetative State)
13/10/2017 – 8 (Vegetative State)

[44] In July 2017, when the conditioning protocol started, 4 years have passed since the acute event.

optimized, both in the montage,[45] and in the type of electrodes used. In particular, at the beginning of the recording 4 electrodes (2 EMG and 2 EOG) were employed to mimic and compare the montage used in healthy subjects. Later on, a montage composed by 2 electrodes (EOG) was employed, to minimize the patient's discomfort, in order not to worsen the blink detection. In this way, the montage is very easy to apply and it is not unpleasant for the patient. In addition, detection has improved through the employment of adhesive electrodes that, unlike those employed in healthy subjects, are lighter, more adherent to the face skin, and faster to apply.

From blink-periphery recording, a population of outlier blinks is obtained. Specifically, in this patient the outlier parameters are those referring to the amplitude. (Figure. 24)

The template is created from the average of those blinks with an amplitude above the average amplitude that usually appears with a frequency of 1/10 with respect to a "normal" event.

An EEG baseline is recorded before the beginning of the conditioning behavioural protocol, so as to compare it with the EEG during conditioning.

The conditioning behavioural protocol lasts for 11 days (with an interval of two days between days 5 and 6), while the EEG recording is performed in days 8-9-10-11.

Description of different days of conditioning:

Day 0: EEG baseline.

Day 1: 5 sessions (4 min, 3 min, 4 min, 11 min, 15 min) amplitude threshold: 1 >500, 2 >500, 3 >400, 4> 380, 5>350.

Day 2: 4 sessions (8 min, 10 min, 15 min, 15 min) amplitude threshold: 1>350, 2>320, 3>320, 4 >320.

Day 3: 7 sessions (3 min, 5 min, 13 min, 11 min, 13 min, 11 min, 11 min) amplitude threshold: 1> 500, 2>400, 3>320, 4>350, 5>380, 6>400, 7>450.

Day 4: 2 sessions (12 min, 13 min) amplitude threshold: 1>400, 2>450.

Day 5: 5 sessions (12 min, 11 min, 12 min,12 min,14 min) amplitude threshold:1>400, 2>450, 3>420, 4>400, 5>400.

[45] The first montage employed is EOG/EMG (4 electrodes), then a belly tendon montage is employed (3electrodes) and finally just EOG (2 electrodes).

Day 6: 6 sessions (5 min, 5 min, 12 min, 12 min, 14 min, 12 min) amplitude threshold:1>450, 2>400,3> 380, 4>350, 5>350, 6>400.

Day 7: 6 sessions (8 min, 12 min, 14 min, 12 min, 20 min, 7 min) amplitude threshold: 1>400, 2>320, 3>320, 4>320, 5>400, 6>450.

Day 8: 6 sessions (30 min – EEG baseline, 10 min, 11 min, 8 min, 20 min, 10 min) amplitude threshold: 1> 400, 2>320, 2>320, 3>320, 4>320, 5>400.

Day 9: 4 sessions (20 min – EEG baseline, 12 min, 7 min, 19 min) amplitude threshold: 1>450, 2>400, 3>350.

Day 10: 2 sessions (20 min – EEG baseline, 20 min) amplitude threshold: 1> 320.

Day 11: 5 sessions (20 min – EEG baseline, 8 min, 5 min, 10 min, 21 min) amplitude threshold: 1>450, 2>480, 3>500, 4>550.

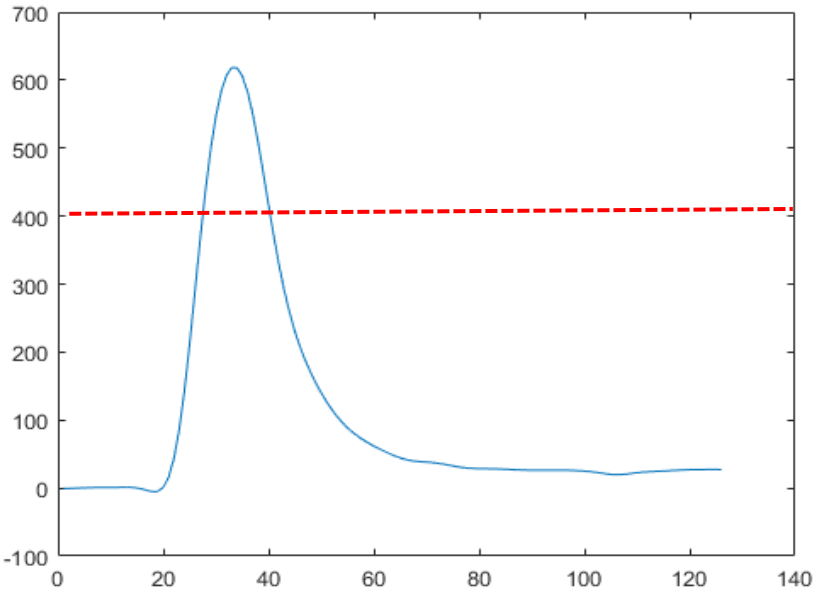


Fig. 24 Blink template with an amplitude's threshold (>400)

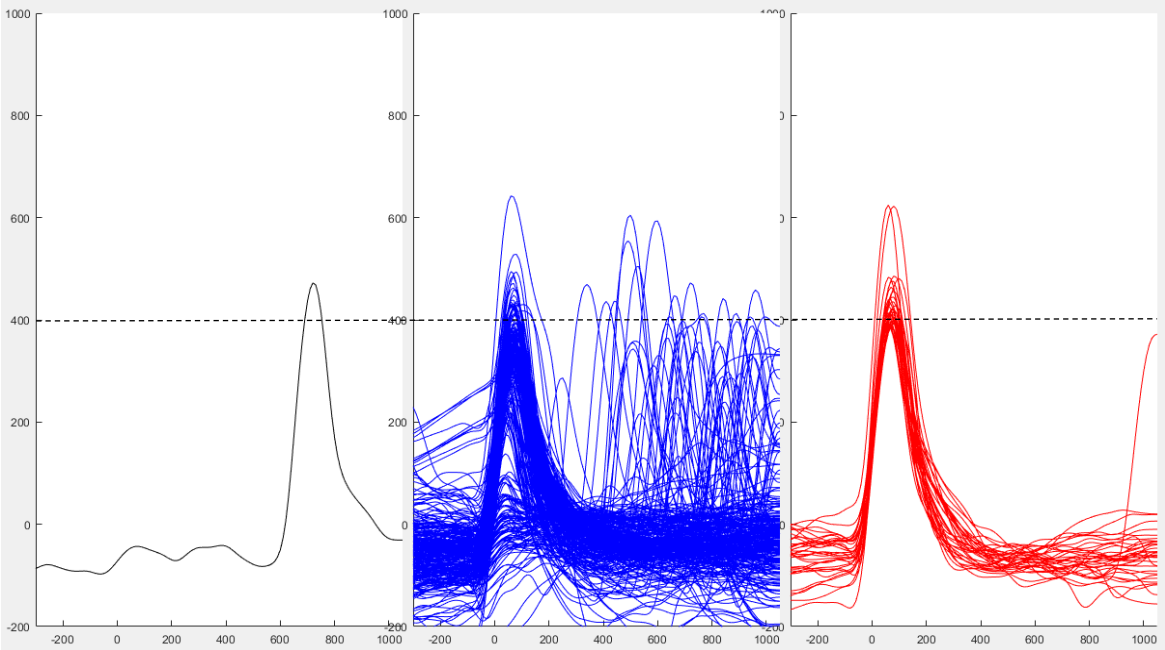


Figure. 25 First column: online blink trace; second column: all blinks detected, reinforced blinks (with a threshold on amplitude > 400)

Stimuli used:

Conditioning stimuli are audio-files created with the help of relatives, close friends, and friends of patient. They are required to record an audio file of the length of approximately 8/10 secs that has to contain the patient's name. Each audio is composed in this way: reference to patient + greeting/encouragement, or memory of past events, or reference to habitual situations (e.g. work or home environment). All audio files are submitted to a closer examination done by a neuropsychologist, who indicates which audio can be suitable for the conditioning.

EEG acquisition and analysis:

EEG recording is done in day 0 (EEG baseline) before the starting of the operant conditioning, and on days 8-9-10-11 during the final days of operant conditioning.

(Baseline)

0 day – EEG 64 channels

(Operant conditioning)

8 – EEG channels FC1 – FCz – FC2 – C1 – CZ – C2 – CP1 – CPz – CP2 – P1 – PZ – P2

9 – EEG channels FC1 – FCz – FC2 – C1 – CZ – C2 – CP1 – CPz – CP2 – P1 – PZ – P2

10 – EEG channels FC1 – FCz – FC2 – C1 – CZ – C2 – CP1 – CPz – CP2 – P1 – PZ – P2

11 – EEG 64 channels

Data analysis was performed with MatLab (Math Works Inc., R2015b) and the SSP BioMedical Data Analysis Package (SiSyPhus Project; Version 2.0e, 2.1e), developed by members and collaborators of TMS-EEG LAB of the Department of Biomedical and Clinical Sciences "Luigi Sacco," University of Milan.

All signals were filtered using a high-pass, 1st order finite impulse response (FIR) filter (corresponding to a detrend).

Filters 0.1-40 Hz was applied, data were downsampled to 500 Hz. Epochs were split to -2000/+1500 ms. The reference was bilobar. Data were baseline corrected to -2000/-1500 ms.

Data were pruned with ICA, only component – the one containing blink artifacts - was removed.

After ICA data were low-pass at 5 Hz. Statistical analysis was done after 5 Hz low-pass filter.

Trigger positioning:

To detect the eye blink onset, the EOG signal was processed as follows:

- 40-2000 Hz band-pass Butterworth filtering;
- Peak interpolation using Hilbert transform function;
- Smoothing using a Gaussian filter;

Amplitude of the EOG signal were then computed.

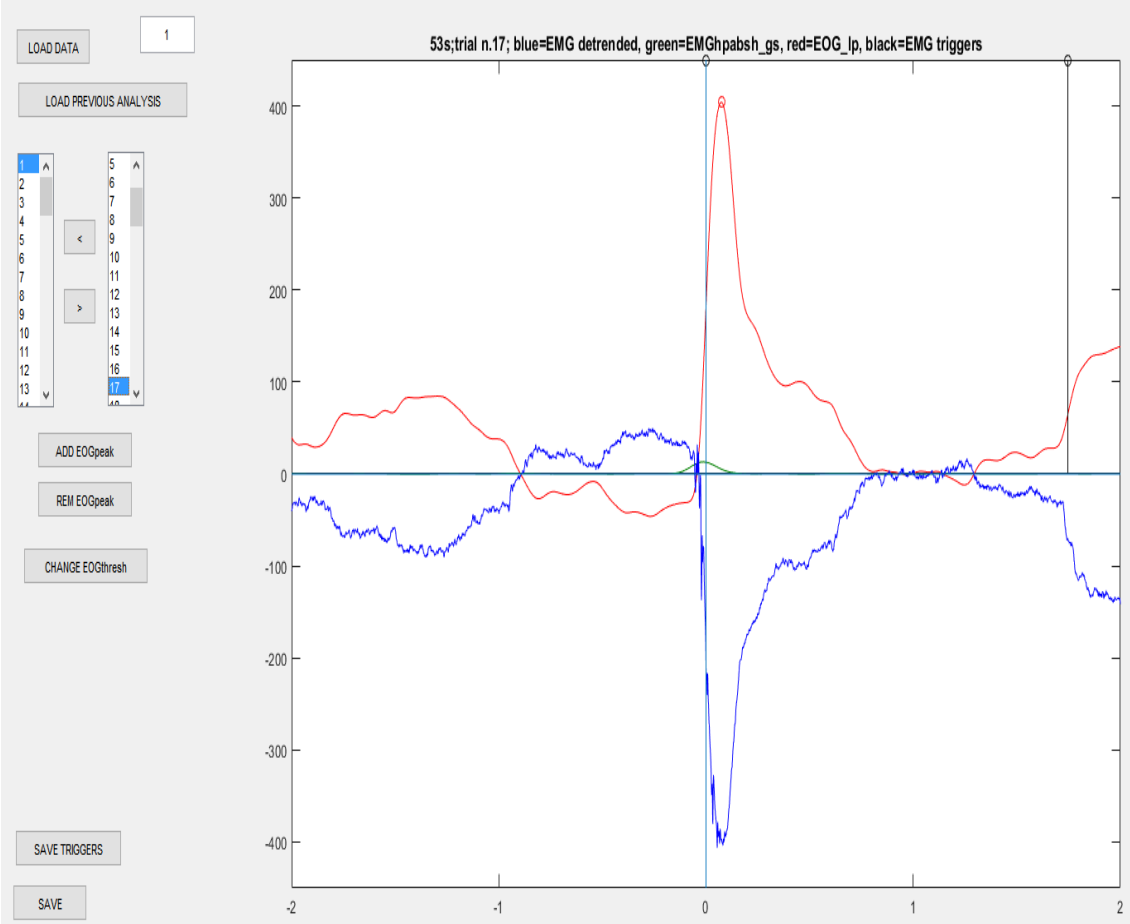


Figure. 25 *Manual GUI for detection and analysis of EOG*

EOG were selected and divided based on amplitudes. In particular, for the EEG analysis blinks with the same amplitude were compared.

These comparisons were done:

1. Epochs in EEG baseline (day 0) containing EOG of the same amplitude of EOG reinforced in day 9 and 10 (amplitude >350). The epochs with EOG >350 of day 0 were compared with epochs with EOG >350 of days 9 and 10.

   Day 0 – 3 sessions – 1 session 17 >350, 2 session 35>350, 3 session 60>350 (after trial rejection)[46]

   Day 9 + Day 10 (59 + 30) 89 reinforced trial >350 (after trial rejection)

[46] Bad trials are considered on the basis of periphery (EOG) and bad EEG signal.

Statistical analysis:

An unpaired t test (one tail: left) is applied to compare both conditions: baseline and operant conditioning.

8 channels were selected (FC1 – FCz – FC2 – C1 – CZ – C2 – CP1 – CPz – CP2).

Both the mean average of 8 channels in baseline and 8 channels in the operant conditioning sessions and single channels were compared. (Figure. 26, Figure. 27)

The results show a significant (p value < 0.05*) difference between baseline condition sessions and operant conditioning sessions.

We also employed the Wilcoxon rank-sum test that is a nonparametric alternative to the two sample t-test, which is based solely on the order in which the observations from the two samples fall. Also, from this non-parametric test we obtained a significant difference between the two conditions. (p value respectively for t-test <0.01 and for rank-sum <0.01)

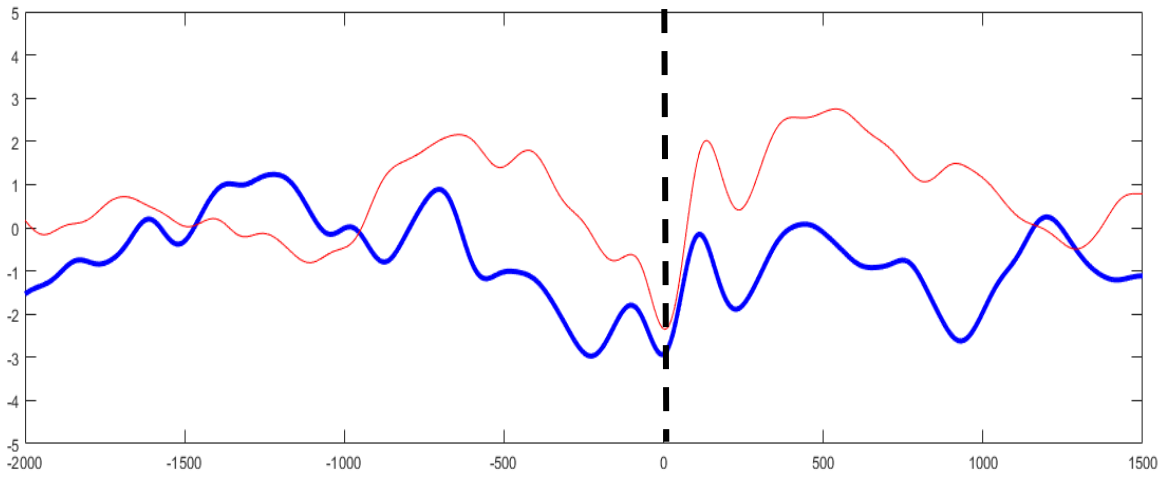


Figure. 26 Average Across Channels (Baseline vs Operant Conditioning sessions)

Baseline session (Day 0)

Operant Conditioning sessions (Day 9-10)

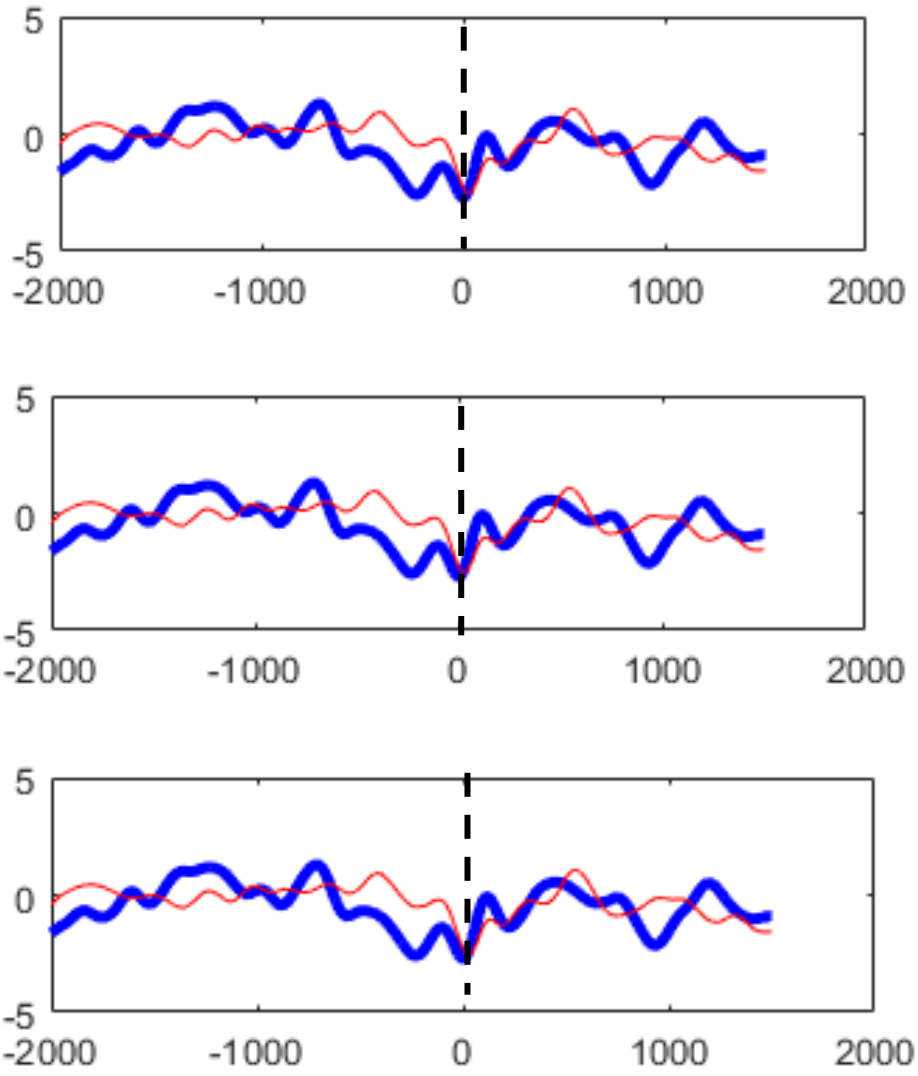


Figure. 27 Single Channels (C1-Cz-C2)

(Baseline vs Operant Conditioning sessions)

Baseline session (Day 0)

Operant Conditioning sessions (Day 9-10)

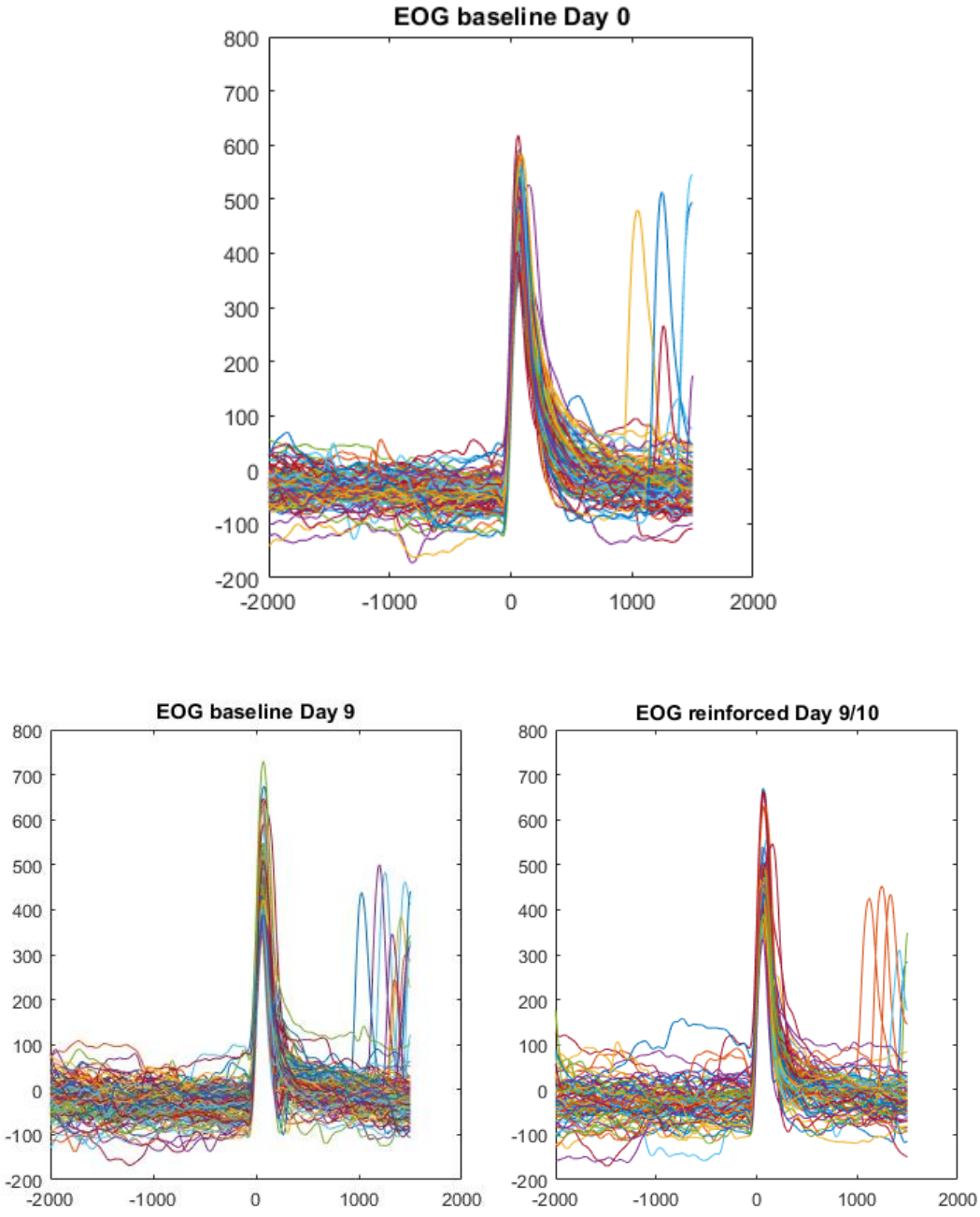


Figure. 28 EOG (blink >350) Baseline (Day 0) VS Reinforced (Days 9/10)

6.5 Case 2: LIS patient

6.5.1 Eye-blink protocol on LIS patient

The Locked-In syndrome (LIS), as explained above, is defined by: 1) the presence of sustained eye opening; 2) preserved consciousness; 3) aphonia; 4) quadriplegia or quadriparesis; and 5) a primary mode of communication that uses saccades or blinking.[47] This state just superficially resembles the vegetative state: the patient in fact appears very impaired at level of motor behaviour except for the eye blink.[48]

Given that the patient is conscious but in highly acute physical condition, he/she is explicitly asked to perform voluntary eye blink each time for several times. Cued protocol is preferable to self-paced one, in this specific case, because the patient, albeit conscious, is in highly severe physical condition and the execution of a self-paced task could be compromised.

Methods:

As for the healthy subjects, a 64-channel EEG amplifier (Nexstim)[49] is employed. Sixty surface electrodes are placed in a cap according to the International 10/20 System. All electrodes are referenced to the linked earlobes, and a ground electrode was placed on the forehead. Impedances were kept below 5 kΩ and the signal was acquired at a sampling rate of 1000 Hz. To detect eye movements and identify the blink artifact, a vertical electrooculogram (EOG) is recorded by two electrodes placed above and below the right eye (in line with the pupil when the gaze was in central position)and the eyelid.

For practical reasons and to try to prevent the patient's discomfort and fatigue,[50] two sessions are acquired: Baseline – recording of spontaneous eye blink movements, and Voluntary session: recording of voluntary eye-blink movements.

[47] Bruno MA., *Rev Neurology* 2008

[48] In some extreme cases defined as Total locked-in syndrome, or completely locked-in state (CLIS), the eyes are paralyzed as well.

[49] Different pieces of equipment are due to the different Hospital in which the EEG is acquired. LIS patient was in San Gerardo Hospital, Monza.

[50] The patient is in intensive care.

EEG recording and analysis: the same analysis applied to DOC patient (A.S.) is performed.

Results:

Unpaired t-test (left) showed a significant difference (p value < 0.01*) between spontaneous and voluntary condition.

Also for the Wilcoxon rank-sum test there is a significant difference (p value < 0.04*) between the two conditions.[51]

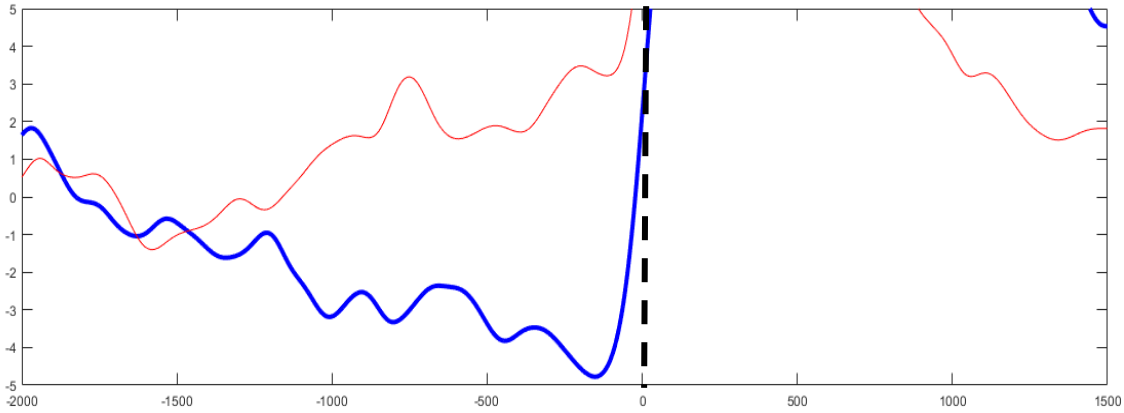


Fig. 29 Average Across Channels

LIS patient (Baseline VS Voluntary Session)

Baseline session

Voluntary session

[51] LIS patient's examination is not meant to explore the potentiality for communication since LIS patient is able to blink in order to communicate his/her preferences when asked to do so (cued protocol). It is just a proof of concept.

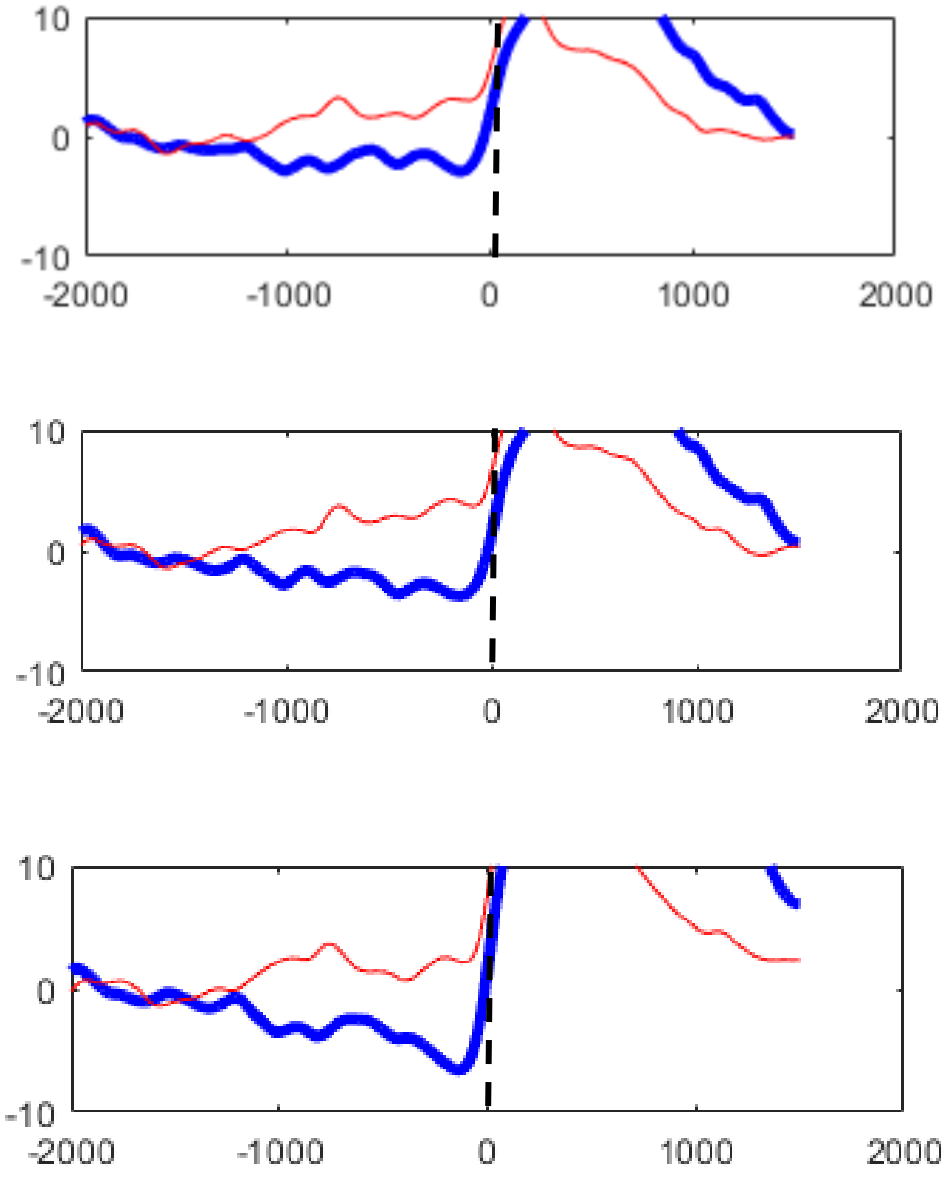
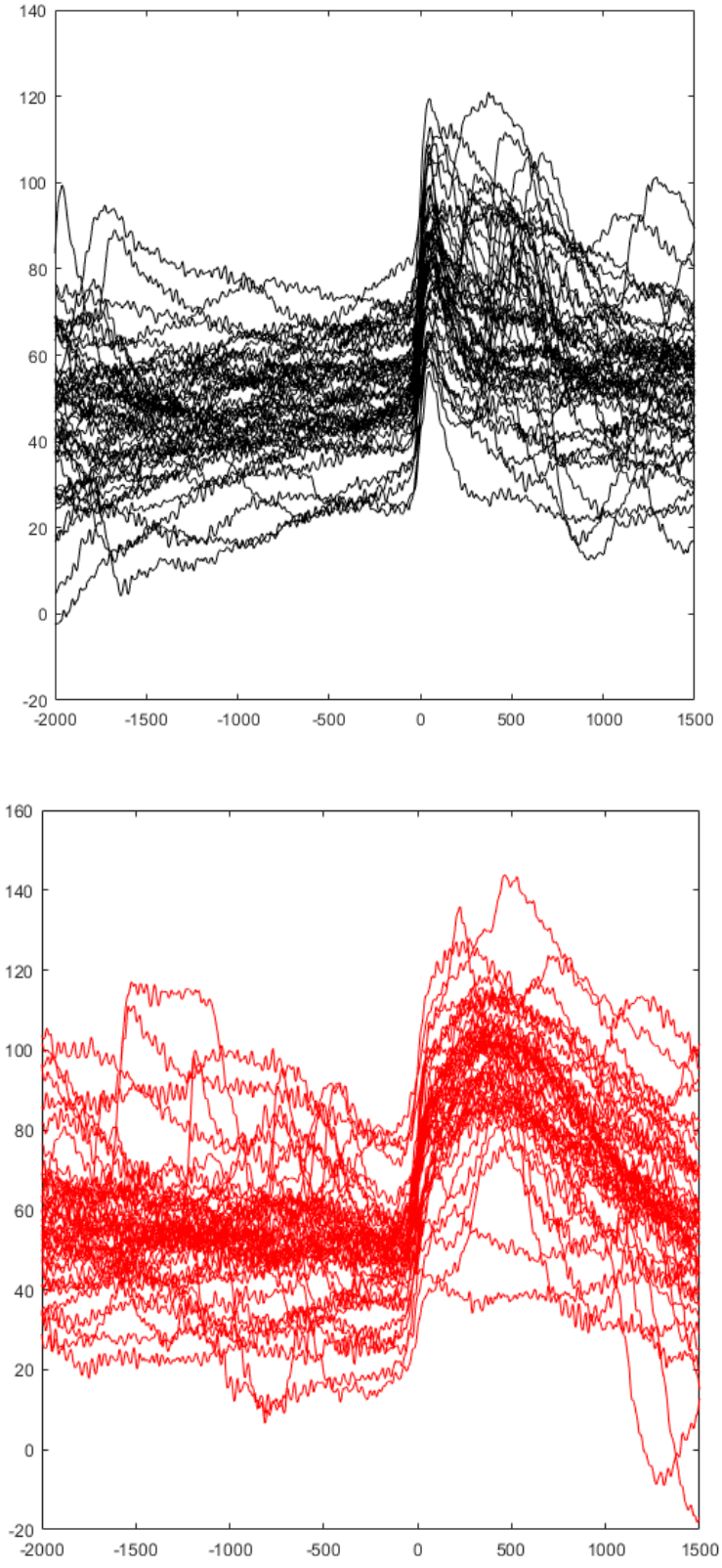


Figure. 30 Single Channels (C1-Cz-C2) (Baseline VS Voluntary Session) LIS patient

Baseline session

Voluntary session


140
120
100
80
60
40
20
0
-20
-2000
-1500
-1000
-500
0
500
1000
1500
160
140
120
100
80
60
40
20
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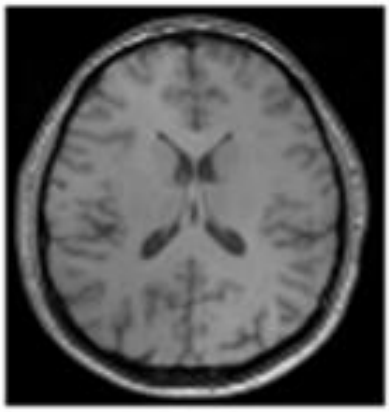
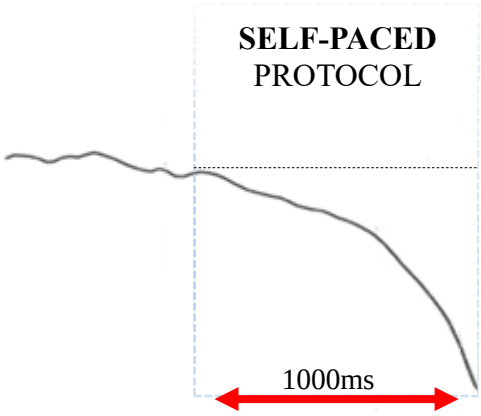
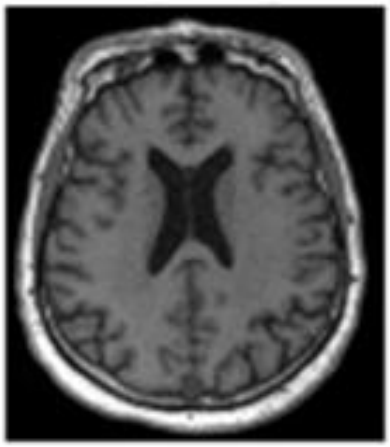
-20
-2000
-1500
-1000
-500
0
500
1000
1500
EOG Baseline
EOG Voluntary

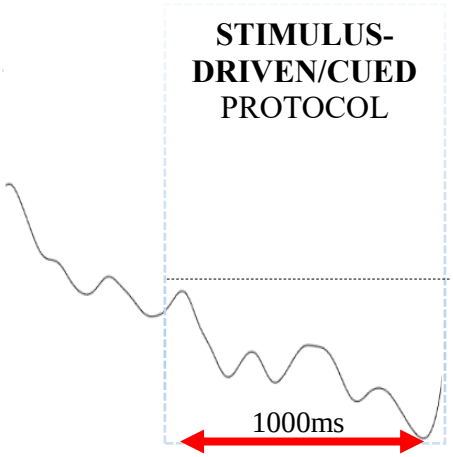


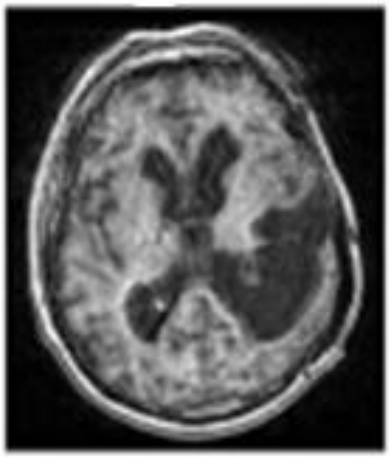
Healthy Subject **PCI: 0.48**





LIS Patient **PCI: 0.47**





DOC Patient **PCI: 0.44**

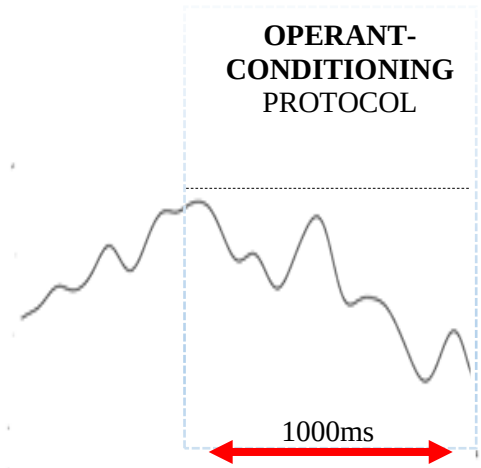


Figure 32. Fingerprints of volition (Readiness Potential) in three representative subjects.

As shown in Figure 32, the Readiness Potential can be elicited with (at least) three protocols:

1. Self- paced protocol
2. Cued/stimulus driven protocol
3. Operant conditioning protocol

In healthy subjects, self-paced protocol is employed to elicit the Readiness Potential through eye blink movement, during a Libet-style experiment: the subject decides when to move based on his/her own free will.

In LIS (locked-in syndrome) patient, the cued protocol is more suitable to overcome the patient's discomfort and to be sure about the voluntary execution of the task. The patient is explicitly asked to make a voluntary eye blink; he/she does not decide when to move.

Finally, in DOC patient, an operant-conditioning protocol is necessary in order to elicit a supposed intentional eye blink. Stimulus-response behaviour is supposed to bring out a volitional response; the patient might decide to receive positive stimulation.

Exploring cerebral dynamics before a voluntary movement is relevant both from a theoretical perspective—because it characterizes the difference, in neural terms, between voluntary processes and spontaneous ones—and from a clinical/experimental perspective, especially in DOC patients. The Readiness Potential, qua neural marker of volition, can be used as an ancillary measure to assess consciousness in DOC patients.

From the perspective of the diagnostic challenge, a more tailored and clearer definition of the disorder of consciousness (DOC) and the possibility of actively conditioning residual movements and of categorizing them from intentional to spontaneous, could add a new dimension to the concept of "consciousness".

From a rehabilitative perspective, as the only preserved channel, the eye blink can be used with a communicative aim as a tool for the patient to interact with the surrounding environment. In addition, the emergence and the increase of a specific motor pattern could determine a global improvement of patient's arousal, vigilance and attention.

Ultimately, from an ethical perspective, the recognition of a voluntary movement is crucial to understand the real possibility for the patient to testify for his/her presence. Which kind of conscious experience is conveyed through an intentional movement? The phenomenal experience, or "what is like" to be in a vegetative state, is not communicable through a subjective report. However, it is worth trying to comprehend if a motor act, appropriately conditioned, might be a channel for an alternative way of communication.

Although, it remains an open question, if neural preparatory processes before a supposedly volitional movement emerged at conscious level. With respect to this, further experiments and analysis are required in order to best characterize the "Readiness Potential" and its relationship not only with conscious processes, but also with intentional ones. Alternative views to the classical interpretation of the Readiness Potential seem to suggest that the early part of RP, usually considered as the brain preparatory development, might not be a necessary condition for a voluntary action to occur (Han-Gue Jo *et al*., 2014), for at least three reasons:

1) The Readiness Potential emerged from the average of at least 30/40 trials (e.g. selected epochs containing movements), but it is not clearly visible from single trials. What is then its actual role in voluntary action?
2) Could the Readiness Potential be a general mechanism not specific for motor initiation, but also for thought and speech initiation? It might be that while for motor initiation, it would be easier to time-locked movements and then back averaging, this cannot be possible for thought and speech.
3) Moreover, from recent studies (Alexander *et al*., 2016) it does not seem neither a sufficient requirement for volition, since Readiness Potential-like negative potentials seem not to be followed by a movement.

The actual nature of the Readiness Potential needs a deeper investigation, especially given the results obtained from Vegetative State patient. Is Readiness Potential-like neural activity a necessary and sufficient condition for an intentional action to emerge? It might be that "slow cortical potentials," including the Readiness Potential, are brain phenomena mainly involved in attention, expectation, preparation, that co-occur with such cognitive processes without causing them. They might be necessary but not sufficient conditions to an intentional action to be carried out.

Part 4. Experimental protocol on epileptic patients

Chapter 7

7.1 Co-registration of HD-EEG and SEEG

Cumulating empirical evidences suggests that the Readiness Potential is not generated in one central brain region, but instead relies on several cortical and subcortical structures that are known to be directly linked with motor control. Cortical sources of the Readiness Potential appear contra-laterally to the movement in the primary motor cortex and somatosensory cortex and bilaterally in the SMA pre-SMA and cingulate cortex.

Subcortical generators of the Readiness Potential are found in in the basal ganglia and in the thalamus (Rektor, 2001). According to Birbaumer (1990) slow cortical potentials such as the Readiness Potential might result as an interplay between cortical and subcortical structures. Furthermore, it is possible that not even all the cortical sources are captured in the scalp-recorded readiness-potential since some dipoles may be hidden from the recording electrodes. Moreover, intracranial data provides a unique tool for exploring information about Readiness Potential timing compared to the common EEG approach.

7.1.1 Experimental Protocol

The data included in the present study was collected during the pre-surgical evaluation of neurosurgical patients with a history of drug-resistant, focal epilepsy.

Five patients were assessed in total. All subjects were candidates for surgical removal of the epileptic focus. During the pre-surgical evaluation, all patients underwent individual investigation with simultaneous hd-EEG and recordings performed by stereotactically implanted depth multi-lead electrodes (SEEG). In the following, we will direct our focus on the SEEG activity.

SEEG activity was recorded from platinum-iridium semiflexibile multi-contact intracerebral electrodes, with a diameter of 0.8 mm, a contact length of 1.5 mm, an

inter-contact distance of 2 mm and a maximum of 18 contacts per electrode. The performed blink protocol was similar to the one described in section 3.

In the spontaneous “baseline” phase patients were given the following instructions:

"In the first phase of the experiment, we will record your cerebral activity at rest, you will need to keep your head still and look at a point / image / object in front of you, think about what you want, just try to relax and avoid sharp movements of the hands and feet, we'll notify you of both the beginning and the end of the recording”.

In the subsequent voluntary phase these additional instructions were given: “"In this new phase of the experiment you will need to keep your head still and look at a point / image / object in front of you, try to relax and avoid abrupt movements of your feet and arms. You will need to make voluntary blinking movements - fast closing and eyelid reopening, do not strain too much, try to do it as naturally as possible - also try to think whether you want to do it, the blink must be intentional. If you blink spontaneously between a voluntary blink, do not worry. Voluntary movements should not be too close; try to wait a bit (at least 5 sec) between a blink and the other without taking in mind the time passing. Do not worry about the number of movements to be performed, but be sure that each blink is performed voluntarily. We will also advise you at this stage and at the end of the recording”.

In the voluntary phase, for these patients, we did not differentiate between brisk and slow execution because of time constraints in clinical setting. Moreover, due to the limitations of the clinical setting only 15 minutes of each condition could be recorded.

7.1.2 Data analysis

Blink onset was detected by the same algorithm as described in section 3.

SEEG and hd-EEG data was segmented -3 to 1 seconds with respect to blink onsets. Note that the slightly longer pre-blink time window compared to section 3 was chosen due to the possibility that the Readiness Potential may have an earlier onset in the SEEG signal compared to the scalp recorded EEG. Hd-EEG data was high-pass filtered for 0.1 Hz and first inspected manually. Noisy channels were interpolated using spherical splines and noisy trials were discarded from further analysis. The data

was then low-pass filtered for 5 Hz. ICA was conducted to reduce the prominent blink artifact and to remove other artifactual components such as eye movements. In order to investigate possible sources of the readiness-potential the SEEG data was analysed in the high-frequency broadband (HFB). Data was first high-pass filtered for 5 Hz. Noisy electrodes were identified manually and discarded. Data was then median referenced and cut into epochs. Line noise and its harmonics were removed. Electrodes and trials displaying frequent or very strong epileptic activity were again identified manually and discarded. After this initial preprocessing, the HFB response was obtained following the same procedure as in Golan et al. (2016). The signal was split into eight 10 Hz frequency bands between 70 and 150 Hz. For each band the absolute value of its Hilbert transform was calculated and divided by its mean across time. Finally, the eight bands were averaged together into a single time course and baseline corrected for mean amplitude between -3 to -2.5 seconds prior to the blink. In order to assess on which contacts voluntary blinks are preceded by changes in HFB activity the single trial traces were averaged over 2 seconds preceding each blink. These mean HFB amplitudes were compared between conditions via unpaired t-tests. Due to the exploratory nature of this analysis no correction for multiple comparisons is currently undertaken. All contacts exhibiting a p-value <= 0.01 are considered significant and will be discussed below.

7.1.3 Results

Of the five assessed patients four had at least one contact exhibiting significant differences in the neural activity preceding spontaneous and voluntary blinks. Patient 1 had a right posterior exploration covering large parts of the visual cortex (see Figure 33). Several of these contacts exhibited very strong effects in the 2 seconds preceding voluntary blinks. The respective contacts were located in the pericalcarine and the lateral occipital cortex, the cuneus and the precuneus. Additional effects were found on contacts in the right lingual and supramarginal cortex. Finally, there was one contact in the right isthmus cingulate also exhibiting RP-like activity (Figure 33).

Patient 2 had a left frontal exploration (Figure 33). Several of these contacts were in the anterior cingulate cortex yet none of them showed the effect of interest. Two interesting contacts were found in the left superior frontal and rostral middle frontal cortex, however. Although the difference between spontaneous and voluntary blinks is not very pronounced the systematic difference between conditions still hints at the possibility that these frontal areas are involved in the RP process.

Patient 3 had a right occipito-parieto-temporal exploration not unsimilar to patient 1 (data not shown). Also for this patient a contact was found in the right cuneus exhibiting a strong preparatory effect for voluntary blinks. A second significant contact was found in the right superior parietal cortex. Patient 4 had a right fronto-temporal exploration (data not shown). The only significant effect was found on a contact in the right supramarginal cortex.

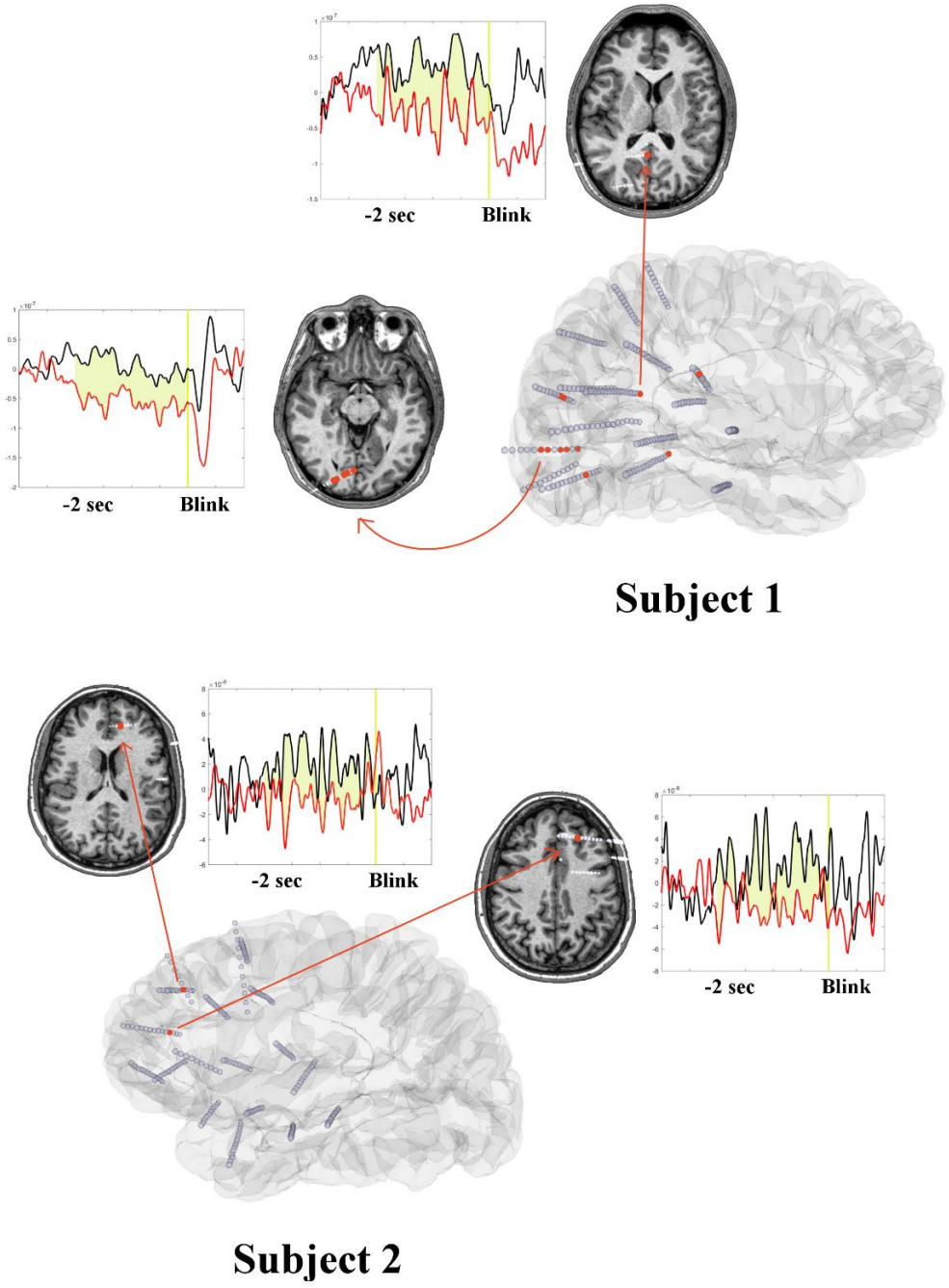


Figure 33. Two representative patients with implanted SEEG. The full exploration for each patient is depicted on a translucent hemisphere. Each gray dot depicts one contact on a SEEG rod. Contacts showing significant differences between spontaneous and voluntary blinks in a 2 second time window prior to the event are highlighted in red. For each patient two of the significant effects are illustrated in

more detail in the insets. Each inset depicts a horizontal slice of the patient's brain with the significant contact once again highlighted in red. Next to it is the average HFB (high-frequency band) activity for the spontaneous (black) and voluntary (red) condition recorded from this contact. Blink onset is marked with a yellow vertical line. The 2 second time window over which mean HFB amplitude was assessed is filled in with pale yellow.

7.1.4 Discussion

From the preliminary results sketched above it is clear that voluntary eye blinks are processed differently compared to spontaneous eye blinks in the few seconds leading up to the event. This confirms the previously described EEG results obtained both in healthy subjects, in the LIS patient and in the DOC patient. However, there are also several noteworthy differences in intracranial activity in respect to the information obtained from EEG recordings.

Specifically, brain activity preceding voluntary movement (eye blink) seems to be spread over diverse areas which are not usually related to movement preparation and execution. As shown in Figure 33, significant differences in HFB between spontaneous and voluntary blinks in a 2 second time window prior to the movement, appeared both in visual and frontal areas that are not usually involved in movement.

It might be possible that the "voluntary" pathway goes through different areas, before reaching the intended movement. Changes in high-frequency power might represent an aspecific brain modulation (e.g. change in neurons firing rate) correlated with intentional processes.

An interesting aspect emerging from the intracranial data is the information relative to "Readiness Potential" timing. HFB decrease seems to start earlier - around 2000 ms before the intentional movement - than the "classical" Readiness Potential observed on the scalp for voluntary eye blink. This might suggest a more spread involvement of different areas in the actual performance of intentional movement. Moreover, since differentiation in brain activity in non-motor areas seems to start earlier in comparison to the "Readiness Potential" in motor areas this might suggest that the "Readiness

Potential" on the scalp could represent the summation of different concomitant processes in different areas.

Another intriguing aspect of the results is the fact that several of the contacts showing a readiness potential effect were also "blink sensitive", i.e., there was a prominent transient of neural activity in response to the blink itself. Most of these contacts were located in the posterior visual cortex. Since HFB difference in spontaneous and voluntary condition it is present specifically in "blink sensitive contacts" this might shape the quest of volition from another perspective. It could be plausible that specific brain region - involved in the production and processing of a certain movement or event - (e.g. visual areas for blink) could be dynamically involved in the volitional process.

A lot of different exploration, in different areas, has to be done in order to confirm this hypothesis, but this is a promising starting point.

In addition, "blink" as a dynamical *continuum* from automatic/spontaneous to intentional movement, represents a new powerful tool that in combination with intracranial exploration, could inform about different neuronal way in which an intentional act can be performed.

Part 5.

Conclusion

In sum, we performed four kind of empirical investigations:

1. Experiment on healthy subjects (EEG)
2. Experiment on DOC patient (EEG)
3. Experiment on LIS patient (EEG)
4. Experiment on epileptic patients (SEEG)

Our study on healthy subjects and LIS patient, characterized and confirmed previous results about the existence of Readiness Potential's like activity preceding voluntary eye-blinks while this activity is absent for spontaneous eye-blinks.

The investigation of DOC patient provides precious findings about the existence of Readiness Potentials' like activity in Vegetative State patient (VS) with an high level of brain complexity (PCI=0.44) that has never been proven to date.

We demonstrated that it is possible to modify the movement "quality" – from spontaneous to intentional – in an operant conditioning protocol as Readiness Potential presence before conditioned blinks might suggest.

Preliminary intracranial explorations, showed that Readiness Potential is not only located in specifically motor-driven areas (Cz and neighbouring electrodes) but it also appears in non-motor areas. This might indicates that the volitional processes is more complex and distributed among different areas. (Figure 34)

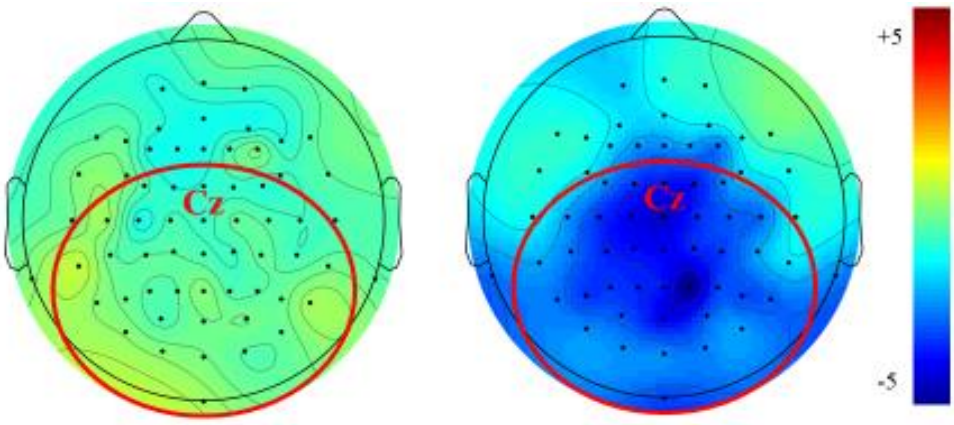


Figure 34. Topoplot of spontaneous blink conditions and voluntary blink condition in a time period between -1500 ms and -100 ms before movement of one representative subject.

In the debate about "Readiness Potential", we added a pivotal dimension: the investigation of the spontaneous movement. Blink, in fact, provides an inestimable tool to explore the movement-related cortical potential taking in account, for the first time, the systematic assessment of spontaneous movements. Starting from Kornhuber & Deecke investigation of brain cortical activity preceding intentional movement, the analysis of movement-related cortical potentials (MRCPs) was always focused on finger or hand movements without taking in account the comparison between intentional and spontaneous movements such as eye-blink movements.

If "Readiness Potential", in the "alternative" framework, is interpreted as the average of stochastic fluctuations time locked with the onset of the movement, why this random fluctuation do not appear before spontaneous movement?

What is missing in Schurger's empirical investigation is a specific claim about non-intentional movements. If spontaneous fluctuations are characteristic of brain activity regardless of the activity that is about to happen, why non-intentional blinks were not preceded by Readiness Potential? In this respect, blink – as one of the few motor act that can be performed either spontaneously and intentionally – is a powerful tool to employ in the deeper investigation of the complex brain dynamics of intentional action.

In DOC patients, "blink" is often one of the few preserved movements. Blink-related cortical activity in this respect became an important and powerful index of brain activity. Detecting a progressive increase of volition (up to the levels obtained in healthy subjects during voluntary blinks) during the course of the conditioning sessions will in fact indicate that patients' blinking might reflect a conscious act.

To which degree intentionality is linked with specific neural activity, and at which moment the "intention to move" emerges, remains an open question of fundamental empirical and theoretical importance.

In conclusion, the novelty of our empirical investigation rely on sketching a new theoretical and empirical framework of an old but gold neuroscientific problem – how intentionality emerges from brain structures – perturbing the brain with the most simple movement: the eye blink.

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