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XXI CICLO

NEW INSIGHT ON THE FUNCTIONS ORGANIZATION OF THE INSULA OF REIL AND THE INNER PERISYLVIAN REGIONS: A MULTIDISCIPLINARY APPROACH

Coordinatore:
Chiar.mo Prof. Vittorio GALLESE

Tutor:
Chiar.mo Prof. Vittorio GALLESE

Dottorando: Ahmad JEZZINI

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OVERVIEW

"The Histology of the Island of Reil. The functions of this part of the brain are unknown, although aphasia, and, again certain disorders of speech in general paralysis have been attributed by some to diseased conditions of its substance. Hidden in the depth of the Sylvian Fissure, there can be no doubt that the Insula is often unnoticed or forgotten in pathological examinations."

This is a note written by J. Crichton Browne and Herbert C. Major in occasion of the issue of Major's paper, "The structure of the Insula of Reil in apes". This note appeared in *The west Riding Lunatic Asylum Medical Reports, Vol. vi.,* published in 1877.

Seventy years after, in their book, "The Neocortex of Macaca Mulatta", Bonin & Bailey wrote: *"Buried in the depth of the lateral fissure lies the enigmatic island of Reil whose functions and affinities are totally unknown*".

More recently Jean Isnard, from the Functional Neurology and Epileptology Unit of the Hospital of Lyon, a world-leading group in the treatment of the insular epilepsy, wrote: *"La notion d'épilepsie Insulaire est longtemps restée inconnue. En raison de sa situation, profondément enfouie sous les opercules et recouverte par le réseau artérioveineux sylvien, l'insula est longtemps restée inaccessible aux explorations intracrâniennes et son rôle dans les épilepsies partielles est demeuré peu connu jusqu'à ce jour."*

From the very early descriptions until these days, two important considerations emerge in all the studies regarding the insula of Reil; the first consideration is the unanimity of opinions about the difficulties of investigations on the insula due to its anatomical localization. The second consideration is the attribution to the insula of a wide range of functions at different levels.

Covering the medial wall of the Sylvian fissure, the insula of Reil reaches a complete opercularisation only in humans.

In spite of a good knowledge of both insula's architectonics and hodology this is not the case for its functions. Actually, the functional study of the insula of Reil knew an alternation between relatively short periods of interest and long periods of forgetting.

From the architectonic point of view, according to the level of granularization, it is well accepted now that the insula of Reil is subdivided in three major fields: the agranular, disgranular and granular insula. Furthermore, the insula of Reil shares these cytological characteristics with the lateral orbitofrontal and temporo-polar regions forming three concentric rings around the prepiriform part of the olfactory cortex (Mesulam & Mufson, 1982).

The insula of Reil has connections with a wide range of cortical and subcortical regions. The connections of the insula vary from principal sensory modalities in the olfactory, gustatory, somesthetic and auditory cortices, to associative modalities. Furthermore, the insula has connections with the polymodal associative cortices and with paralimbic areas in the orbital, temporo-polar and cingulate areas. Besides, the insula has important thalamic connections as well as with the amygdala and the hypothalamus.

Our understanding of the functions of the insular cortex is poor since its anatomical position buried in the depth of the Sylvian fissure, its opercularisation, and the high vascularisation make very difficult any kind of neurophysiological investigation on monkeys. As a consequence, the interest in the functions of this structure knew a long period of neglect despite a few single neurons recording studies, and few intracortical microstimulation studies (ICMS) that are very old and obsolete, or performed in non ecological situations such as surgical conditions or general anaesthesia. However, these few studies and the topographic distribution of the insular connections suggest two wide functional fields.

The anterior insula, along with the adjacent frontal operculum, are thought to be related to gustatory and olfactory processing. Nevertheless the sensory processing could not be the primary role played by the anterior insula (Augustine, 1996). The results obtained by early electrical stimulation experiments suggested that some kind of motor response can be electrically evoked from the whole insular cortex (Frontera, 1956; Showers & Lauer, 1961). Integrating the data concerning the few gustatory responses found with single unit analysis and the motor responses found by early stimulations it is possible to propose the hypothesis that the anterior insular function is unlikely limited to gustatory processing, but most likely related to an integration of sensory-motor information in order to produce complex behaviours.

In addition, the data from ICMS studies suggest a key role of the orbito-insular complex in the control of the functions of the autonomic nervous system since ICMS evoked changes of the instantaneous cardiac frequency and the blood pressure as well as alteration of respiration (Kaada et al., 1949; Hoffman & Rasmussen, 1952).

The posterior insula, along with the adjacent parietal operculum, are thought to be predominantly somatosensory cortical regions containing a coarse representation of the body, characterized by wide receptive fields (Robinson & Burton 1980, Schneider et al., 1993). Moreover, Zhang et al. (1998) found that middle-posterior insular cortex of the macaque contains neurons modulated by nociceptive stimuli and baroreceptor stimulation, suggesting that somatosensory processing most likely represent only a part of the functions of posterior insula. More recently, Remedios et al. (2009) demonstrated that the posterior insula of macaque monkeys contains neurons responding to conspecifics' vocal stimuli, suggesting that the insula is also involved in complex auditory processing functions.

In humans, despite some indications in few neurosurgical studies, the insula of Reil remained at the margin of the interests, mostly confined to its relation with some forms of temporal epilepsy. But this coarse view of the insula began to change in the last two decades. The general improvement concerning techniques and diagnosis, in particular related to the develop of new electrophysiological and neuro-imagery techniques, enabled to shed light on the notion of insular epilepsy, that was forgotten for long time because of the inaccessibility of the region. The electrical microstimulation represents an essential phase of exploration prior to the surgical treatment enabling a better comprehension of the functional properties of the insular cortex. Isnard & collaborators found responses related to somato-sensory functions (paraesthesia of large cutaneous sectors sometimes associated to thermal or painful sensations) mainly evoked by stimulation of the posterior half of the insula, viscero-sensitive functions (strangulation sensation, abdominal weight, thoracic oppression, nausea…) evoked in the anterior insula, and auditory and language-related (disartria or blocking of mental evocation of words) functions found in the most posterior part of the insula. Other responses were evoked varying from sensation of vertigo or illusion of displacement in space to feeling of derealisation.

Most of these attributed behavioural functions are confirmed by clinical observations regarding patients with tumours, stroke, lesions and epilepsy involving the insular cortex.

The role of the insular cortex in the control of the autonomic nervous system functions in humans is reported in several clinical cases where a variety of cardiovascular alterations is described, among which sinus tachycardia, atrial fibrillation, atrio-ventricular block, as well as a correlation between stroke restricted to the right hemisphere and the phenomenon of the "sudden death" occurring about three month after the ictus (Christensen et al., 2005). Moreover, by means of intracortical microstimulation performed in the insular cortex of pharmaco-resistent epileptic patients, Oppenheimer and colleagues

(1992) reported the existence of lateralization of the sign of autonomic response between left and right insular cortex. they demonstrated that the left insula is predominantly responsible for parasympathetic cardiovascular effects while the right insula has mainly sympathetic cardiovascular effects.

Besides all these "organic functions", the insula of Reil is thought to be involved in the processing of emotional and social behaviour as well as being elated to several psychiatric disorders. The development of new techniques of investigations on humans and, specifically, the introduction of functional magnetic resonance (fMRI) imaging focused the attention on the relationship between the insula and variety of psychological functions. Imaging studies suggested that the insular cortex is involved in the processing of the emotional states both when lived in first-person and when experienced by others. The coding of sensory information (visceral, somesthesic, nociceptive, gustatory, etc…) suggests the implication of the insular cortex in interoception and self-awareness by the mapping of the internal bodly states (Craig, 2002-2003-2009). Furthermore, recent neuroimaging studies suggest the insular cortex to be involved in various neuropsychiatric disorders such as mood disorders, panic disorders, PTSD, obsessive-compulsive disorders, eating disorders, and schizophrenia (Nagai et al., 2007).

Because of the scarcity of single neurons studies on the functions of the insula of Reil, it is urgent to shed light on the neurophysiological aspects of insular functions in order to clarify its role and provide solid physiological bases and interpretation of findings obtained in humans with less finely grained techniques.

In this context, the present research was undertaken in order to deepen our neurophysiological knowledge of this region and to evaluate its contribution to behaviour. In order to obtain converging evidence, the plan of the research project is to combine electrophysiological and anatomical approaches performing: 1) Intracortical Microstimulation (ICMS) of the insula of Reil and adjoining opercular regions (upper and lower bank of Sylvian fissure) in rhesus monkeys (macaca *mulatta*); 2) Study of the neural connectivity of distinct regions within the insula, previously functionally characterized by ICMS.

The first study investigates the functional organization of the insula and inner perisylvian regions in macaque monkeys in order to assess a possible somatotopic organization. ICMS experiments were carried out on two awake free-moving rhesus monkeys (macaca *mulatta*). ICMS was performed with 50 Hz biphasic waves (0.2 msec of phase width) lasting from 50 msec up to 3 sec. Intensity was varied in a range up to 4 mA. During experiments, overt behavior and cardiac activity (ECG) evoked by ICMS have been monitored. The results showed that ICMS of inner perisylvian regions evokes a wide range of behavioral responses, which appeared to be roughly somatotopically arranged.

In the rostral part a representation of oro-alimentary behaviour is present; responses like chewing, mouthing and deglutition prevail dorsally (frontal operculum and dorsal insula). In the ventral part (anterior ventral insula), strong viscero-motor responses (vomiting) are evoked. In the middle part (fronto-parietal operculum and middle dorsal insula), complex behaviours are evoked. In the dorsal caudal part (parietal operculum and posterior dorsal insula), simple motor responses involving distal and proximal effectors are evoked. Moreover, in the ventral intermediate sector of the insula, ICMS evoked communicative responses: the stimulations induced the monkey to lip-smack only when facing the experimenter. In the ventral insula and the lower bank, a miscellaneous of stereotyped and repetitive responses was also present.

For what concerns the effects of ICMS on the autonomic system, a heart rate variability (HRV) analysis was carried out. The results showed different responses (bradycardia and tachycardia) along the rostro-caudal axis: bradycardia was evoked by stimulation of the rostral portion, showing an increase of the effect along the dorso-ventral axis. The posterior part of the studied regions showed a segregation of spots where stimulation induces bradycardia, tachycardia and no-effect.

The present results show the involvement of inner perisylvian regions in the control of behavior as well as in the control of autonomic nervous system functions. Moreover, they show that such control obeys to a coarse somatotopically arranged segregation of functions within the explored regions.

In the second experiment, we investigated the cortical and subcortical connections of the insular cortex. Three anatomical tracers were injected in three different sites where the functional properties were studied by mean of ICMS. On the one hand, the findings of this experiment are in agreement with what had been reported in the literature. The anterior insulo-orbital regions where oro-alimentary behaviours were evoked are connected with orbito-frontal areas (area 12, 11, 13 and 14), the rostral ventral prefrontal cortex (area 46), the precentral opercular area (PrCO), anterior cingulate areas (24b\c and 24a, 32), temporal pole, superior temporal pole (STP), inferior temporal gyrus (TEm, TEa\d), entorhinal cortex, baso-lateral amygdaloid nuclei, hypothalamus and ventral tegmental area (VTA). The ventral middle insula, where communicative responses were evoked, shows connections with areas 12r\m, 13l\m and 11 of the orbitofrontal cortex, area 45a of the prefrontal cortex, with area 44, area F5c of the premotor cortex, disgranular opercular area (DO), areas 24c and 24b of the cingulate cortex, temporal pole, TEa and TEm of the inferior temporal cortex, IPa and amygdala. Injection in the most medial part of SII, bordering with the posterior dorsal insula, where simple movement of lower limbs were evoked, is connected with area F3 of the premotor cortex, primary motor cortex, posterior cingulate areas (32, 24d, 23c), primary and secondary somatosensory areas, superior parietal cortex (PE, MIP) and inferior parietal lobule (AIP, PFop, PGop). On the other hand, these findings are in agreement with the functional properties of the injected sites, since the connected areas are functionally involved in different aspects of the behaviours evoked by ICMS performed in the injected loci.

Taken together, the findings of these two experiments not only confirm a role of the insular cortex and the inner perisylvian regions in a wide range of behaviours and in the control of the autonomic functions, but also improve our understanding of the dynamics of the involvement of the stimulated regions within neural networks responsible of complex behaviours. In the following sections, an introduction on the anatomy and functions of the insula of Reil and the adjoining opercular regions will be presented, before describing data from each of the two experiments. The overall contribution of this research to current understanding of functional organization of the studied regions will be then discussed in a final General Discussion section.

A. INTRODUCTION

SECTION 1 - INSULA AND INNER PERISYLVIAN REGIONS ANATOMY

I. ISLAND OF REIL CONCEPT: HISTORICAL EVOLUTION

The first identification of the island of Reil or commonly the insula, this portion of the human brain lying in the depth of the Sylvian fissure, was published by the German physician, anatomist and physiologist Johann- Christian Reil in 1796 (Figure A1), in his Latin treatise *Exercitationum anatomicarum fasciculus primus de structura nervorum* (*First Volume of Anatomical Practice: On the Structure of Nerves*) (Figure A3). Interestingly, the insula was only mentioned in the text; the figures were primarily dedicated to the structure of cranial and spinal nerves and plexi. Reil stated that these convolutions are the seat of the human mental processes. Reil's discovery was immortalized later as *insula Reili* or island of Reil in Henry Gray's textbook *Gray's anatomy* from the edition published in 1858 through the current edition (Figure A3). Since then the insula is known under different terms like the "fifth lobe of the brain", the "central lobe", the "intersylvian convolutions" and the "intralobular gyri"

The insula became later the subject of anatomo-morphological studies (Eberstaller, 1887; Guldberg, 1887; Cunningham, 1891; Clark, 1896), whose description of the insula and nomenclatures of gyri and sulci are still used in contemporary anatomy. Other studies focused on the embryological development of the insula and the Sylvian fissure noted that the insula is the first cortex to differentiate (Cunningham, 1891; Retzius, 1902; Streeter, 1912; Kodama, 1926, Lockard, 1948). Comparative and phylogenetic studies were initiated early (Cunnigham, 1897; Holl, 1899; Landau, 1919-1934; Ariens Kappers, 1908-20-21-36; Petronievics, 1932; Friant, 1952) and established that only three mammals orders have insular cortex. Cytoarchitectonic studies were initiated early on apes in 1877 with Major. After most of work carried out in the first half of last century regarded the human brain (Brodmann, 1909; Economo, 1925-27; Brockhaus, 1940), studies on monkeys followed with Bailey and von Bonin (1947), Roberts and Akertrs (1963), Sanides (1968), Jones and Burton (1976) and Mesulam and Mufson (1982).

Experimental studies coupled with observations in humans began mostly during the second half of the 20th century, suggesting that the insula is involved in several functional aspects like gustatory, visceral, sensory-motor, vestibular, somatosensory, and auditory functions. There is, however, growing evidence that the insula also contributes to interoception, self-awareness, emotions and empathy.

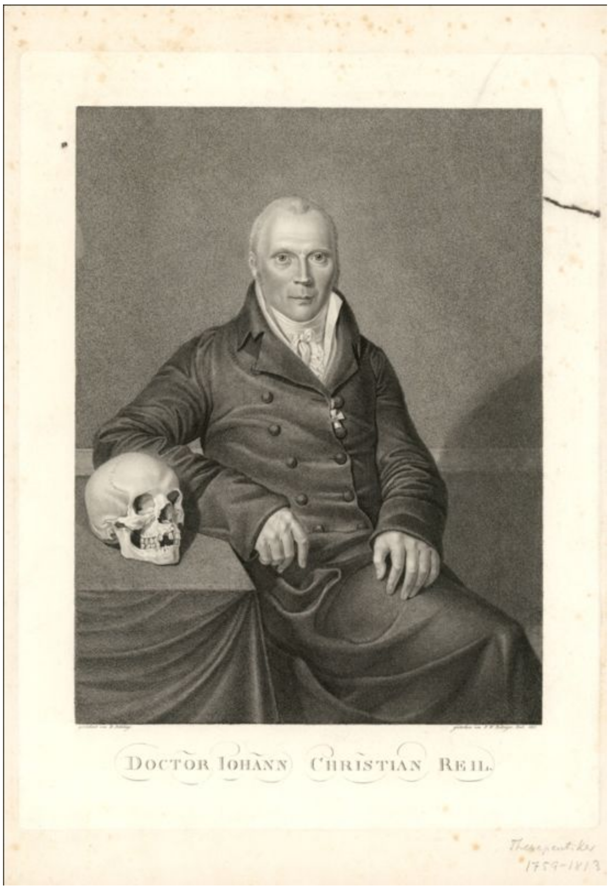
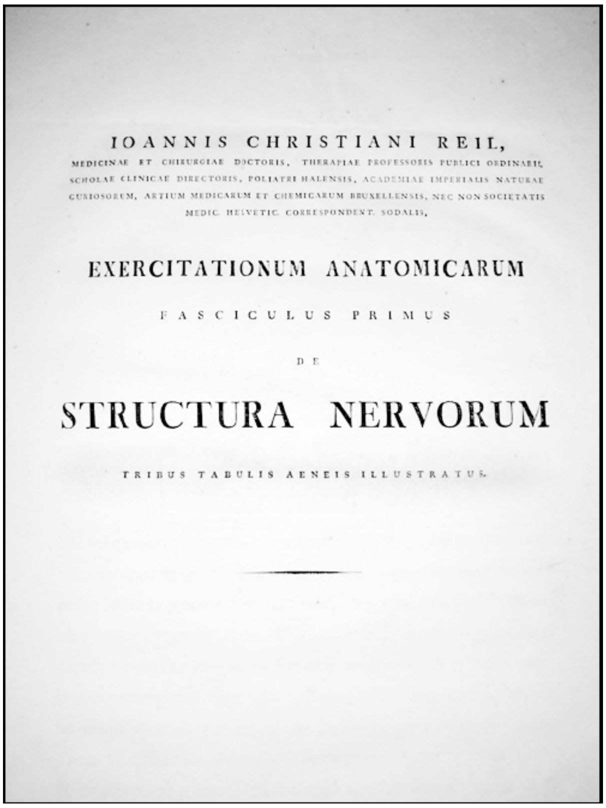


Figure A1: Johann-Christian Reil portrait. In 1796, Johann-Christian Reil published the first description of the Insula, later *Insula Reili* or Island of Reil.
(Source: http://www.sammlungen.huberlin.de/dokumente/7415/)

"JOHANN-CHRISTIAN REIL (1759–1813) was a prominent German physician, anatomist, physiologist and a professor at the Universities of Halle and Berlin. Anatomic features named after him include the **island of Reil (insula)**, Beau-Reil cross furrows, Reil's finger, the fillet of Reil, and Reil's triangle. In 1795, he founded the first German journal of physiology, Archiv für die Physiologie. Later, he would also be considered the founder of German psychiatry (the "German Pinel") and coined the term "psychiatry." In addition, he was a private confidante and physician to Johann Wolfgang von Goethe. Reil died in 1813 from typhus while treating soldiers wounded in the Battle of the Nations at Leipzig during the Napoleonic Wars."

Binder at al., 2007.



IOANNIS CHRISTIANI REIL,
MEDICINAE ET CHIRURGIAE DOCTORIS, THERAPIAE PROFESSORIS PUBLICI ORDINARII,
SCHOLAE CLINICAE DIRECTORIS, POLIATRI HALENSIS, ACADEMIAE IMPERIALIS NATURAE
CURIOSORUM, ARTIUM MEDICARUM ET CHEMICARUM BRUXELLENSIS, NEC NON SOCIETATIS
MEDIC. HELVETIC. CORRESPONDENT. SODALIS,

EXERCITATIONUM ANATOMICARUM

FASCICULUS PRIMUS

DE

STRUCTURA NERVORUM

TRIBUS TABULIS AENEIS ILLUSTRATUS.

Figure A2: Title page of Reil's 1796 32-page Latin treatise *Exercitationum anatomicarum fasciculus primus de structura nervorum* (*First Volume of Anatomical Practice: On the Structure of Nerves*). This work contains the Reil's first description of the insula.

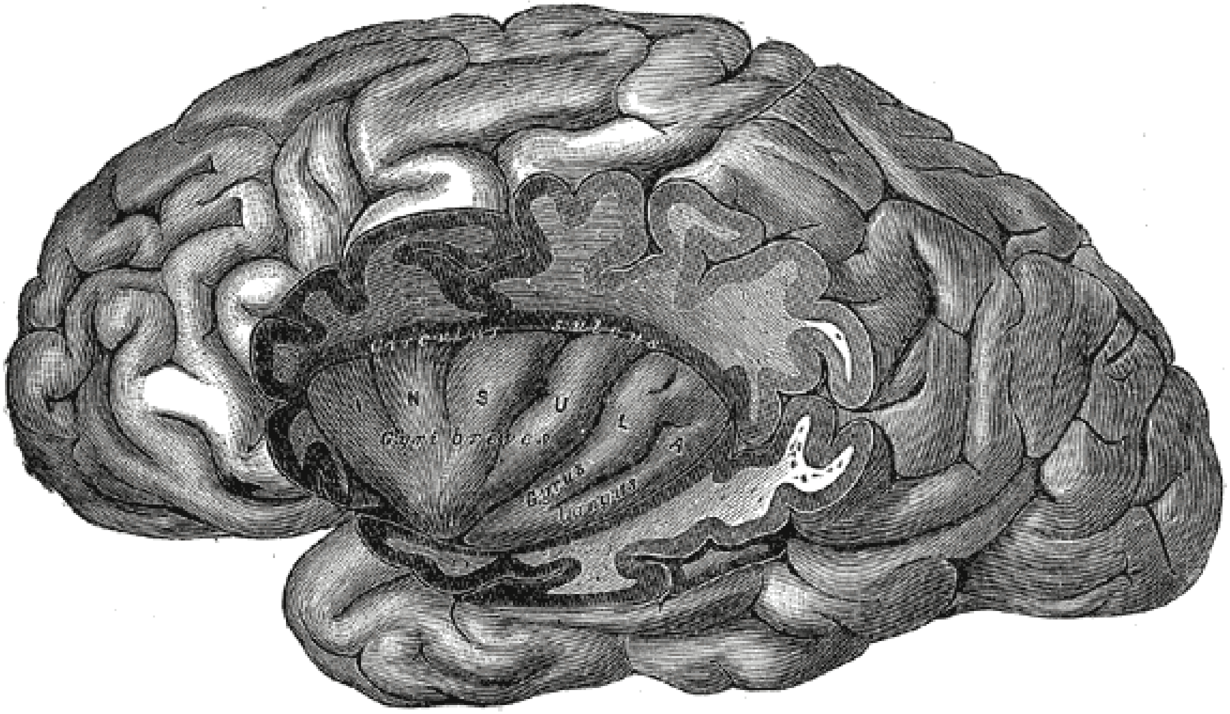


Figure A3: The human insula of the left side, exposed by removing the opercula. This faithful reproduction of a lithograph plate from Gray's Anatomy, a two-dimensional work of art, is from the 20th U.S. edition of Gray's Anatomy of the Human Body, originally published in 1918.

II. Morphological and topographical aspects

Earlier researchers were mostly interested in the morphology of the human insula and the homologies of the different sulci and gyri described by Reil. They pointed out that the insula is divided into an *"insula anterior"* and *"insula posterior"* (Eberstaller; 1887) by the *"sulcus centralis insulae"* (Guldberg; 1887) that lies in the same plane and presents the same direction as the fissure of Rolando and is covered by three opercula: an upper or parieto-frontal, a lower or temporal and an anterior or fronto-orbital operculum. The anterior operculum is further divided into two parts, the frontal operculum or pars triangularis and the orbital operculum. These four opercula are separated from each other by the limbs of the sylvian fissure. The posterior horizontal limb lies between the temporal and parieto-frontal operculum, the anterior ascending limb separates the parieto-frontal from the frontal operculum and the anterior horizontal limb intervenes between the frontal and orbital operculum. Such organization suggests that the anterior insula is entirely connected with the frontal lobe while the posterior insula is connected with the parietal and temporal lobes. The anterior insula contains three gyri named by Eberstaller in the rostro-caudal axis, the "gyrus brevis primus" (anterior short gyrus), the "gyrus brevis secundus" (medial short gyrus) and the "gyrus brevis tertius" (posterior short gyrus). The three short gyri are separated by two sulci. Cunningham (1891a) proposed to attribute the Guldberg's term "sulcus praecentralis Reilii" to the "sulcus anterior", given that the second gyrus consists of a superficial depression between the medial and the posterior short gyri.

In the present anatomy books the anterior sulcus is called the short insular sulcus and the posterior sulcus is the precentral sulcus. In connection with the anterior insula Eberstaller describes two additional gyri, the "gyrus transversus" (transverse insular gyrus) and the "gyrus accessorius" (accessory insular gyrus). The transverse gyrus extends forwards from the pole of the insula connecting its lower part with the inferior or orbital surface of the frontal lobe. On the outer side of the transverse gyrus is placed the accessory gyrus, which frequently becomes continuous with a corresponding gyrus on the deep surface of the outer part of the orbital operculum (Eberstaller,1887). The posterior insula is divided in two portions by a sulcus called by Cunningham the "postcentralis Reilii" (postcentral insular sulcus). The anterior gyrus, named the "gyrus lungus", and the posterior gyrus, named "gyrus posterior secundus" (Eberstaller, 1887) are currently known as the anterior long gyrus and the posterior long gyrus, respectively.

Modern anatomy describes the human insula (Figure A4) like a triangular shaped area buried in the depth of the Sylvian fissure. The human insula counts five to seven sulci and seven gyri. It is separated from the surrounding areas by the circular sulcus (posterior limiting sulcus), the anterior limiting sulcus and the posterior limiting sulcus. Complete exposure of the insula becomes possible by removing the fronto-parietal and temporal opercula.

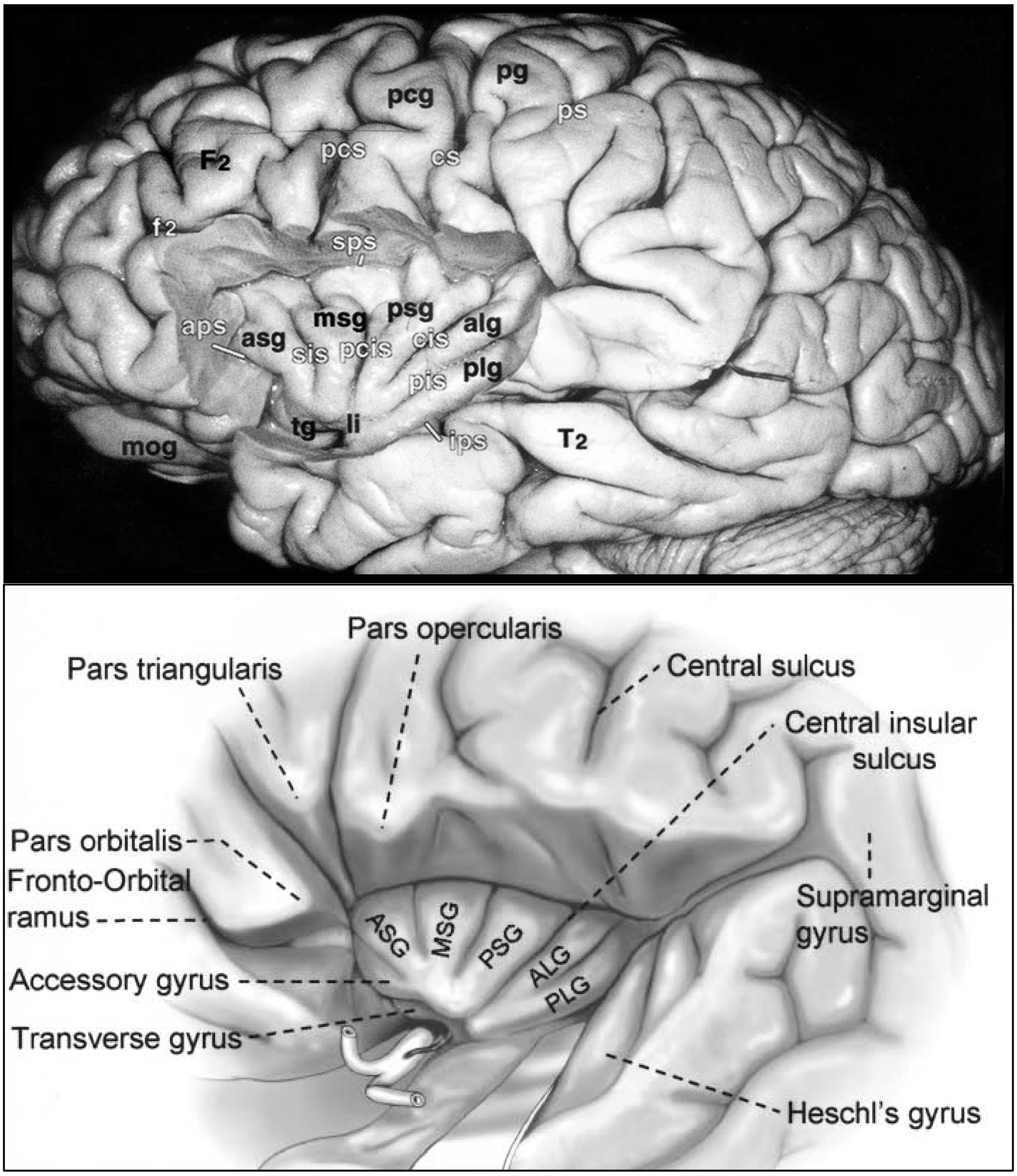


Figure A4: lateral view of the human insula of the left hemisphere. Top: Photograph of brain specimen (Bamiou et al., 2003). Bottom: Drawing from Tanriover et al., 2004. aps: anterior periinsular sulcus; alg: anterior long gyrus; asg: anterior short gyrus; cis: central insular sulcus; ips: inferior periinsular sulcus; li: limen insula; mog: medial orbital gyrus; msg: medial short gyrus; pcis: precentral insular sulcus; pis: postcentral insular sulcus; plg: posterior long gyrus; psg: posterior short gyrus; sps: superior periinsular sulcus.

In 1897, Cunningham included in his studies the man-like apes and concluded that in the latter the insula consists of hidden part covered by a fronto-parietal and temporal operculum, and an anterior exposed part due to the total absence of the frontal and orbital opercula. Cunningham noted another characteristics of apes that is the presence of the fronto-orbital sulcus which limits the exposed insular part and can correspond to the anterior limiting sulcus in the human brain.

In 1963 Roberts and Akerts presented a schematic unfolding (Figure A5) of the components of opercular and insular cortex in the macaque monkeys. Four topographical entities were identified in the insula and the fronto-parietal operculum: the outer, inferior and inner face of the operculum and the insula. The frontal and parietal portions of the operculum was identified with the sulcus subcentralis anterior and not anymore with the imaginary line drawn in continuation with the central sulcus.

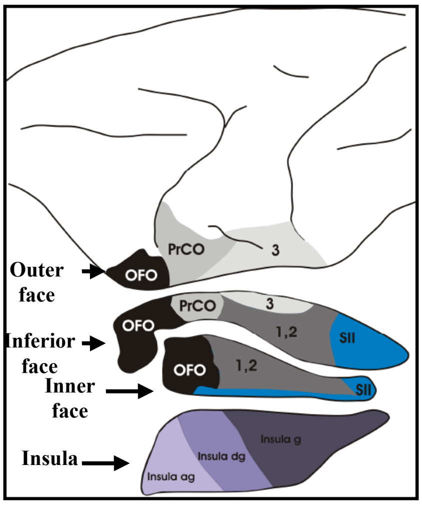


Figure A5: Unfolding of the fronto-parietal operculum and Insula of macaque monkey (modified from Roberts & Akert, 1963).

In rhesus monkeys the insula (Figure A6) is an elliptical shaped area covering the medial wall of the Sylvian fissure and separated from bordering areas by the inferior and superior limiting sulci. The insular cortex in the monkey presents no evidence of true gyri and sulci. It can be divided into a dorso-rostral part, covered by the fronto-parietal operculum and a ventro-caudal part covered by the temporal operculum. The insula has an estimated surface of 160 mm².

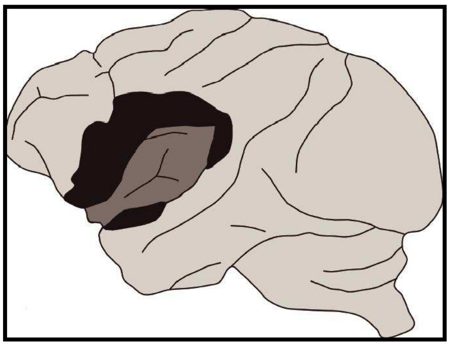


Figure A6: Lateral view of the insula of the left side. Modified from Showers & Lauer, 1961.

III. Embryological development

In 1891, Cunningham noted that in humans the insula remains perfectly smooth up to the half of the fifth month of gestation, but long before this period there is a marked indication of its division into a frontal and a parieto-limbic portion. He noted that the first sulcus to appear is the central one in the second half of the fifth month followed by the precentral and poscentral sulci, respectively, in the first and second half of the six month. He advanced the hypothesis that the formation of the insula is due to the accelerated growth first of the temporal operculum and secondly of the fronto-parietal operculum. He correlated the late development of frontal operculum in the human fetuses with the fact that phylogenetically they are new acquisition of man.

In 1934 Landau suggested that the development of the insula, due to a general flexion of the "whole pallium", is inversely proportional to that of the rhinencephalon, emphasizing the fact that species with well-developed insula are microsmatic.

In 1912, Streeter suggested that the insular cortex is the first cortex to differentiate in the fetus, beginning from the sixth week, in a cortical region that will later become the limen insula. He observed the first appearance of the sylvian fissure during the third month. Lockard in 1948 pointed out – contrary to Cunningham's description- that the first sulcus to appear is the inferior limiting sulcus at about 15 weeks. Between 16 and 17 weeks, the superior limiting sulcus appears and shortly joins the inferior limiting sulcus to form the circular insular sulcus. According to Lockard, the formation of the insula is due mostly to the development of the temporal lobe.

Recently, Afif et al. (2007) proposed a 5-stage macroscopic classification of development of the insular and central cerebral regions from the 13th to 28th growth weeks (GWs) (Figure A7): The first two stages (13-17 and 18-19 GWs, respectively) are characterized by the appearance of first sulci. The first sulcus to appear (Stage 1) will later become the posteroinferior periinsular sulcus and not the sylvian fissure as previously suggested (streeter, 1912). Afif and coworkers noted that all latter developments of the insular cortex occur above this sulcus. The observation of the central insular sulcus and central cerebral sulcus is possible at 18 GWs (stage 2) in agreement with Cunningham findings. One discrepancy is that the two sulci develop independently, suggesting that the two sulci are not strictly continuous. In the last three stages the opercularization takes place as the result of important development of the surrounding insular cortex. In agreement with earlier findings, the opercularization is due in the first phases to the development of the temporal and parietal opercula (stage 3; 20-22 GWs). In second phase the development involves the frontal lobe and finally the orbital lobe (stage 4; 23-26GWs). The development proceeds gradually over the sylvian region become a real fissure and the insula assumes the adult trapezoid-like shape (stage 5; 27-28 GWs).

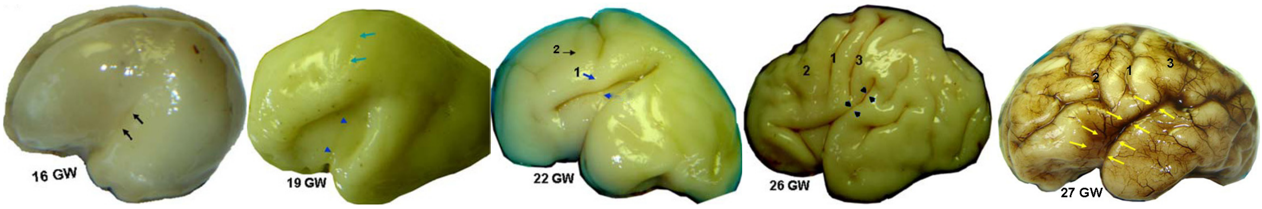


Figure A7: photographs of the lateral view of the human fetal brain at the different stages of development (from Afif et al. 2007). Stage 1 at 16 GWs, stage 2 at 19 GWs; stage 3 at 22 GWs, stage 4 at 26 GWs, and stage 5 at 27 GWs: black arrows: posteroinferior periinsualr sulcus; blue arrowheads: central insular sulcus; green arrows: central cerebral sulcus; 1: the central cerebral sulcus; 2: the precentral cerebral sulcus; 3: the postcentral cerebral sulcus.

IV. Phylogenetic aspects and comparative anatomy

In 1956 Frontera presented in his paper an excellent summary regarding phylogentic aspects and comparative anatomy of the insula based on earlier studies (petronievics; Holl; Cunningham; Marchand; E. Smith; Turner and Landau). According to the definition of Ariëns Kappers that the insula is the cortex overlying the claustrum only three orders of mammals have such structure: carnivores, ungulates and primates. In all these orders, the opercularization of the insula follows similar pattern proceeding progressively from an exposed insula in the more primitive forms (the opercularization is never complete in carnivores or ungulates) to a total opercularization only in humans.

In monkeys there is a progressive enlargement of the insular region in comparison with those of the non-primate forms and despite the extent of the opercularized part monkeys have always an exposed portion and still no insular central sulcus. In apes, the opercularization has farther progress and the insular region expands to some adjacent areas both rostrally and ventrocaudally. Furthermore,in apes it still present an anterior exposed portion. Moreover, it is possible to identify the appearance of the central insular sulcus. In humans, the insula achieves the peak both in opercularization and extension. In his paper, Frontera stressed the hypothesis that "all the opercularized island in monkeys is actually the posterior part of the anterior insula of men".

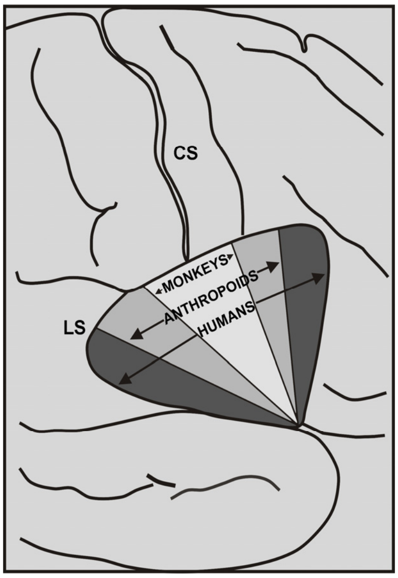


Figure A8: Drawing illustrating the progressive enlargement of the insula in primates (modified from Frontera, 1956). CS: central sulcus; LS: Lateral sulcus.

V. CYTOARCHITECTONIC PARCELLATION OF THE INSULA OF REIL AND THE INNER PERISYLVIAN REGIONS

1. The insula of Reil

In 1877 Herbert C. Major published the first systematic histological study on the insula of apes. He concluded that layers structure of the insula agree in number, order and general arrangement with those of neocortex except a third layer with smaller cells in insula than in other cortical parts. He also noted no difference between various gyri forming the insula as well as between left and right insula. Since then several histological studies were performed with different staining techniques. Bonin and Bailey (1947) stated that a granular cortex covers the largest part of the insula in monkeys and only a small rostral allocortex portion is constituted by agranular cortex.

More recently, Roberts and Akerts (1963) and Jones and Burton (1976) published Cytoarchitectonic maps based on the perikarya distribution within the insula and surrounding regions (Figure A9). They subdivided the insula into three major sectors: an anterior agranular, an intermediate disgranular and a posterior granular sector. Sanides (1968) proposed an alternative schema based both on perikarya and myelin distribution. He described an anterior peripaleocortical portion with two agranular strata and a proisocortical portion with gradual granularization.

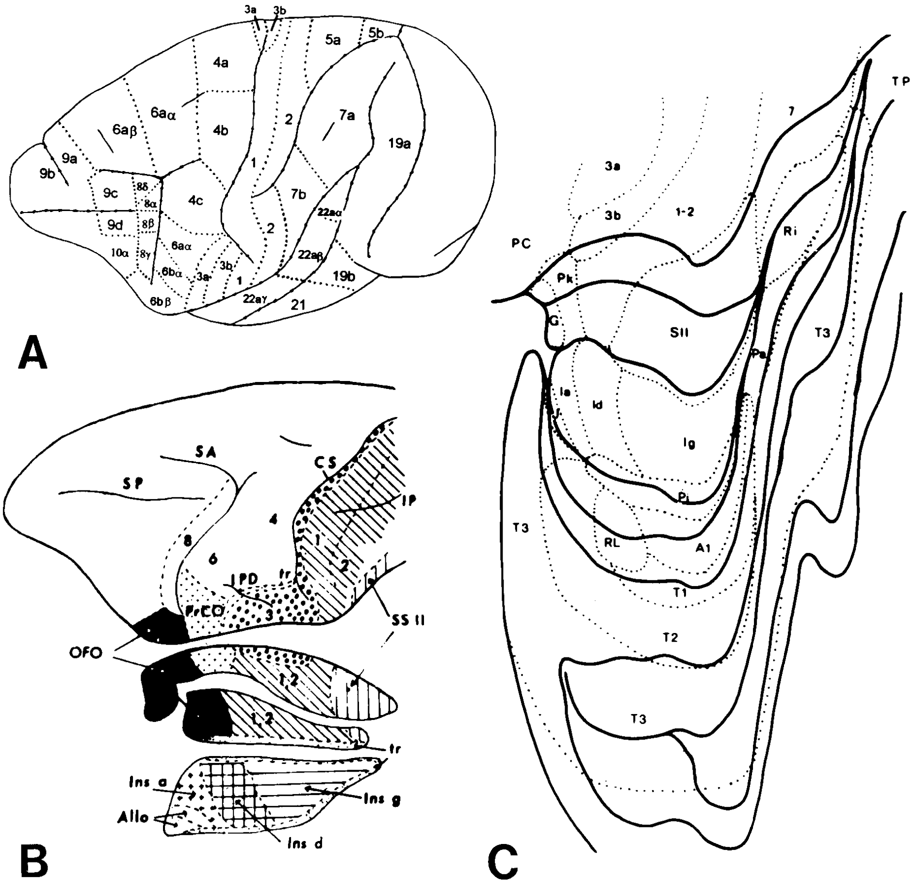


Figure A9. A–C: Architectonic maps to show the dorsal Sylvian operculum and adjacent areas of the monkey cerebral cortex according to: A: Vogt and Vogt (1919). B: Roberts and Akert (1963). C: Jones and Burton (1976). Abbreviations (B): Allo, allocortex; CS, central sulcus; Ins a, agranular insular cortex; Ins d, dysgranular insular cortex; Ins g, granular insular cortex; IP, intraparietal sulcus; IPD, anterior subcentral sulcus; OFO, orbitofrontal cortex; PrCo, precentral opercular cortex; SA, arcuate sulcus; SP, principal sulcus; SSII, second somatosensory cortex; tr, transitional zone. (C) A1, primary auditory cortex; G, gustatory area; J, transitional juxtallocortex; Ia, agranular insular cortex; Id, dysgranular insular cortex; Ig, granular insular cortex; Pa, postauditory cortex; PC, precentral cortex; Pi, parainsular area; Pk, prokoniocortex; Ri, retroinsular field; RL, rostrolateral area; SII, second somatosensory area; T1, first temporal field; T2, second temporal field; T3, third temporal field; TP, temporoparietal field.

The most preeminent and exhaustive study on the cytoarchitectonic and connections of the insula is due to the excellent work of M.M. Mesulam and E.J. Mufson who in 1982 published in three part their observations on the architectonic composition and cortical connections of the insula of the old world monkey. On the basis of their observations on the distribution of perikarya, myelin and acetylcholinesterase and in agreement with previous data, Mesulam and Mufson subdivide the insula into three architectonic sectors (Figure A10):

Periallocortical agranular sector (Ia-p): covers the anterior ventral portion of the insula is continuous with the preperiform cortex. Three cellular strata form this sector, characterized by the lack of aggregates of granule cells:

- The outer layer is composed by small pyramid cells and is in direct continuity with the pyramidal layer of the prepiriform cortex.
- The intermediate layer consists of larger hyperchromic pyramidal cells and is contiguous with the fifth layer of the more dorsal dysgranular cortex.
- The inner layer contains polymorphic cells and displays continuity with the underlying claustrum and the deep cellular layer of the prepiriform cortex.

In this agranular sector the presence of cortical myelin is limited to myelinated fibers in layer I. Cortical AChE reaches its highest concentration over the whole insula in the inner and intermediate layers of this sector.

Dysgranular sector (Idg): is dorsocaudal to the agranular sector, and covers the largest portion of the insular territory. This sector contains five to six layers:

- Layer 2 contains spread granule cells and it is difficult to differentiate from layer 3 in the vicinity of the agranular sector.
- Layer 3 is sparsely populated by Pyramidal cells and assumes a laminar or columnar organization only in its more dorso-caudal part.
- Layer 4 contains granule cells arranged in clusters in the rostral part and in continuous lamination in the more dorso-caudal part.
- Layer 5 displays a decreased prominence (because of the hyperchromic cells) going from rostral to caudal part.
- Layer 6 is difficult to demarcate both from layer 5 and the white matter.

In this sector, the cortical myelin is still relatively low and mostly present in radial fibers. The cortical AChE is present in the infragranular layers but in lower concentration than that of the agranular sectors.

Granular sector (Ig): this sector covers the dorso-caudal part of the insula and presents higher degrees of granularization and layer differentiation. Layer 2 and 4 are clearly granular and well demarcated from other layers. Layer 3 displays an incipient sublamination. Layer 5 is better defined with a clear demarcation between layer 6 and layer 5 as well as between layer 6 and the white matter.

The granular sector contains the highest concentration in cortical myelin of the entire insular sectors. Cortical AChE is lower than in the other sectors.

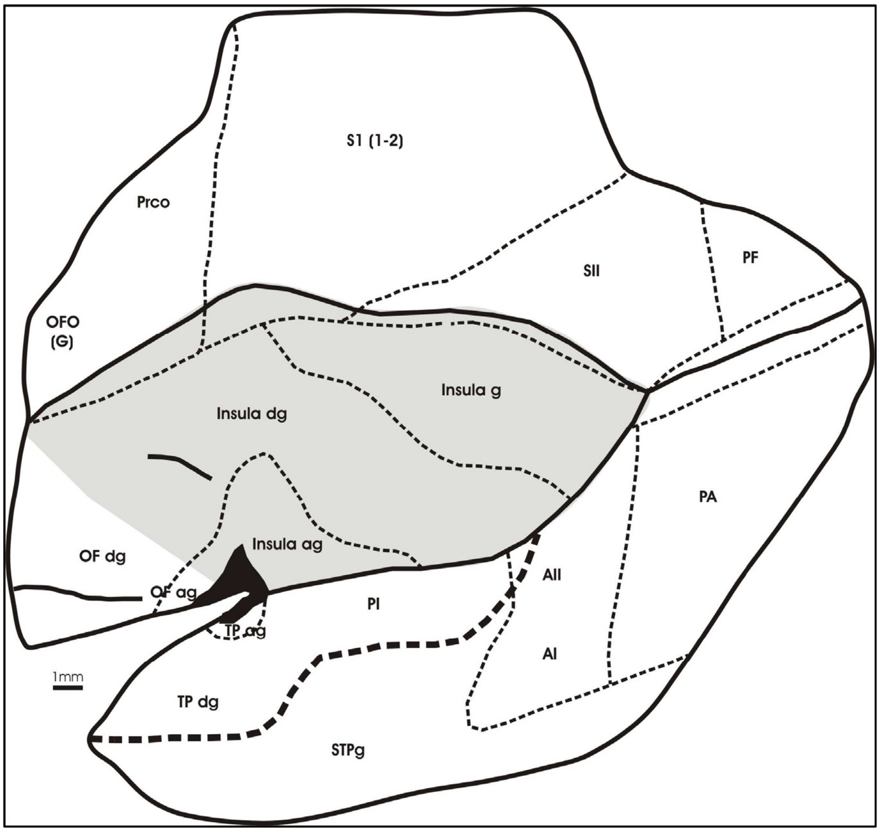


Figure A10: Cytoarchitectonic map of the insula, the inner face of the fronto-parietal operculum, the supra-temporal plane and immediately adjacent orbital and temporopolar areas.. Modified after Mesulam & Mufson ('82). AI: primary auditory area; AII: secondary auditory area; G: Gustatory area; insula ag: agranular insula; insula dg: dysgranular insula; insula g: granular insula; OF ag: agranular orbitofrontal cortex; OF dg: dysgranular orbitofrontal cortex; OFO: opercular cortex; PA: postauditory cortex; PF: anterior inferior parietal cortex; PI: parainsular cortex; STPg: granular supratemporal cortex; SI (1-2): primary somatosensory cortex; SII: secondary somatosensory cortex; TPag: agranular temporopolar cortex; TPdg: dysgranular temporopolar cortex.

2. The fronto-parietal operculum

A cytoarchitectonic map of the parieto-frontal operculum was proposed in 1963 by Roberts and Akerts (Figure A5). They subdivided the opercular region in three principal sectors: an orbitofrontal sector (OFO), a precentral sector (PrCO) and a postcentral sector.

The orbitofrontal operculum occupies the most anterior part of the three topographical portions of the fronto-parietal operculum and displays a similar architectural pattern with respect to the frontal granular cortex and is formed by inconspicuous six strata: Layer 2 is relatively well developed with granule cells. Layer 3 is sparsely populated by small sized pyramidal cells without a clear sublamination. Layer 4 is thin but well demarcated. Layer 5 is populated by small and medium sized pyramidal cells. Layer 6 is not clearly differentiable from layer 5 and the white matter.

The precentral operculum (PrCO) is situated in the inferior and outer face of the parieto-frontal operculum and caudally to the orbitofrontal operculum. Roberts and Akerts noticed the difference between this sector and area 6 of Brodmann. The PrCO is thinner.

Layer 4 is more differentiated. Layer 3 is thin and sparsely populated like in the frontal granular cortex. Layer 5 is well demarcated and filled with medium and large sized pyramidal cells. Layer 6 is subdivided in inner and outer sublayers. The latter contains small cells that bind with the white matter.

The cytoarchitectonic organization of the anterior part of the lateral orbital sulcus before the insula makes appearance was described by Mesulam and Mufson (1982). They identified from rostral to caudal: A granular sector (OFg) with moderate but well demarcated granular layers 2 and 4. A dysgranular sector (OFdg) that makes appearance posteriorly at the emergence of the superior limiting sulcus. This sector has a layer 4 with clusters of granule cells; layer 2 is no more granular and fused with layer 3 and deep layers hardly differentiated from the white matter. An agranular sector (OFa-p) situated posteriorly lacks granularity. It consists of an inner lamina in continuity with the claustrum and an outer lamina in direct continuity with the prepiriform cortex. Carmichael and Price (1994) classified the most caudal part of the orbital surface as the orbital part of the agranular insula that cab be subdivided by topographical positins into five areas; medial (Iam), intermediate (Iai), lateral (Ial), posteromedial (Ipm), and posterolateral (Ipl).

The Postcentral region is situated caudally to the PrCO and is formed by the areas 3, 1-2 and SII. Area 3 covers more than two third of the outer opercular face. It is one of the most defined areas among the opercular subdivisions. Layers 2,3 and 4 are hardly discernable and form a broad lamina. Furthermore, layer 5 is lightly populated and layer 6 is confused with the white matter.

Areas 1-2 of the primary somatosensory cortex occupy more than the two third of the inferior and inner faces of the operculum in direct continuity with the area 1-2 of Brodmann. In these areas, layers 2 and 4 are well developed and display a rich granularization. Layer 3 displays a gradient sublamination of small and medium pyramidal cells from the base to the top of the lamina. Layer 5 is relatively thin with no large pyramidal cells. In their posterior portion, layers 3 and 5 contain larger pyramidal cells. Layer 6 is easily separated from layer 5 and the white matter.

Posterior to the areas 1-2, the opercular field is occupied by area SII. Roberts and Akert extend the territory of SII caudally until the end of the Sylvian fissure. Because of the width of SII, they argued a similarity with the precentral motor cortex. Architecturally, SII follows the pattern of the postcentral granular cortex. Layers 2 and 4 are hypergranularized. Layer 3 has a clear sublamination based on size gradient with large sized pyramidal cells at the base. Layer 4 is less well developed than in areas 1-2. Layer 5 is filled by small and medium sized pyramidal cells. Layer 6 is scarcely distinguishable from layer 5 and the white matter. In 1995 Krubitzer and colleagues proposed an alternative subdivision of the parietal operculum based on anatomic and functional criteria. He suggested that the posterior portion of the parietal operculum contains two distinct anatomical fields: the parietal ventral field (PV) located in the most anterior part while the most posterior part is defined as the secondary somatosensory area (SII).

3. The temporal operculum

Most rostrally at the limen there is an agranular periallocortical sector, the TPa-p superficial stratum, in continuity with the prepiriform cortex. This is an internal stratum in continuity with a cell's sub-group that represents the temporopolar extension of the claustrum. The TPa-p lacks an intermediate pyramidal stratum. Laterally to the TPa-p there is a disgranular sector (TPdg). In this sector, layer 2 lacks granule cells but contains the same cellular type of the layer 2 of orbital and the insular agranular-disgranular sectors. Layer 4 has an incipient granularity. Caudally to the limen, is situated the parainsular cortex (PI), a dysgranular field which extend on the supratemporal plane. In PI layer 2 still lack granularity but there is a gradual increase of granularization in the more posterior part. A longitudinal granular sector occupies the sector lateral to the supratemporal dysgranular band of cortex, the supratemporal granular cortex (STPg): this sector is characterized by the presence of well demarcated and granularized layers 2 and 4. The STPg contains the Ts3 and PaAlt of Sanides and T1 and T2 of Jones and Burton. More posteriorly, the supratemporal plane contains the auditory cortex subdivided by Sanides into proA (AII), Kam (medial AI) and Kalt (lateral AI). AI is hypergranular with layers 2,3 and 4 unified in one broad layer. AII is less granular with more discernable six layers. Posterior to the insula, the supratemporal plane is covered by a homotypical cortex identified as the postauditory cortex by Jones and Burton.

On the basis of their data, Mesulam and Mufson underline the striking similarity of the organization between the insula and the supratemporal and the lateral orbitofrontal regions. And therefore it *"becomes desirable to approach the insulo-orbital-temporopolar region collectively and as a single entity"*.

In this perspective, the prepiriform olfactory cortex represents the centre around which the insulo-orbital-temporopolar complex is developed in concentric rings of agranular, dysgranular and granular cortex.

The first ring is a periallocortical cortex and contains the agranular sectors of the orbital sector (FOa-p), the insular cortex (Ia-p), and the supratemporal pole (TPa-p). The second ring is covered by the dysgranular areas of the orbital (FOdg), the Insula (Idg) and the supratemporal pole (PI and TPdg). The third ring consists of the granular sectors of the lateral orbital cortex (OFg), of the granular sectors of the parieto-frontal operculum (OFO, 1-2 and SII), and the granular sectors of the supratemporal pole (STPg, AII and PA). Furthermore, an incomplete fourth ring can be identified as hypergranular koniocortex formed by area 3 and AI.

In light of these observations, Mesulam and Mufson in accord with Sanides advanced the hypothesis that there is a concentric development around the prepiriform cortex following a granularization gradient, and that this *"arrangement reflects the stages of cortical development in phylogeny and ontogeny".* **This finding will become a barycentre of the interpretation of the functional aspects of all these regions.**

Another important consideration comes from the work of Mesulam and Mufson, that is, the comparison between human and monkey's insula. They noticed a remarkable similarity of the general cytoarchitectonic organization between the two species, in spite of the difference regarding the extension of the territory covered by the insula between these two species. These data were recently confirmed by a comparative anatomy study performed by A. Morel and colleagues (2008) in the laboratory for Functional Neurosurgery. Their results suggested additional subdivisions within the classical architectonic fields. Moreover, they noted the striking correspondence between the humans and monkeys subdivisions. **Such observation will be very helpful when we will try to extend to humans some functional considerations stemming from our exploration of the insula of macaque monkeys.**

VI. ANATOMICAL CONNECTIONS OF THE INSULA AND THE INNER PERISYLVIAN REGIONS

Pribram and colleagues in the early fifities performed one of the first studies on the connections of the insula with strychnine neuronography methods· These studies showed reciprocal connections with the adjacent orbital, Temporopolar, opercular and supratemporal areas. Nevertheless, these results should be considered with reservation given that the methods employed in these works present non-negligible intrinsic limits, like the possibility of trans-synaptic diffusion and the unresponsiveness of some neurons to strychnine.

In the following years hodological studies were mainly based on anterograde degeneration method, .i.e., detection of the neuronal degeneration (assessed on the basis of cell loss, atrophy of cells and gliosis) in the areas presumably connected with a given removed area. Few studies focused specifically on the connections of the insula (Krieg, 1963; Turner et al, 1980; Showers and Lauer, 1961; Wirth, 1973). These authors described anterograde degeneration subsequent to insular ablation and identified insular outputs to the frontal cortex, the cingulate cortex, the striatum, the nucleus accumbens, the hypothalamus and the amygdala. Other connections of the insula were reported in few works where the main target was not the insula. In these studies the authors described cortical input to the insula from the inferior prefrontal convexity (Johnson et al., 1968; Pandya et al., 1971), orbitofrontal cortex (Van Hoesen et al., 1975), area 6 (Showers, 1958), precentral gyrus (Pandya and Vignolo, 1971), somatosensory cortex (SI) (Jones and Powell, 1970), inferior parietal lobule (Pandya and Seltzer, 1982) and frontoparietal operculum (Showers and Lauer, 1961; Pandya and Vignolo, 1971). One of most important study based on anterograde degeneration was performed by Roberts and Akert in order to assess the thalamic connections of the insula and the opercular cortex.

Besides these studies, few studies mapped the insular connections by mean of axonal transport methods. Insular connections were reported with area 24 of the cingulate cortex (Pandya et al.,1981), the thalamus (Burton & Jones, 1976), the frontal lobe (Jacobson & Trojanowski, 1977; Potter & Nauta, 1979). The most important studies in this series are those realized by Mufson et al. (1981) where they described reciprocal insular connections with the amygdala, and by Mesulam and Mufson (1982) where they reported the results on the cortical inputs and outputs of the insular cortex by mean of anterograde and retrograde transport (HRP, TAA). The importance of these studies resides in the fact that insular connections were viewed on the basis of the architectonic organization and topography of the insular cortex described by the same authors together with physiological data, thus allowing the identification of the organization of the functional circuitries in which the different sectors of the insular cortex are involved.

Concerning the connections of the frontoparietal operculum, few studies were dedicated especially to the inner and inferior faces of the operculum. In 1986 Friedman et al. described both anterograde and retrograde connections of the somatosensory fields of the lateral sulcus (SII) as well as of the retroinsular cortex (Ri) and area 7b. These connections were re-described by Disbrow et al. (2003) taking in consideration the two different subdivisions of the former SII in PV and SII. The connections of the more rostral regions were described by Cipolloni and Pandya (1999) in a study concerning the entire frontoparietal operculum.

Connections of the temporal operculum regions were studied by Moràn, Mesulam and Mufson (1987) by mean of anterograde tracers.

1. Cortical connections of the insula of Reil

Mesulam and Mufson showed that the insula has reciprocal connections with a wide range of cortical areas in the frontal lobe, parietal lobe, cingulate cortex and the temporal lobe (Figure A11).

a. Anterior insula

The anterior insula contains the agranular and the anterior dysgranular portions of the insula. The anterior insula has reciprocal connections with the frontal lobe mainly in the lateral and orbital regions: labeling was found in areas 46 and 12 of the granular prefrontal cortex, in the anterior granular part of the lateral orbital region (OFg) corresponding to areas 11 and 13, in the caudal part of the orbitofrontal cortex including the dysgranular (OFdg) and the granular (OFg) sectors. Labeling was also found in the orbitofrontal operculum (OFO) and the precentral cortex (PrCO).

Heavy labeling was found in the frontal, orbital and temporal extensions of the prepiriform cortex (POC). Connections with the parietal lobe are limited to sparse labeling in the primary somatosensory areas (3, 1 and 2) and in the anterior part of the inferior parietal lobule (PF). Sparse labeling was found in the anterior cingulate cortex limited to the lower bank of the cingulate sulcus corresponding to area 24 and to a transitional area between area 24 and 6. The anterior insula has connections with a wide range of the temporal areas. Heavy to moderate Labeling was found in the agranular (TPa-p), the dysgranular (TPdg) and the granular (STPg) areas of the dorsal temporal region. Moderate labeling was found also in the posterior part of the superior temporal plane in the parainsular cortex (PI). Labeling was also found in the dysgranular anterior part of the superior temporal sulcus (STS) and of the inferior temporal gyrus (TEm) as well as in the banks of the rhinal fissure (prorhinal and prerhinal cortex).

b. Midanterior insula

The midanterior insula is entirely dysgranular. The connections of this sector with the frontal lobe are almost identical to that of the anterior insula. Labeling was found in OFg, OFdg and OFa-p as well as in the areas 46 and 12 of the granular prefrontal cortex. In contrast with the anterior insula, no labeling was detected in OFO. Only PrCO is scarcely marked. Labeling is extended to the posterior bank of the inferior arcuate sulcus in a cortical region corresponding to area 6 (likely F5). In the prepiriform olfactory cortex, the labeling is limited to the temporal extension. The midanterior insula shows parietal connections in part similar to that of the anterior insula, but with more intense labeling. These connections include the primary somatosensory cortex and the rostral part of PF. In addition, labeling was found in SII and area 5 of the superior parietal lobule (granular). Labeling in the cingulate cortex is moderately present in the anterior part of the cingulate gyrus and the ventral bank of the cingulate sulcus (areas 24 and 23) and extended dorsally to SMA (area F3). Similar to the anterior insula, the midanterior insula has connections with TPa-p, TPdg, STPg and PI. In addition, labeling was found in the retroinsular cortex (RI). In the case of the midanterior insula, the connections with STS and TEm are extended to the more caudal granular sectors. Labeling was found in AI and AII as well as in the banks of the rhinal fissure.

c. Posterior insula

The posterior insula contains the most caudal part of the dysgranular insula and the granular insula. This sector shows weak connections with the frontal lobe in respect of the anterior and middle sectors. Sparse labeling is present in areas 46, 45, OFg, OFdg, OFa-p, PrCO and area 6 (likely F5). Connections with the parietal lobe include SI and SII in the opercular region, PF, PG and PFG in the inferior parietal lobule, area 5 in the superior parietal lobule. The cingulate cortex is moderately marked in the mid-posterior part of the cingulate gyrus (areas 23 and 24) and in upper banks of the sulcus corresponding to the area MII of Woolsey (F3). The connections with the temporal lobe include TPa-p (light labeling), STPg (moderate labeling), PI (moderate labeling). Moderate labeling was found in the more caudal parts of the supratemporal plane in the retroinsular (RI) and postauditory (PA) cortex. Moderate labeling is present in the granular and dysgranular parts of the upper bank of STS and the inferior temporal gyrus (TEm), as well as in AI and AII. The banks of the rhinal fissure are lightly marked.

d. Topographic and cytoarchitectonic considerations

The cortical connections of the insula suggested both topographical and cytoarchitectectonic patterns. From the topographic point of view, it was noted that the anterior portion of the insula has preferential connections with the orbital and fronto-opercular areas (OFO, PrCO, OFa-p, OFdg), with the prepiriform olfactory cortex (POC), with the entorhinal cortex, and with the anterior cingulate cortex (area 24). The posterior portion has preferential connections with parietal lobe areas (SI, SII, PF, area 5), with lateral and mesial area 6 (likely F3), with AI and AII, and with mid-posterior cingulate cortex (area 23).

From the cytoarchitectonic point of view, it seems that the agranular insula is connected with allocortical, dysgranular and agranular areas, while the granular insula is connected exclusively with granular areas. The dysgranular insula appears to represent a transitional pattern of connectivity due to its transitional cytoarchitectonic aspects. The connections of the anterior part are similar to those of the agranular insula whilst the connections of the posterior part are similar to those of the granular insula.

2. Cortical connections of the fronto-parietal operculum (Figure A12)

a. Anterior fronto-parietal operculum (OFO, PrCO)

Even though Cipolloni and Pandya (1999) in their study considered separately the regions of the anterior frontoparietal operculum into gustatory (rostral OFO) and proisocortical motor (ProM; OFO and PrCO), nevertheless we can conclude that these regions have a very similar pattern of connectivity. Additionally to intrinsic connections, this region is connected with the opercular portions of areas 3, 1 and 2 of the primary somatosensory cortex, with the ventral premotor cortex (areas F5c-a) and with areas 46v, 11, 12 and 13 of the frontal lobe and with the anterior cingulate cortex (area 24). This region has connections as well with the anterior and mid-insula with a rostro-caudal gradient.

b. Postcentral opercular areas 3, 1and 2

the connections of these areas with the prefrontal cortex are limited to the ventral portion of area 46, while connections were extended to a wide field of the ventral part in the post-arcuate regions where labelling was found in the bank of the arcuate sulcus (areas F5a and F5p), in the convexity of the ventral premotor cortex (areas F5c and F4) as well as in the most lateral part of the primary motor cortex. Connections were also described with the ventral postcentral primary somatosensory cortex (areas 3, 1 and 2) as well as with area SII. In the parietal lobe connections were found with the inferior parietal lobule and the lower bank of the intraparietal sulcus (areas PF and 7b). Labelling was found rostrally in the frontal operculum and the dorsal insula.

c. Parietal operculum

The parietal operculum is subdivided into a parietal ventral part (PV) rostrally and secondary somatosensory cortex (SII) caudally. Both sectors have connections with the primary somatosensory cortex (areas 3, 1 and 2) both in the opercular and postcentral portions. In the frontal lobe connections were found in the bank of the central sulcus (area F1), in the ventral premotor cortex (area F5) and PrCo. In the cingulate cortex labelling was found in area 24. In the parietal lobe, connections were described in the inferior parietal lobule in area PF. The parietal operculum has also connections with the posterior part of the dysgranular insula and the anterior part of the granular insula as well as with the retroinsular cortex (Ri). Disbrow et al. (2003) reported that PV has additional connections with other sensory systems such as the auditory belt area.

In their study Friedman et al. (1986) reported that the Ri is connected with SII, Ig, and area 5, and that area 7b is connected to SII, area 5, PMv, and the claustrum.

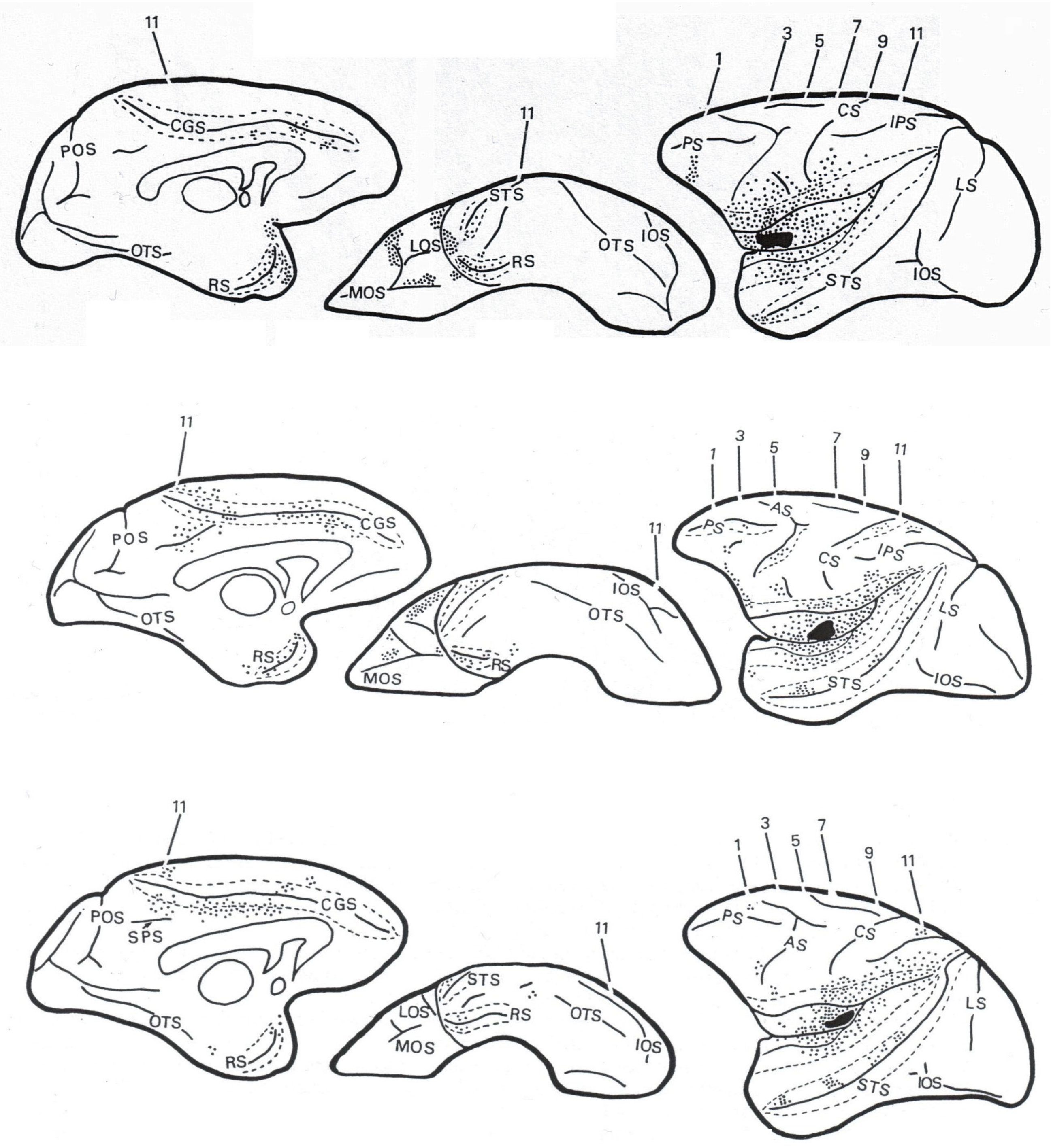


Figure A11: distribution of neuronal labelling observed following tracers injection in the anterior, mid-anterior, and posterior insula (Mesulam & Mufson, 1982). The mesial, ventral, and lateral surface of the monkey brain is shown form left to right. The injection sites are shown as solid black. The blacks dots correspond to the labelled neurons. AS: Arcuate sulcus; CS: Central sulcus; CGS: Cingulate sulcus; IOS: Inferior occipital sulcus; LOS: Lateral orbitofrontal sulcus; LS: Lunate sulcus; MOS: Medial orbital sulcus; OTS: Occipitotemporal sulcus; POS: Parietal occipital sulcus; RS: Rhinal sulcus; SPS: subparietal sulcus; STS: Superior temporal sulcus.

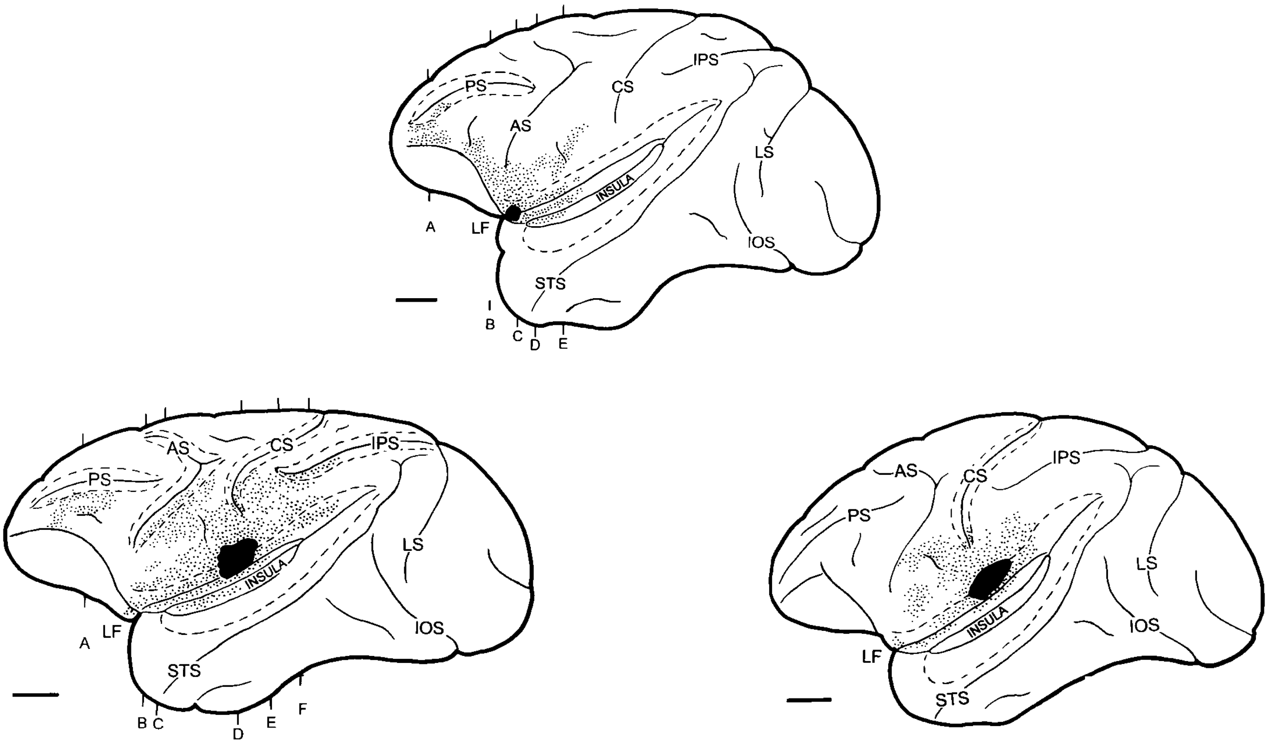


Figure A12: distribution of neuronal labelling observed following tracers injection in three different regions of the fronto-parietal operculum. The labelling is shown on a lateral view of the monkey brain. The injection sites are shown as solid black. The blacks dots correspond to the labelled neurons. AS: Arcuate sulcus; CS: Central sulcus; IOS: Inferior occipital sulcus; IPS: Intraparietal sulcus; LF: Lateral (Sylvian) fissure; STS: Superior temporal sulcus.

3. Cortical connections of the temporal operculum

The prevalent cortical connections of the dysgranular temporopolar regions are those with the insula and the frontal lobe. Labelling was found in the agranular (Ia-p) and dysgranular (Idg) fields. Connections with the prepiriform olfactory cortex (POC) were as well described. Connections with the frontal lobe include essentially the orbitofrontal region and the medial prefrontal areas (area 10, paraolfactory and subcollosal cortex). Less important are the connections with the lateral area 10 and the anterior supracollosal cingulate gyrus. The dysgranular region and generally the entire temporal pole is an enigmatic structure and rarely studied. It is considered as a transition zone from the olfactory allocortical cortex to the granular associative cortices of the temporal lobe and it is considered commonly as a convergence site of sensory inputs (auditory, visual and olfactory) and the limbic system.

4. Subcortical connections

The next sections will consider the subcortical connections of the insula and the surrounding opercular regions in order to complete the picture of the connections and to best understand the functions of the insula, especially his role in the control of the autonomic nervous system.

a. Thalamic connections:

The first studies on the thalamic connections of the Sylvian fissure regions (Roberts and Akert, 1963; Jones and Burton, 1976) reported a correlation between the thalamic inputs and the cytoarchitecture of the sylvian fissure regions. They proposed a one to one relationship between a given thalamic nucleus and the cortical field to which it projects. This schema was contradicted by more recent thalamo-cortical connection studies (Mufson and Mesulam, 1984; Friedman and Murray,1986), which support the point of view that a cortical field can receive projections from several thalamic nuclei.

a.1. Anterior insula (Ia-p, Idg)

This sector is connected to the thalamic ventromedial basal nuclei with the heaviest labelling in the parvicellular aspect of the ventral posterior medial nucleus (VPMpc) and lesser in its principal aspect (VPM). Light labelling was also found in the ventral posterior inferior nucleus (VPI). In the posterior and medial nuclei, the connections include the pulvinar (Pul), suprageniculate (SG), limitans (Li), parvicellular medial geniculate (MGpc), magnocellular and parvicellular of the medial dorsal (MDmg and MDpc), reuniens (Re), centralis inferior (Ci) and centralis intermedialis (Cim) nucleus.

Anterior insula has connections also with the parafascicularis (Pf) and the centromedian (CM) nucleus in the intralaminar and reticular nuclei complex.

a.2. Mid insula (Idg)

The dysgranular mid-insula has similar connection pattern as in the anterior insula. Connections was described with VPMpc, VPM and VPI of the ventromedial basal nuclei complex. In the posterior and medial nuclei complex the connections were found with the oral and medial portions of the pulvinar nucleus (Pulo and Pulm), with Re, MDpc and MDmg nuclei. Unlike the anterior insula, no connections were observed in SG and Li and

MG nuclei. In the intralaminar and reticular nuclei complex light labelling was found in CM, Pf and reticular (R) nucleus.

a.3. Posterior insula

Contrary to the anterior and middle insula, the connections of the posterior insula with the VPMpc and VPM are more or less insignificant, whereas it is striking the connection with VPI. In the posterior and medial nuclei complex more intense then in the mid-anterior insular regions as well are the connections with Pulo, Pulm, SG, Li nuclei, while MG has light labelling. The connections with the MD complex are minor in comparison with the relatively dense labelling in the case of the anterior insular portions. No connection was observed with Cim. The intralaminar and reticular nuclei complex has virtually no connections with the granular posterior insula.

a.4. Fronto-parietal operculum

In their study Roberts and Akert (1963) correlated the degeneration of the medial dorsal nucleus (MD) to the ablation of the orbito-frontal opercular cortex. Degeneration of the medio-basal portion of the ventral posterior medial nucleus (VPM) occurred after ablation of the opercular aspect of area 3, while lesions in PrCo led to a degeneration in the medial basal portion of the ventral lateral nucleus (VL). On the basis of these observations, Roberts and Akert advanced the hypothesis that the MD nucleus projects upon the orbito-frontal cortex, while the medial basal portion of the VPM projects primarily to area 3 and secondarily to areas 1 and 2.

Friedman et al. (1986) described the thalamic connections of SII. They reported that the principal connection is with VPI suggesting that this connection may be somatotopically organized. Other connections but less important were described with VPLc, Pulo, SG and Li nuclei.

a.5. Functional considerations

Findings on the thalamic connections of the insular and opercular regions suggest a rostro-caudal gradient. This organization is very informative with respect to the comprehension of the functions of the insula and opercular regions (Figure A13) reported in the next chapters.

The anterior portion of the insula as well as the orbitofrontal and precentral opercular portions have more pronounced connections with the ventral medial posterior nucleus, in particularly with its parvocellular aspect (VPM, VPMpc) and with the medial dorsal complex (MDmc, MDpc) and with some midline (Ci, Cim) and intralaminar (CM, Pf) nuclei. These connections suggest that the anterior agranular-dysgranular insular portions and the orbito-frontal and precentral opercular portions are related more to olfactory, gustatory, visceral, autonomic and limbic functions, since that the VPMpc receives projections from the nucleus of the tractus solitarius (NTS), which is the first central relay nucleus for gustatory information. Moreover, the NTS is involved in the regulation of several autonomic reflexes. Mufson and Mesulam (1984) suggest a neural circuit that includes the anterior insula, the VPMpc and the NTS and in which occurs the processing of information related to gustatory , visceral and autonomic functions. According to Roberts and Akert, the mediobasal portion of the VPM nucleus can be correlated with the transmission of sensory information regarding the lung and the oro-pharyngeal cavity. The mediodorsal complex has wide connections with the limbic system, particularly with the hypothalamus, and appears to be involved in a neural circuit that underlies the processing of motivational and affective information.

The posterior insula has the most pronounced connections with the suprageniculate (SG) nucleus and the ventral posterior inferior (VPI) nuclei. VPI has also extensive connection with the parietal operculum. It was shown that VPI receives somatic input from pacinian receptors (Dykes et al, '81), spinal cord by means of the spino-thalamic tract (ST) (Berkley, 1980) and somatosensory cortex (Jones and Powell, 1970), suggesting that the VPI may be involved in the processing of somesthetic information. Craig (2002) described this thalamic nucleus as a relay of information about the sympathetic system coming from the lamina I of the spinal cord. Moreover homeostatic information like temperature, nociception, hormonal and immunological activity is thought to be retransmitted by the VPI. The SG nucleus receives information from the superior colliculus. This latter receives from somesthetic, auditory and visual areas. It is proposed that SG may be a relay for multimodal sensory information to the posterior insula. The connections with VPI and SG agree with the observations that the posterior insula is prevalently related to visual, auditory and somesthetic functions.

The pulvinar (Pul) nucleus has reciprocal connections with the entire insula. The same nucleus has connections with other paralimbic areas, like the temporopolar and the orbitofrontal cortex, with limbic structures such as the amygdala and the hypothalamus as well as with high order polysensory association areas. These data suggest that it may be a region for convergence of high order sensory association information and limbic information. Stimulation of the pulvinar nucleus evoked respiratory arrest and alteration in the vascular tone furthermore it was shown that the activity of neurons in the pulvinar nucleus of cats are related to different phases of the operant behavior in particular increasing of the neural activity occured during the reward phase (Wei & Marczynski, 1979). These few studies suggest that the pulvinar nucleus plays a role in both autonomic and motivational behaviors.

Regarding the autonomic nervous system, the thalamo-insular connections show a rostro-caudal organization. The anterior insula through the insula-VPMpc-nTS circuit seems to be involved in a parasympathetic control system while the posterior insula through the insula-VPI-Lamina I circuit seems to be involved in sympathetic control system.

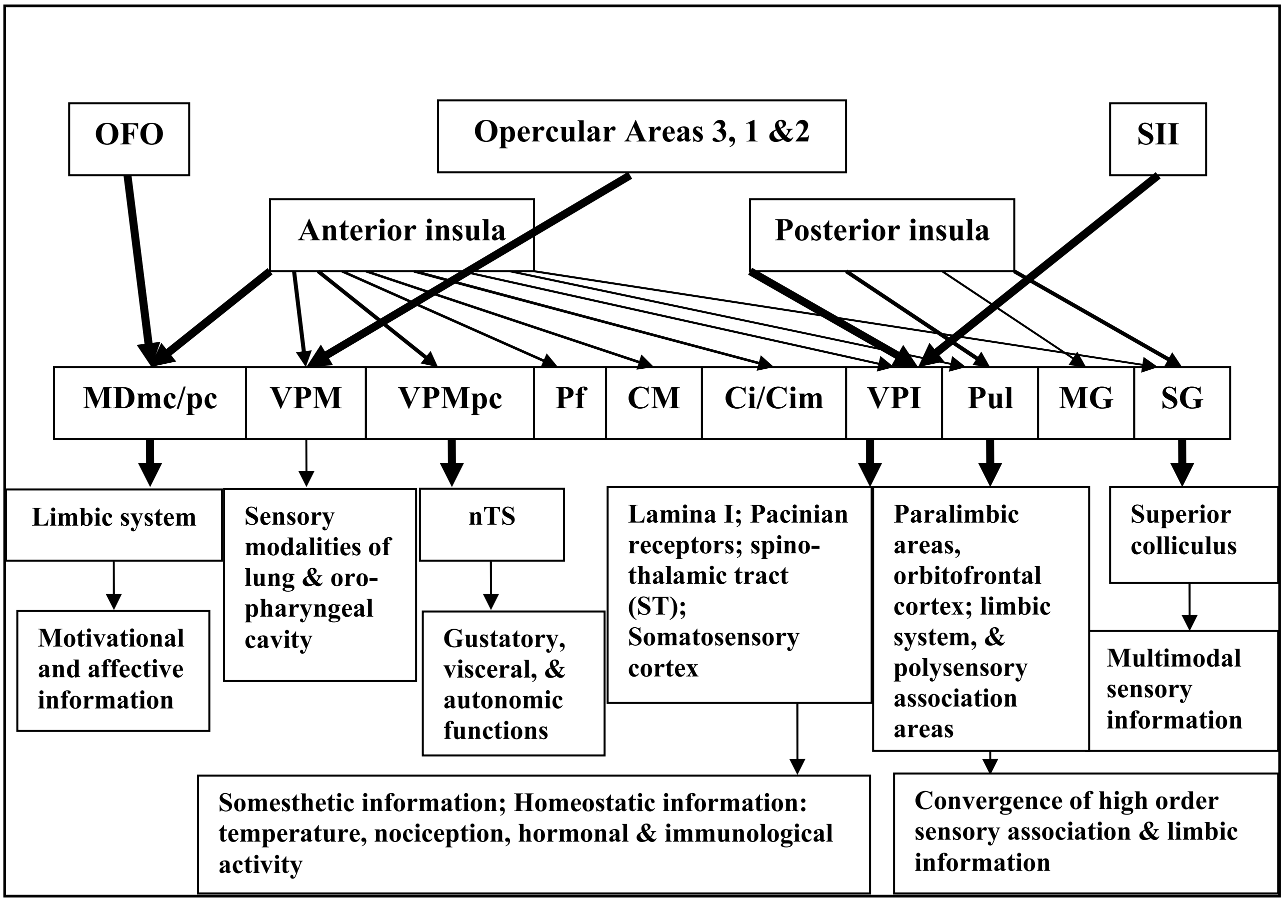


Figure A13: Diagram of principal thalamic connections of the insula and fronto-parietal operculum, neural networks, and functions. After Roberts & Akert (1963), and Mesulam & Mufson (1984).

b. Amygdalo-insular connections

In anterograde-retrograde connections study, Mufson and his colleagues (1981) showed that the amygdala has reciprocal connections mostly with the anterior insula. Injections in the anterior agranular-dysgranular insula showed labelling in the anterior amygdaloid area (AAA), the medial (M), the accessory basal magnocellular (ABmc) nucleus, the cortical nucleus, the parvicellular aspect of the baso-lateral nucleus (Bpc) and the lateral nucleus (L). Tracers injections in the posterior dysgranular-granular insula resulted in labelling limited to the dorsal portion of the lateral nucleus (L) and the central nucleus (CE). The dysgranular temporal pole, studied by Moràn, Mufson and Mesulam (1987), is connected with the lateral nucleus, the accessory basal nucleus, basal lateral and medial nuclei, the central nucleus, the amygdaloid anterior area and with the cortical amydaloid nucleus. Similar results were shown in a recent study (Hoistad & Barbas, 2008). These results demonstrated that the medial temporal pole and the anterior agranular-dysgranular insula have extensive connections with the amygdala, while the connections become sparse in the case of the lateral temporal pole and the posterior dysgranular-granular insula.

The basal lateral nucleus appears to be connected with several cortical areas such as the polysensory areas as well as the frontal lobe. The baso-lateral nuclei are involved in the modulation of the central nuclei responses in relation to associative process, moreover they are involved in a neural circuit including the basal ganglia (especially the ventral striatum) and several prefrontal regions such as the orbitofrontal cortex (OFC), the anterior cingulate cortex (ACC), and the medial prefrontal cortex (mPFC). The central nuclei, phylogenetically older, seem to be related to more primitive functions, generally in relation to the modulation of brain stem responses in relation to behavioural, autonomic and neuroendocrinal responses by mean of hypothalamic connections as well as connections with the reticular area, and several other regions of the brain stem. It was demonstrated that the central nuclei play a role in the control of the release of dopamine (through the ventral tegmental area and the pars compacta of the substantia nigra), of serotonin (through the raphe nuclei), of noradrenalin (through the locus ceruleus), and of acetylcholine (through the basal nuclei). Accordingly, the central nuclei are involved in the control of a battery of autonomic responses. Therefore the fact that the insula has a direct access to the central nuclei emphasizes the hypothesis of a direct insular control on the autonomic functions.

c. Hypothalmus

The relation between the anterior insula and the autonomic nervous system emerges from the analysis of the complicated network of interactions between the prefrontal cortex and the hypothalamus. The hypothalamic-prefrontal connections were studied by Öngür & Price (1998). They demonstrated that a wide region of the anterior insula projects to the caudo-lateral portion of the hypothalamus. The rostral projections of the hypothalamus emerge from a restricted number of prefrontal areas denominated the "medial prefrontal network" by Ongur & Price (Charmichael & Price, 1996; Öngür & Price, 1998). However, besides the medial prefrontal regions, this network includes the intermediate agranular insula, a small field of the rostral agranular insula lying in the most rostral orbitofrontal region and anterior to the aperture of the lateral sulcus. This network is involved in the control of the regulation of autonomic and homeostatic responses through the connections with the brain stem nuclei. Furthermore, the other fields of the anterior agranular insula together with the posterior orbito-frontal regions belong to the so-called "orbital prefrontal network" that conveys information to the medial prefrontal network. The orbital prefrontal network and, consequently, the anterior insula are thought to be strongly connected to the posterior hypothalamus.

According to the hypothesis of Öngür & Price (1998), the orbital prefrontal network can receive and process sensory information, then send it to the areas of the medial prefrontal network. This latter sends the received information to the amygdaloid basal nuclei and to the periaqueductal grey matter. At the same time, the orbital prefrontal network is connected to the posterior lateral hypothalamus that is strongly connected with the brain stem centres involved in the control of the autonomic nervous system, in particular way by means of the dorsal nucleus of the vagus nerve and the nucleus ambiguous, showing a specific role in the modulation of the parasympathetic system. This network seems to play a role in the modulation of the autonomic nervous system by means of the hypothalamus in relation to the orbitofrontal areas and the anterior insula. It was demonstrated that the activation of the lateral hypothalamus evokes a decreasing of the blood pressure and of the heart rate (Lowey, 1991). Moreover, the medial, lateral and posterior hypothalamus are connected with the dorsal portion of the temporal pole. Electrophysiological studies have shown that the stimulation of the lateral hypothalamus evokes an increasing of the animal food intake, while lesion of this region evokes a decreasing of food intake. Furthermore, the lesion of the lateral hypothalamus leads to modulation of the taste sensibility; an individual with such lesions is less sensitive to taste and odours of food. According to what will be detailed in the next chapters about the functional aspects of the anterior insula, the information on the lateral hypothalamus supports the hypothesis of a neural circuit connecting the anterior insula to it.

SECTION 2: FUNCTIONAL PPROPERTIES OF THE ISLAND OF REIL AND INNER PERISYLVIAN REGIONS

I. DATA FROM NEUROPHYSIOLOGICAL STUDIES ON MONKEY

Notwithstanding the relatively good knowledge of the anatomy of the insula and the surrounding perisylvian regions both as for architectonic and hodological data, knowledge of the functional properties didn’t keep pace with the anatomical progress because of a general scarcity of electrophysiological functional data, both in term of recording studies of single units activity and in term of intracortical microstimulation studies (ICMS). Such scarcity of information can be due to several reasons. The work by Frontera (1956) supports the idea that these reasons should be sought in the technical difficulties to reach the insula and, more generally, all the inner perisylvian regions. Lying in the depth of the sylvian fissure and being hidden by opercularization these regions are difficult to reach. Their high vascularization make any experimental approach to these regions particularly delicate, thus posing additional difficulties. Furthermore, the negative results of the first stimulation studies on macaque monkeys and apes (Ferrier, 1885; Grünbaum & Sherrington, 1903; Leyton & Sherrington, 1917) made these regions less attractive.

For all these reasons there is a scarce number of electrophysiological studies on the insula and the surrounding regions. More particularly, in the case of ICMS experiments, the studies are mostly rare and outdated and performed in inadequate conditions, since these studies were almost all performed on anesthetized monkeys, thus calling into question the interpretation of both behavioral and autonomic responses. Furthermore, in these studies a part of the fronto-parietal operculum was removed in order to make accessible the insular cortex. This procedure compromised an important part of the neural circuitry underlying the insula and consequently jeopardized the investigation of important functions of the insula. Nevertheless, it is still possible to build a picture of the functional properties of the insula and the inner perisylvian regions. Based on anatomical and functional data it is possible to subdivide the insula and the inner perisylvian regions principally into two functional fields: a mid-anterior field, including the mid-anterior insula and the orbito-frontal operculum, thought to have gustatory, olfactory, as well as visceral and autonomic functions, and a mid-posterior field, including the mid-posterior insula and the parietal operculum, thought to have auditory, somesthetic and skeleto-motor functions.

1. Mid-anterior insula and orbito-frontal operculum

The anterior insula and the orbitofrontal operculum are classically considered as the primary gustatory cortex. Moreover, the most anterior sector of the agranular insula along with the most anterior portion of the orbitofrontal operculum and the temporal pole are a part of the prepiriform olfactory cortex (POC). This functional view is supported by the pattern of connectivity of these regions as well as by electrophysiological experiments. The groups of Edmund Rolls and Thomas R. Scott studied for many years the gustatory function of several regions of the central nervous system including the anterior insula and the orbito-frontal operculum. In these studies various aspects of the gustatory function such as taste, texture, viscosity, temperature, were tested. In these studies, besides the four prototypical taste stimuli (glucose, NaCl, HCl and quinine), a wide range of stimuli were tested, like fruit juice, amino acids, water and various combinations of the four basic tastes. A very important finding common to all these studies is the low percentage of neurons in relation to the total number of tested neurons responding to one or more gustatory aspects. In one of these studies (Yaxley et al., 1990) gustatory responses were studied in the rostral dorsal insula of the cynomolgus macaque monkey, *macaca fascicularis*: out of 2925 recorded neurons only 65 (2.2%) were taste responding neurons. Moreover, given that the same stimuli were tested also in the frontal operculum and the NTS, the authors were able to analyze the degree of generalization of the responsiveness to the taste in the three areas. Taking into consideration a metric scale in which 0.0 corresponds to the responsiveness to one stimulus and 1.0 correspond to the responsiveness to all four basic stimuli, the insula, the frontal operculum and the NTS scored 0.56, 0.67 and 0.87, respectively. These authors advanced the hypothesis that the neurons of the anterior insula tend to be more selective and respond to single taste than those of the frontal operculum, where an integrative processing takes places and the neurons are mostly less committed to a single taste. This view is in agreement with the consideration that the anterior insula is a primary gustatory cortex while the frontal operculum represents a shift towards the secondary gustatory cortex. In another study Verhagen and colleagues (2004) showed that neurons in the anterior insula and the frontal operculum respond not only to taste but also to the physical properties of stimuli such as viscosity, texture and temperature. Out of 1122 tested neurons, only 62 (5.5%) responded to oral stimuli. 50% of these neurons were uni-modal while the other were bi- or multi-modal neurons. More interestingly, none of these neurons responded to the smell or sight of food. Previous data showed that taste neurons in the anterior insula and the frontal operculum are not modulated by the transition from hunger to satiety (Yaxley et al, 1988; Rolls et al., 1988). These data are in agreement with the hypothesis that neurons in the primary gustatory cortex (anterior insula) seem to map the quality of taste and not hedonic and reward values, which seem to be coded in the orbito-frontal cortex (OFC) .

Since the percentage of neurons of the anterior insula and the rostral part of the frontal operculum responding to taste is very low, it is reasonable to suppose that these regions are not limited to the gustatory function but can be involved in some other functions. Smith-Swintosky et al. (1991) and Plata-Salaman et al. (1992) found that out of 1863 and 1129 neurons recorded in the insular and opercular cortex 2.7% and 4.8% were taste cells, respectively. Within non-taste cells, 20.7% and 11.1% responded to mouth movement, 9.6% and 3.8% responded to somatosensory stimulation, 1.7% and 2.2% responded to approach or anticipation of the taste stimulus and 0.6% and 0.4% responded to tongue extension. The properties of the other neurons could not be identified in these two studies. In addition to the convergence with other studies regarding the percentage of taste cells, these studies suggest that the role of primary gustatory cortex assigned to the insular-opercular cortex should perhaps be reconsidered.

Previous microstimulation studies performed on the insula and the surrounding opercular regions demonstrated various autonomic and sensory-motor outcomes.

In 1949 Kaada, Pribram and Epstein realized a microstimulation study on anesthetized rhesus monkey. Stimulations interested the medial anterior perisylvian regions including the insula, the temporal pole and the orbital surface. In this study, autonomic parameters such as respiratory and vascular responses (Figure A14) were studied. Regarding respiratory responses, microstimulations evoked decrease of both frequency and amplitude of respiration. A variation of the responses in relation to the variation of pulse duration and/or frequency of the stimulation was observed. Concerning vascular responses, microstimulation evoked the increase of arterial blood pressure. In this study, responses could be evoked exclusively from the anterior regions, while no autonomic responses could be observed following the stimulation of more caudal regions. Furthermore, lesion of the connections between the stimulated areas didn't produce any alteration of the evoked responses, while lesion of the connections between the stimulated areas and sub-cortical centers produced an immediate stop of every response. These data suggest that autonomic responses evoked in each of the studied regions is not due to the other cortical regions even if interconnected. Each one of these regions has its independent sub-cortical autonomic output that can generate the observed responses following electrical microstimulation.

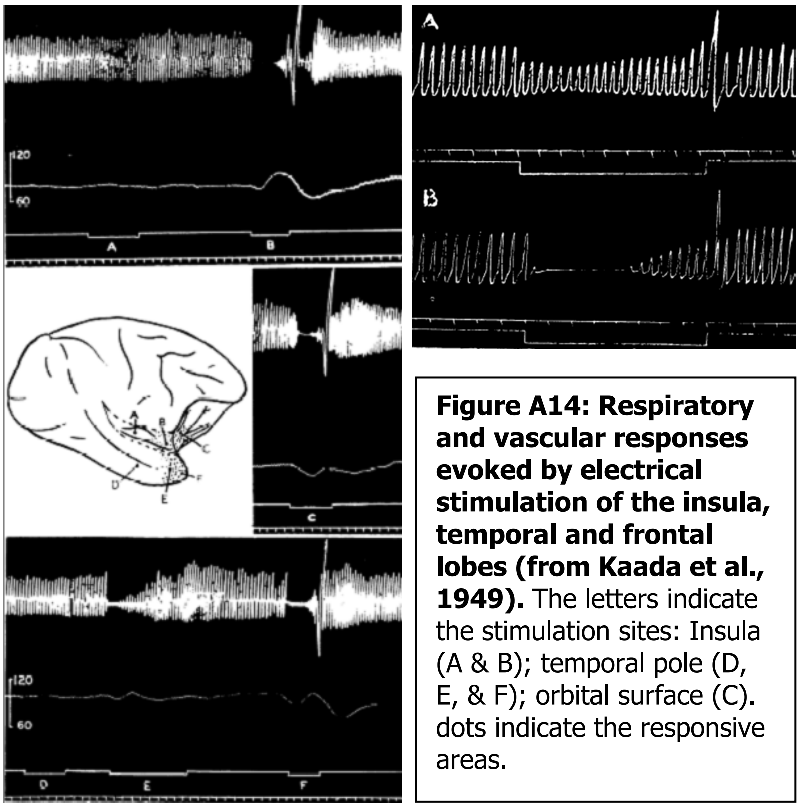


Figure A14: Respiratory and vascular responses evoked by electrical stimulation of the insula, temporal and frontal lobes (from Kaada et al., 1949). The letters indicate the stimulation sites: Insula (A & B); temporal pole (D, E, & F); orbital surface (C). dots indicate the responsive areas.

In 1952 Hoffman and Rasmussen performed another microstimulation study of the anterior perisylvian regions on anesthetized macaque monkeys. Like in the previous study, behavioral responses have been ignored while the authors were interested only to the variation of blood pressure, respiration and gastric activity. Stimulations (even at low voltage) of the insula evoked inhibition of gastric peristalsis (muscular contraction), decrease of intragastric pressure, as well as of stomach tonus and decrease of blood pressure. At higher voltage, stimulations evoked inhibition of respiration during the expiration phase. The same effects were replicated applying various types of anesthesia. Following a bilateral section of the vagus nerve, stimulations could not evoke inhibition of gastric peristalsis or gastric tone, while the inhibition of respiration and decrease of blood pressure persisted. Following section of the trigeminal nerve (retrogasserian neurectomy) no variation was observed on the effect of stimulations. Being a section of a sensory root, it follows that responses evoked in the insula are genuine cortical responses and not the indirect outcome of reflex. The same experiment was performed in the temporal pole and the posterior orbital surface. Unlike the anterior insula, stimulations in the temporal pole evoked an increase of blood pressure while in the posterior orbital surface it was observed both an increase and a decrease of blood pressure.

In 1956 Frontera performed electrical stimulation experiments on anesthetized macaque monkeys in order to assess the hypothesis of an extrapyramidal second somatic motor area lying within the fronto-parital operculum and the posterior portion of the insula. Stimulations were applied by means of surface macroelectrodes after insula exposure. Frontera reported negative results following stimulation in the fronto-parietal operculum, while some movements could be evoked following stimulation of ventral insula. These responses were segregated along the rostro-caudal axis into a corporeal map (Figure A15). Ipsilateral and controlateral face movements (ipsilateral wink, wrinkling of the forehead, retraction of the angle of the mouth producing a sneer, contralateral sneer and contraction of mouth and face muscles) were evoked in the anterior portion of the insula. Upper extremity (flexion of the controlateral upper extremitiy, particularly at elbow, wrist and finger joints) and lower extremity (flexion of the controlateral lower extremity at hip, knee, and ankle, with plantar flexions of the toes) movements were represented in middle and posterior ventral insula, respectively. Frontera reported that these responses could not be systematically evoked, thus rejecting the hypothesis of a somatic motor area. Furthermore, even if autonomic responses were not systematically assessed in his experiments, Frontera documented responses such as occasional arrest of respiration at inspiration, salivation, and shivering preceded by pilo-erection. Based on these data and on previous studies, Frontera advanced the hypothesis that besides the anterior insula, which seems to be strongly involved in visceral control, the insula contains "other centers, which although strictly speaking are non-visceral, are nevertheless related to such functions as respiration, and to such reactions as vomiting, shivering, and other which might be called the somatic expression of visceral phenomena" (1956, page 390). Regardless of non-constantly evoked behavioral responses, likely due to the experimental procedure, the insula shows a complete corporal representation, with the head located rostrally and lower limbs caudally.

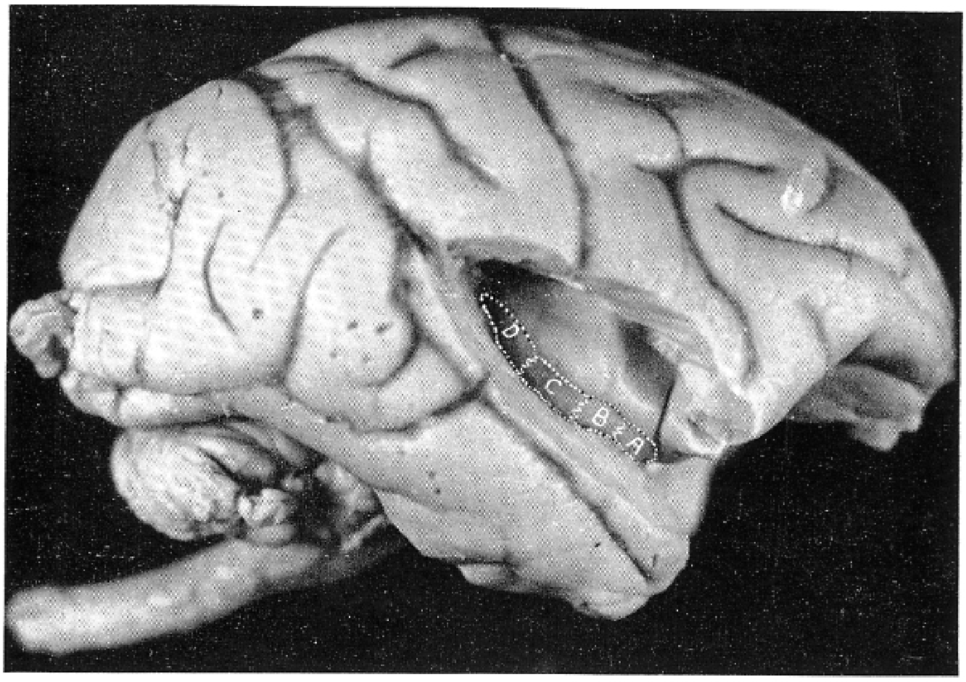


Figure A15: Lateral view of the right hemisphere of macaque monkey showing the insula. From Frontera, 1956.The letters indicate the insular stimulation sites and the responses evoked by the electrical stimulations. A: Ipsilateral wink, wrinkling of the forehead and retraction of the angle of the mouth producing a sneer. B: controlateral sneer with contraction of the muscles of mastication and face muscles. C: Flexion of the contralateral upper extremity, particularly at elbow, wrist, and finger joints. D: flexion of the contralateral lower extremity at thehip, knee, and ankle.

The electrical stimulation experiment performed by Showers and Lauer (1961) on anesthetized macaque monkeys showed both somato-motor and visceral effects. Stimulations generally evoked gross body movements that appeared to be organized into a somatotopical pattern (Figure A16). Facial movements (muscular contractions of the lips, nose, eyelids and mouth muscles) could be evoked from all insular regions. Laryngeal, neck, upper extremities, trunk, lower extremities and tail movements were evoked following a rostro-caudal order within the insula. Showers and Lauer reported that in the dorsal part the evoked movements tended to be homolateral and bilateral, while in the ventral portion evoked movements tended to be controlateral and bilateral. Unlikely to the somatomotor effects, visceral responses were elicited only in the dorsal portion of the insula. The evoked visceral responses included: decrease of respiration frequency up to apnea, increase of salivation and secretion of mucus, increase of heart rate and blood pressure and increase of gastro-intestinal motility.

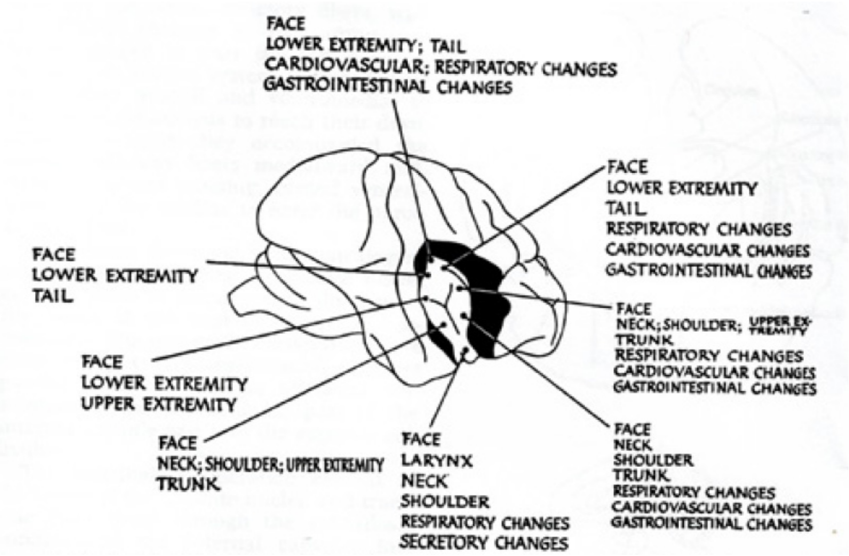


Figure A16: Drawing summarizing the somato-motor and visceral responses evoked by the electrical stimulation of the insula. From Showers & Lauer, 1961.

Looking to all the data described above, it becomes more plausible to suppose that the functional role of the mid-anterior insula and the frontal operculum is not limited to the sensory aspects of gustatory function. Instead, these regions can be involved in more complex behaviors such as motor and cognitive aspects of feeding in addition to a key role in the control of the visceral and autonomic functions.

2. Posterior insula and parietal operculum

In the 1948 Sugar, Chusid and French described a second motor area lying on the lateral and medial wall of the fronto-parietal operculum on the posterior part of the insula. Such hypothesis was rejected by Frontera even if it was possible in a series of electrical stimulation experiments performed by Frontera and others to evoke various gross movements (see above). Successively, anatomical and functional studies focused instead on somatosensory functions both for the posterior insula and the parietal operculum.

Although hodological studies (described in section 1) reported that in addition to connections with the somatosensory areas posterior insula has connections with visual areas of the temporal lobe and auditory areas (AI &AII), single cell recording studies aimed mainly to study the different tactile properties and modalities (Robinson and Burton; 1980, Shneider at al, 1993.; Fitzgerald et al., 2004-2006; Thakur et al., 2006). On the other hand, rare studies were dedicated to other modalities such as the vestibular

(Akbarian et al., 1989; Grüsser et al., 1990a-1990b, Zhang et al., 1999), or auditory ones (Remedios et al., 2009).

The adjacent parietal operculum is considered as the second somatosensory cortex although recent anatomical and functional studies subdivided the parietal operculum into anterior parietal ventral area (PV) and posterior second somatosensory area (SII).

In 1980 Robinson and Burton published a series of three papers where they described the neuronal response properties to somatic and non-somatic stimuli and the somatotopic organization of the somatosensory receptive fields within the lateral sulcus regions: SII, Ig, 7b, Ri and PA. In SII 86.5% of tested neurons were activated by innocuous mechanical stimuli. Noxious stimuli were rarely responded to while none of recorded neurons were non-somatic responding neurons. The majority of tested neurons had predominantly controlateral, small to medium sized receptive fields. A somatotopic organization was demonstrated. Different body parts were represented within SII region in successive anterior to posterior strips following an anterolateral to posteromedial orientation. Trigeminal receptive fields were the only ones to be mostly bilateral and were found in the anterior portion of SII. Digits and hand were the most represented body parts in SII with mostly contralateral receptive fields situated posterior to the face ones. Digits and hand sector was followed respectively by the arm, the upper and lower trunk, and hindlimb territories.

In the granular insula 70% of tested neurons responded to innocuous somatic stimuli. These neurons were characterized by large bilateral receptive fields without an explicit somatotopic organization. Unlike the parietal operculum, neurons in the granular insula responded to auditory, thermal, or noxious stimuli. Neurons of the granular insula responded to a wide range of somatic modalities. Some neurons responded to gentle hair, skin and deep stimulation, while other neurons responded exclusively or additionally to other modalities.

In 1993 Schneider, Friedman and Mishkin published the results of an electrophysiological study where single cell recording was performed in the posterior insula presumably in the granular sector. 68% of the tested neurons showed somatosensory properties. Half of these neurons responded to superficial corporal stimulations while the remaining neurons responded to intraoral stimulations. On a total of 159 recorded neurons, 7 neurons showed visuo-tactile bimodal responses and 1 neuron showed auditory-tactile responses. None of these neurons responded to taste stimuli. The authors suggested that the granular insula is a modality-specific somatosensory area that "serves as a higher-order, modality-specific link in the somatosensory-limbic pathway". Even though the hypothesis that the granular insula is a relay between the sensory and limbic areas is plausible and interesting, the hypothesis of its specific-modality is less convincing. The fact that the investigators found often somatic-responding neurons does not exclude the presence of neurons that respond to visual or auditory modalities. Actually, in the cited experiments the visual and auditory properties were tested only by applying simple visual and acoustic stimuli. Since anatomical connections suggest that visual and auditory stimuli could be effective in relation with emotional aspects, facial expressions or communicative sounds should have also been tested. This last supposition was supported by the findings of a recent work (Remedios et al., 2008), in which the authors described auditory responses to natural sounds in the posterior insula. In the insula neurons responded to acoustic stimuli with greater latency than the neurons in auditory cortex and the response didn't represent the temporal evolution of the sound. Furthermore, the insula showed a higher selectivity to naturalistic sounds than the auditory cortex and preferentially responded to conspecific vocalizations. This vocalization selectivity depends on the spectral and temporal features of the sound suggesting that the insula plays a role in the processing of vocal communication sounds. Moreover, individual neurons showed a high selectivity to a few vocalizations suggesting that these neurons identify individual vocalizations in order to use this information for behavioral reactions. These findings agree with the hypothesis that the posterior insula is an area of convergence of sensory and emotional responses and disagree with the hypothesis of being a modality-specific somatosensory area.

In 1990 Grüsser, Pause and Schreiter recorded from the so-called parieto-insular vestibular cortex located in the upper bank of the lateral sulcus at the end of the insula and the upper posterior end of the insula. 15.2% (152 out of 1000 neurons) of the recorded neurons were found to be activated by vestibular stimulation (sinusoidal rotation of the head or the trunk). Most of the vestibular neurons showed multimodal integration responding also to somatosensory stimulation. In 1998 Zhang and colleagues described in the mid-posterior insula neuronal responses to nociceptive stimulation as well as to stimulation of baroreceptors. The nociceptive receptive fields were large as described above. More than half of the recorded neurons responded to variation of the blood pressure, and the majority of those responded also to the nociceptive stimulations. It is interesting to note that baroreceptive responses seem to prevail in the right hemisphere with respect to the left hemisphere (62% against 14%). The findings of these studies provide further supports to the hypothesis of key role of posterior insula in the integration of sensory information with the corresponding autonomic modulation.

II. DATA FROM NEUROPHYSIOLOGICAL STUDIES ON HUMANS

Even though the experimental part of this thesis was performed on monkeys, it is necessary to know the state of art of neurophysiologic studies on humans since the understanding of the functions of the human brain is the ultimate goal of neuroscientific research.

Electrophysiological investigations of the human perisylvian regions are quite frequent. The electrophysiology and in particular way the electrical stimulation covers an important role as a useful pre-surgical evaluation technique and this enables, in spite of significant limitations, the comprehension of human insular functions and to compare the obtained data with those obtained in the monkey. Since the goals at the basis of the experiments on humans are different from those of basic research, on the one hand these studies don't have the same systematic character of studies on animal models, with the studied sites usually limited and non homogeneous. On the other hand, there are undoubted advantages: intracortical stimulation performed in many cases on awake patients can benefit from the first person judgments reported by the patient on the effect of the stimulation. This allows to obtain information about the modality of the evoked responses (for example if it is tactile or thermal) or about the localizations of the evoked responses (for example, if it is a sensation on the hand or the arm). Furthermore, electrophysiological human studies allow addressing themes that can't be approached on animal models, first of all, obviously, language.

The insula was the subject of important studies by Wilder Penfield (1955), the famous Canadian neurosurgeon who preformed cortical stimulation of the insula on alert patients since the thirties of last century. The work of Penfield is mostly known for the elaboration of the motor and sensory maps that led to the definition of the concept of *"homunculus"* (Penfield & Rasmussen, 1952), as well as for the methodological aspects employed in the neurosurgery (Penfield, 1947; 1949; 1952; 1955). However, the findings of the stimulation experiments of the temporal lobe and the insula are equally important.

During the stimulation of various portions of the insula, the patients reported sensory, motor and visceral responses such as nausea, noise of the gastrointestinal tract, desire to defecate and desire to vomit. It was reported the frequent lack of responses and, more interestingly, occasional emotional responses.

Similar responses were reported recently. In 2003 Krolak-Salmon and colleagues evoked emotional responses related to disgust following the stimulation of the ventral anterior insula. Patients reported unpleasant sensation in the throat spreading up to the mouth, lips and nose. Patients reported that this sensation was not painful but "difficult to stand". More interestingly, the authors recorded by means of the same electrodes the intracerebral event-related potentials (EPRs) to human facial emotional expression. They reported significant differences between ERPs elicited by disgusted faces and by other emotional faces suggesting a crucial role of the ventral anterior insula in the processing of facial emotional expressions, with tendency to selectivity for disgust.

The correlation between the insular cortex and the visceral responses is supported by the results of the stereo-encephalographic exploration of the perisylvian regions for pre-surgical evaluation of drug-resistant epilepsy. In this case the clinical protocols consist in the suspension of the pharmacological treatment in order to elicit spontaneous epileptic seizures that allow the localization of the epileptogenic focus. During the period that follows the pharmacological suspension, the cortical activity is monitored by means of electroencephalography (EEG), by means of sub-dural grids or intra-parenchymal electrodes. In this way Catenoix and colleagues (2008) showed that ictal vomiting manifestation is related to the discharge affecting exclusively the anterior part of the insular lobe. The electrical stimulation of this site evoked epigastric sensation, nausea, or abdominal pain. Furthermore it is noted (Isnard et al., 2004) that the clinical manifestations reported during epileptic insular lobe seizures are generally described as unpleasant sensations and/or paresthesiae involving restricted peri- or intraoral areas during a first phase of the seizure, and spread to large cutaneous territory in a second phase. These manifestations include: laryngeal discomfort described as "unpleasant sensation of constriction", sometimes turned into a sensation of strangulation with a feeling of suffocation, sensation of electrical paresthesiae, tingling or warmth in lips, cheek, or whole face, paresthesiae of the gums, sensation of tension in the sublingual salivary glands, painful abdominal sensation, paresthesiae and unpleasant sensations in the throat, oro-alimentary movements (chewing, licking or swallowing movements) and hypersalivation. Paresthesiae, clonic jerks, tonic contraction, dystonia of the face and the upper and lower limbs were observed in the second phase of the seizures. During this phase the authors reported the manifestation of dysartria progressing up to complete muteness.

Like in monkeys, electrophysiological studies on humans suggest that neuronal modulation of the posterior portion of the insular cortex is predominantly related to somatosensory and nociceptive modalities. These modalities were studied in a series of electrical stimulation works performed by the group of neurosurgery of the Neurological Hospital of Lyon. In 2002, Ostrowsky and colleagues studied the somatosensory and nociceptive responses (Figure A17) evoked by means of intracortical microstimulation experiments in the insular lobe. A total of 188 stimulations were performed in 93 sites in 43 patients. Clinical responses were evoked only in 72% of sites (59% of stimulations and 81% of patients). Painful responses were evoked in the upper posterior portion of the insula in 18.2% of the responding sites (32.5% of patients) mostly located in the right hemisphere. The painful sensations were described as electrical shock, disabling sensations, stinging sensation, and burning sensation, and were located controlateral to the stimulated sites or bilaterally when midline parts of the body were involved. The non painful responses (22.5% of responding sites and 37% of patients) were evoked in the posterior insula of both hemispheres and overlapping with the painful-evoked sites. The somesthetic responses were described as warmth, cold or tingling sensations.

In 2006 Mazzola and colleagues described the somatosensory and pain responses in SII and adjacent SI and insular cortex evoked by means of intracortical microstimulations performed on 14 patients. Non-painful Somatosensory responses (cutaneous paresthesiae, temperature sensations) were evoked in all the stimulated regions (93.5%, 72.7%, 54.8% of responding sites in SI, SII and insula, respectively) while painful responses were exclusively and equally evoked in the upper posterior insula (9.2%) and SII (10.3%) and never from stimulation of SI. Furthermore, few non-somatosensory responses were evoked in SII including mostly pharyngo-laryngeal constriction and sudden interruption of speech while it was possible to evoke various types of non-somatosensory responses in the insula including viscero-sensitive, auditory, speech disturbances, vestibular sensations and fear responses.

The electrical stimulations reported differences in the receptive field extension and lateralization. While in SI receptive fields seemed to be restricted and controlateral, those of SII were larger and could be ipsilateral or bilateral like in the case of the insula, which has larger receptive fields.

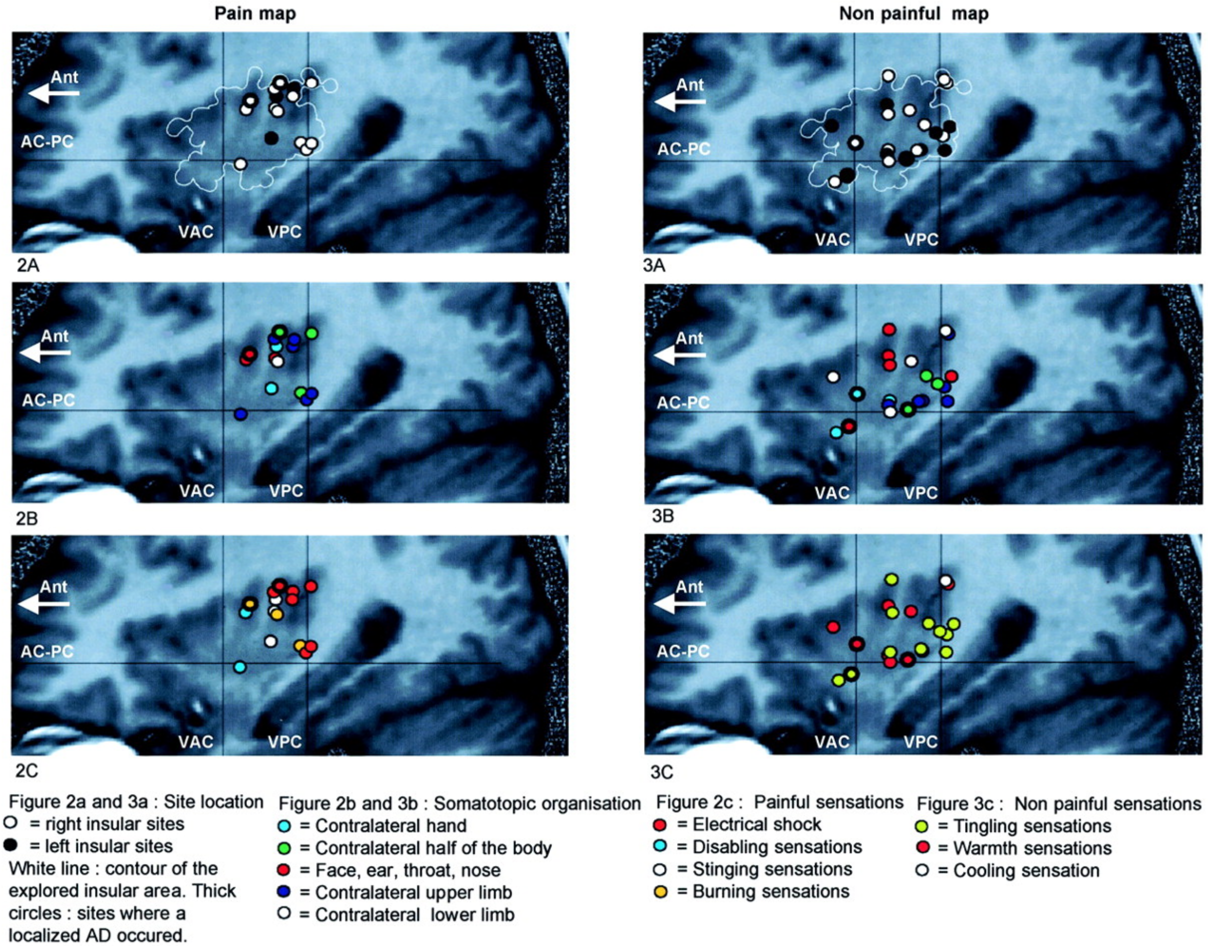


Figure A17: maps of Painful and non painful responses evoked by electrical stimulation in epileptic patients. Ostrowsky, K. et al. Cereb. Cortex 2002.

These findings were recently confirmed by the Lyon group (Mazzola et al., 2009). On a total of 472 electrical stimulations in 273 sites in 142 patients, only 10% of stimulations (in 38 patients) evoked pain sensation (Figure A18). These responses were mostly evoked in the posterior two third of the insula. 51 of the responses were evoked by stimulations in the right hemisphere. In 55% of the pain-evoked stimulations, pain was restricted to one body area (face, upper limb or lower limb). In the other cases, the pain sensation covered large skin areas including different body parts (trunk and lower or upper limb, face and upper limb, face and lower limb). Pain sensations evoked in the limbs were mostly controlateral while facial pain sensations could be contro-, ipsi- or bilateral. The results of this study suggest a somatotopic organization (Figure A19) of the painful responses along the antero-posterior and the vertical axis. The face is represented rostral to the upper and lower limb, and upper limb is represented dorsal to the lower limb.

The non-painful responses evoked following electrical stimulations included paresthesiae (35%), warmth (10.1%), laryngeal constriction (7.3%), auditory sensations (6%), cold (3%), vertigo (4%), dysarthria (2.8%), hypersalivation/nausea (2.6%), fear/anxiety (2.4%) and gustatory sensations (2.4%).

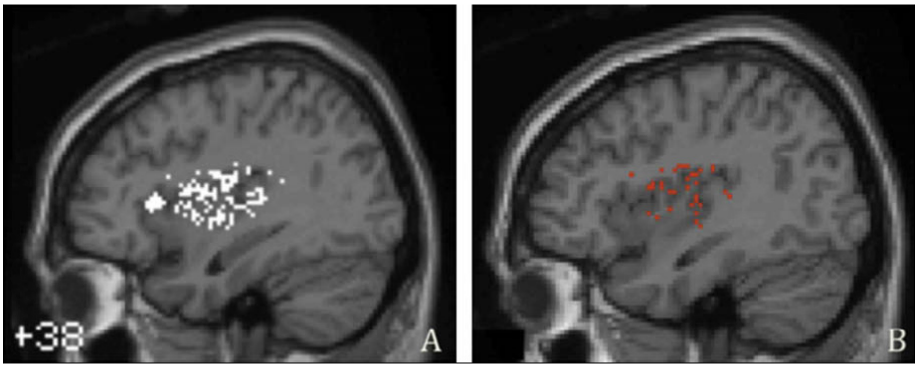


Figure A18: Plotting of insular stimulation sites (from Mazzola et al. 2009). A: All the stimulation sites. B: Painful responses sites

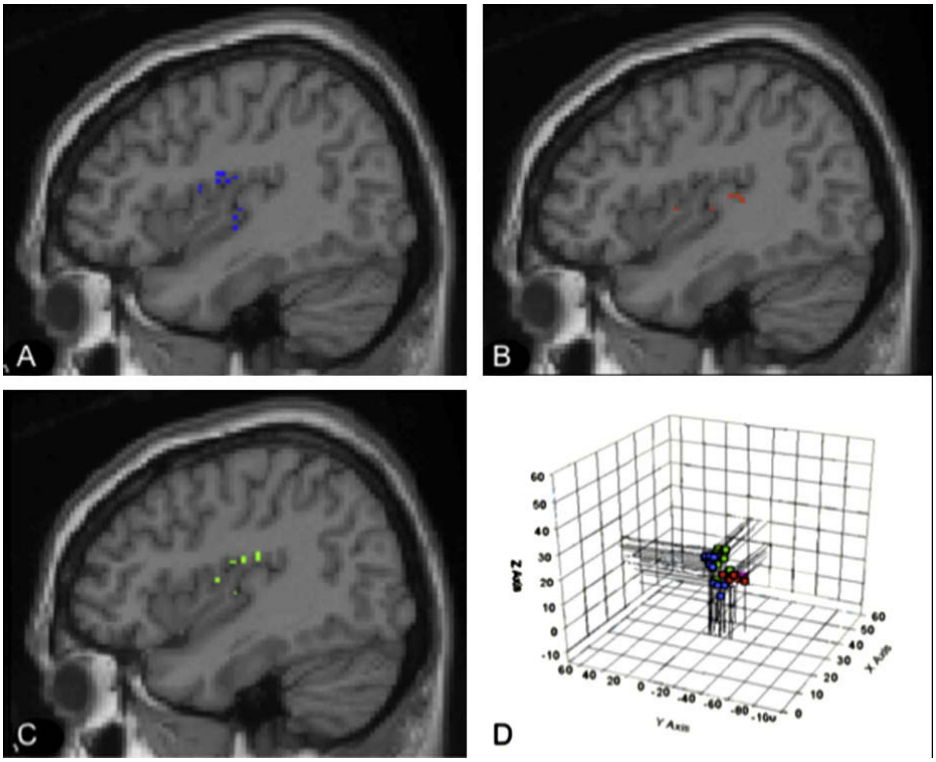


figure A19: Somatotopic organization of the painful responses restricted to one body area (from Mazzola et al., 2009). A: face, B: Lower limb, C: Upper limb. D: three-dimensional representation of the three body parts; face (blue), lower limb (red) and upper limb (green).

Another work of the Lyon group (Isnard et al., 2004) is of a great interest for the objectives of our study, for the reason that this work provides a detailed description of all the responses evoked by the electrical microstimulations of the insular cortex as well as a topographic localization of each evoked modality. On a total of 144 stimulated sites in 50 patients, clinical responses were evoked in 125 sites. The authors subdivided these responses into five main categories: somatosensory responses, viscerosensitive responses, auditory responses, language deficits, other types of responses (Figure A20).

Somatosensory responses constituted 43% of the evoked responses. They were mostly evoked in the posterior part of the insula. The evoked sensations were described by the patients as neutral or unpleasant non-painful paresthsiae such as pins and needles or slight electric current (Figure A20), or as warmth sensation, or as painful electric discharges. These responses were predominantly controlateral and covered either restricted fields (lips, cheek, face, tongue, pharynx) or large areas (hand and upper limb, neck and face, hand and leg).

Viscerosensitive responses represented 22% of the evoked responses and were evoked in the anterior part of the stimulated area. Viscerosensitive sensations were mostly situated in the pharyngo-laryngeal region and were reported as unpleasant sensations of constriction of various degrees, from a simple breathing discomfort to a painful sensation of strangulation. Besides, various types of viscero-sensitive responses were evoked such as abdominal heaviness, thoracic constriction, unpleasant ascending epigastric and retrosternal sensation, sudden flush, or nausea. In few cases it was reported abdominal pain.

Auditory responses were evoked after stimulation of the lower posterior part of the insula below the temporal operculum. These responses represented 10% of the evoked responses and were described as buzzing or whistling sounds, diffuse or coming from the space contralateral to the stimulated sites. Only one response was reported by the patient as typical of what perceived during spontaneous seizures. This response consisted in complex auditory illusion described as muffling of sounds as if “there were no echo”.

Language deficits consisting in dysartria and missing words during spontaneous speech were evoked in 6% of the responding sites. If stimulations occurred during reading or during loud voice counting task, patients interrupted the task and reported the sensation that their jaws were blocked. These responses were distributed in the posterior part of the insula. Recently Afif and colleagues (2009) reported speech arrest or lowering of voice intensity by electrical stimulation in the middle short gyrus of the insula.

Other types of responses represented 10% of the evoked responses. These responses included sensation of unreality evoked in about 4% of the stimulated sites and occurring in full consciousness; whole-body sensation consisting in vestibular sensations described as a sudden sensation of displacement of the body in space, such as a brisk forward projection, a vertical or horizontal rotation of the body or a sensation of levitation; olfacto-gustatory responses evoked in 2% of the responding sites; vegetative responses consisting of facial rubefaction and sweating evoked in 2% of stimulated sites.

This latter work of the Lyon group is particularly important for the purposes of our experiments for the reason that this work gives us important interpretation instruments of our findings through the first person description reported by the patients. This is particularly relevant when stimulations evoked sensations without any apparent behavioural outcome or in the case of behavioral outcomes that are meaningless in term of classical sensory-motor repertoire.

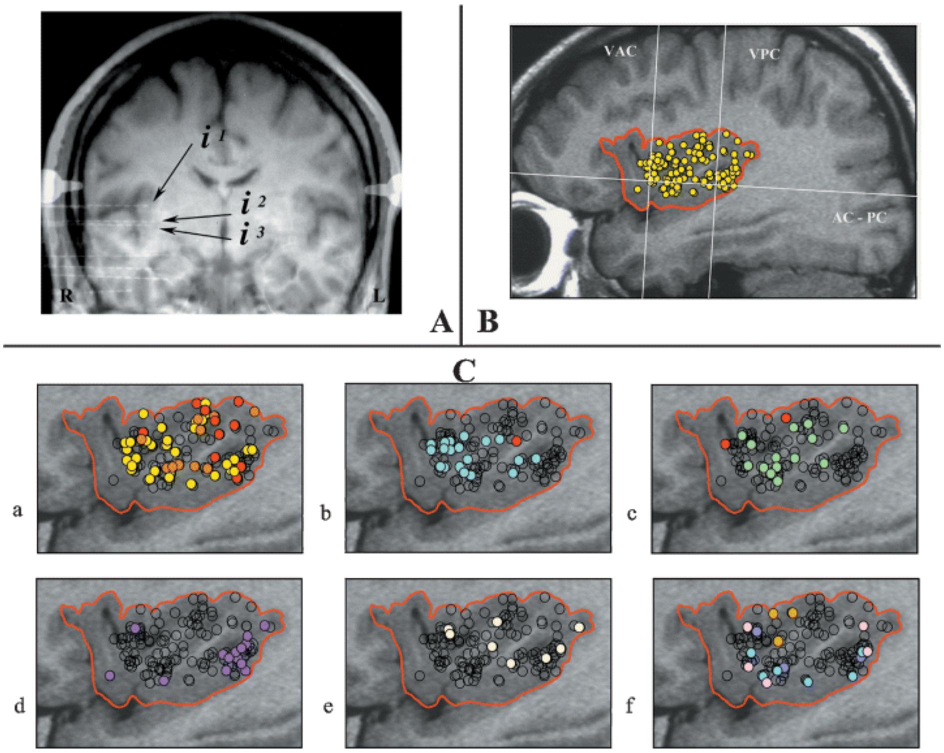


Figure A20: Results of the electrical stimulation of the insula (from Isnard et al., 2004). A: Illustration of the position and trajectories of the electrodes within the insular cortex. B: plots of the all insular stimulation sites (yellow dots) on a sagittal MRI section. The res line indicates the borders of the insula. C: Functional map of the insula; Ca: Somatosensory responses; Simple paresthesiae (yellow), warmth sensations (orange), and painful sensations (red). Cb: Viscero-sensitive responses; non-painful (blue) and painful (red). Cc: Sensations of laryngeal constriction; non-painful (green) and painful (red). Cd: Auditory responses (lilac). Ce: Dysarthric speech and missing words (white). Cf: Miscellaneous responses; sensation of unreality (light blue), olfactogustatory responses (orange), vestibular responses (pink), and vegetative responses (purple).

The effects of the microstimulations presented in the cited studies agree with the clinical description of symptoms following ictus of the posterior insula (Cereda et al., 2002). Clinical manifestations include stereoagnosia and sensory deficit of the tactile modality, of the superficial nociceptive modality and thermal modality located on the face, trunk, and the leg; gustative disorders such as the inability to discriminate between saline and acid solutions; vestibular syndromes such as the loss of equilibrium, instability and walking difficulty; cardiovascular disorder such as episodes of hypertension; neuropsychological disorders such as aphasia, dysartria, somatoparafrenia.

Electrical stimulation experiments on humans allowed also to investigate other functional aspects of the insula. in a paper published in 1992 Oppenheimer and colleagues described the cardiovascular effects obtained by the electrical stimulation of the human insula. Out of a total of 70 stimulations (39 in the right hemisphere and 31 in the left hemisphere) in 4 patients, 50% of stimulations evoked modulation either in heart rate only or in heart rate and in blood pressure; 23% of stimulations evoked only changes in blood pressure; the remaining stimulations didn’t evoke any cardiovascular effect. The cardiovascular responses were distributed along the antero-posterior axis. In the right hemisphere, responses were more evocable from the anterior portion than from the posterior portion (58% against 38%) and in the left hemisphere, the posterior part showed more cardiovascular changes (61% against 39%). Moreover, the amplitude of the evoked bradycardia was greater in the posterior portion than in the anterior portion.

Within the entire insula, in the left hemisphere bradycardia represented 93% of evoked heart rate responses (100% in the posterior portion against 86% in the anterior portion); while in the right hemisphere, bradycardia represented 50% of the evoked heart rate responses (53% in the anterior portion against 40% in the posterior portion). Based on these findings, the authors suggested a lateralization of the cardiovascular responses with a sympathetic dominance in the right hemisphere. A further reading of these data suggest that the hypothesis of an hemispheric lateralization needs more in-depth examination since both bradycardic and tachycardic responses are equally represented in the right hemisphere suggesting a vagal dominance in the left hemisphere against a dual control pattern in the right hemisphere. Equally interesting, the difference in the number of responsive sites and the amplitude of the responses along the antero-posterior axis suggests an intrahemispheric segregation with dual autonomic dominance within the same hemisphere. Such hypothesis is supported by the sub-cortical connection pattern of the insula (see section 1). The hypothesis of the lateralization of autonomic nervous system modulation was investigated at the level of the insula on animal models by Zhang and colleagues (cited above). The correlation between the modulation of the autonomic nervous system and the activation of the insula and the inner perisylvian regions was confirmed by several clinical studies. Christenssen and colleagues (2005) reported important cardiovascular consequences following to ictus of the insula. They described the electrocardiographic trend of 43 patients during the 24 hours following the ictus. Lesion of the insula evoked in the patients sinus tachycardia, with heart rate over 120 bpm; more than 10% of ectopic beats, and dislevelling of the ST tract. In this case, the authors reported the presence of a possible lateralization due to the prevailing of atrial fibrillation, atrio-ventricular block, ectopic beats and inverted T wave following lesions located in the right hemisphere. Furthermore, the authors advanced the hypothesis of the correlation between the lesions located in the right hemisphere and the phenomenon of sudden death occurred three month after the ictus. Blanke, Seeck and Zaim (2001-2002) described cases of temporal epilepsy that led to behavioral disorders and palpitations accompanied with state of anxiety. Such state is due to the presence of a sinus tachycardia secondary to a concomitant syndrome of postural orthostatic tachycardia (POTS, is a condition of orthostatic intolerance in which a change from the supine position to an upright position causes an abnormally large increase in heart rate, often, but not always accompanied by a fall in blood pressure), that can be treated by beta-blocking drugs. The majority of epilepsies leading to cardiac arrhythmia take place in the temporal lobe, adjacent to the insula. However, the majority of studies don’t describe significant lateralization.

B- INTRACORTICAL MICROSTIMULATION STUDY ON MONKEY

I. INTRODUCTION

The findings of the human studies show strong convergence with both functional and hodological data obtained from studies on animal models. The anterior portion of the insula is implicated in oro-alimentary and viscero-motor behaviors. These functional roles are suggested by the data obtained both from single cell recording studies on monkeys, the stereoencephalographic observation on epileptic patients and from the intracortical microstimulation experiments performed on these patients. The connections of the anterior insula support the hypothesis of oro-alimentary and visceral components by means of the connections with the lateral hypothalamus and the ventral posterior medial parvocellular nucleus (VPMpc) that retransmits vagal information coming from the cranial nerves. The posterior portion of the insula is predominantly a multi-modal region; somatosensory, nociceptive and thermoceptive modalities are represented in a dorso-caudal portion, presumably corresponding to the granular insula reached, among others, by sub-cortical connections transmitting homeostatic information of spinal origin. Also in this case, human and monkeys data have a certain degree of convergence. The auditory modality was reported in the ventral caudal portion of the insula both in humans and monkeys in agreement with the cortico-cortical connections of the posterior insula with the auditory areas.

In the present study we addressed these hypothesis by means of an intra-cortical microstimulation (ICMS) experiment.

The goal of this experiment was the realization of a functional map of the insula and the adjacent inner perisylvian regions. The detectable behavioral outcomes and the modulation of the autonomic nervous system were monitored and studied jointly.

Behavioral responses. This study was interested in dual aspect of the behavioral responses evoked by ICMS:

- The first aim was to investigate the behavioral responses depending on neuronal circuits involving the perisylvian regions. Such responses were evoked by means of long train stimulations or behavioral train scale stimulation (BTS-ICMS). The application of BTS-ICMS allows evoking complex behavioral responses that can be dependent on the experimental context, recruiting specific neural circuits.

- The second aim of our study was to assess the presence, localization and disposition of a corporal map in the insula and the perisylvian regions. To this purpose, we employed short train stimulations evoking brief twitches in order to determine which body part is involved. Furthermore, the excitability of these regions was evaluated establishing the intensity threshold.

Modulation of the autonomic nervous system. The evidence of a correlation between the nonstandard function of the insula (due to an hypoactivation, like in the case of stroke or to an hyperactivation, like in the case of electrical stimulation or insular epilepsy) and the variation of heart activity suggests the need of a detailed study on the control of the autonomic nervous system at the cortical level. In this study we addressed the following points:

- To assess the effect of stimulation on instantaneous heart rate, in term of sign (increase or decrease), amplitude (percentage of the change in respect to the rest condition), and trend (dynamicity of the heart rate change and recovery).
- To assess the hypothesis of a rostro-caudal segregation of sympathetic and parasympathetic responses, suggested by thalamic connections, but without any striking evidence from neurophysiological studies.
- To assess the hypothesis of rostro-caudal segregation of the amplitude of responses, suggested by human stimulation studies, but with no evidence coming from animal studies.
- To assess the hypothesis of an hemispheric lateralization of sympathetic and parasympathetic responses, suggested by data of electrical stimulation studies and clinical observations on humans. No evidence of this lateralization was ever suggested from studies on monkeys.
- To assess the hypothesis of lateralization of the amplitude of the responses, suggested by human stimulation studies and clinical observations (more cardiovascular complications derive from right insular ictus than left ictus). This hypothesis was never studied on monkeys.
- To assess the presence of a possible correlation between the evoked behavioral responses and the heart rate.

II. MATERIALS AND METHODS

1. Subjects

Two male rhesus monkeys (*Macaca mulatta*), MK1 and MK2, weighting 7 and 11 kg, respectively, were employed in this study. Animal handling as well as surgical and experimental procedures complied with the European guidelines (86/609/EEC and 2003/65/EC Directives) and Italian laws on the care and use of laboratory animals, and were approved by the Veterinarian Animal Care and Use Committee of the University of Parma and authorized by the Italian Ministry of Health. Before the beginning of the experimental sessions, each monkey was trained to sit quietly in a primate chair and to be handled by the experimenters.

2. Training

No specific training was employed in this study but a shaping to get monkeys used to the experimental situation. The shaping procedure took few months. In a first phase each monkey staying in its cage was habituated to the presence of the experimenters and to interact with them. The second phase was achieved with monkeys seating in a primate chair. In this phase each monkey was habituated to perform various basic movements and to receive various tactile or proprioceptive stimulations done by the experimenters. The monkey’s collaboration and progress were rewarded by pieces of fruit and juice. In the early phase of the shaping, monkeys worked with their head free to move. In the late phase, the head was fixed using a head fixation device surgically implanted.

3. Surgeries

Surgeries were performed under aseptic conditions and general anesthesia by means of periodic intra-muscular induction of Ketamine hydrochloride (6.4mg/kg) and Medetomidie (0.08mg/kg). During surgery, hydration was maintained with saline delivered intravenously and temperature was maintained constant by using a heating pad. Heart rate, blood pressure, respiratory depth, and body temperature were continuously monitored. Upon recovery from anesthesia, the animals were returned to their home cages and closely monitored. Dexamethasone and prophylactic broad-spectrum antibiotics were administered pre- and postoperatively. Furthermore, analgesics were administered intra- and postoperatively.

In a first surgery, a head-restraining device was implanted. This system consisted in four titanium cave cylinders positioned in the frontal depression and on the parietal bones. Evarts screws were implanted on the skull in proximity to the cylinders. The whole system was fixed using acrylic cement. The fixation implant was left untouched for one month after the surgery in order to favorite his stabilization.

In a second surgery, two recording chambers (25*20mm rectangular titanium chamber) were implanted on the regions of interest in both hemispheres. During this surgery, the skull over the regions of interest was removed and the dura exposed. The recording chamber was fixed on the skull by means of screws covered by hydroxyapathite to facilitate osteo-integration and acrylic cement with antibiotic. One of these chambers allowed the access of the microelectrode to the studied region, while the other one was used for the visualization of the microelectrode into the brain by means of ultrasound imaging (see below). The stereotactic coordinates were calculated for one angle of both chambers. These points were used as reference points for calculation of the stereotactic coordinates of the penetration sites.

4. Estimation of recording chamber coordinates

Since the target of this study – the insula and the adjacent perisylvian regions – was never studied in our institution, no information was available regarding the localization of these regions in term of stereotactic coordinates. Such information is crucial in order to determine the position for the implantations. For this purpose, we integrated information coming from the analysis of anatomical cases, brain atlases and morphologic MRI.

a. Estimation of chamber coordinates by mean of anatomical cases

Four rhesus monkey anatomical cases taken from the archives of the laboratory of Neuroanatomy of our Department were examined in order to identify the position of the insula along the antero-posterior axis. The positions of the presumable anterior and posterior boundaries of the insula were calculated with respect to the stereotactic zero positioned at the anterior commisure. The measures are reported in the table below.

Case	Anterior limit	Posterior limit
Case n° 10	28.5 mm	7.2 mm
Case n° 14	22.8 mm	5.1 mm
Case n°20	24.0 mm	7.2 mm
Case n° 21	26.1 mm	5.1 mm
Conservative measures	28.5 mm	5.1 mm

b. Estimation of chamber coordinates by mean of brain Atlas

The stereotactic coordinates of the employed atlas (Paxinos G.; Huang X.F.; Toga A.W. The Rhesus monkey brain in stereotaxic coordinates. 2000, Academic press) were calculated with reference to the stereotactic zero positioned at the anterior comissure level. The insula begins four mm. rostral to the anterior comissure (three mm anterior to the intersection between the superior and inferior arcuate sulcus at the level of the caudal extremity of the principal sulcus). Caudally, the insula ends at about 20mm from the anterior comissure, (we considered as anatomical reference the rostral extremity of the intraparietal sulcus (IPS), situated 13 mm caudal to the anterior commisure). The anatomical reference to the arcuate sulcus and the intraparietal sulcus were pertinent during the surgery given the experience of Parma group in the stereotactic localization of these sulci and surrounding regions.

c. Estimation of the chamber coordinates by mean of MRI cases

Further information on the stereotactic coordinates of the recording chambers was obtained by means of morphologic magnetic resonance imaging (MRI) performed on the experimental subjects. The calculation of the stereotactic position of the insula was performed in reference with the stereotactic coordinates of two screws implanted before the scanning.

Based on the three analyses, we performed a triangular openings in the skull according to the following coordinates:

- MK1: right AP (+30; +3); ML (+5; +23) and left AP (+28; +1); ML (+7; +24).
- MK2: right AP (+30; +5); ML (+12; +26) and left AP (+30; +5); ML (+12; +26).

5. Experimental setup and procedures

During the daily microstimulation sessions, tungsten microelectrodes (FHC instruments®, hypoxide insulation, impedance < 200 kΩ) were inserted into the brain parallel to the vertical plane, through the dura mater, and advanced with a hydraulic micromanipulator into the insula and the surrounding opercular regions. The antero-posterior and medio-lateral stereotactic coordinates for each penetration was calculated with respect to the reference point on the recording chamber. These coordinates were then putted on a topographic map that can be used for the post-mortem anatomical reconstruction of penetration sites. In MK1, penetrations were spaced at 500-µm and 1000-µm intervals in the mediolateral and rostrocaudal directions respectively. While in MK2, penetrations were spaced 1000- µm and 2000-µm intervals in the mediolateral and rostrocaudal directions respectively.

a. Functional localization of the penetration sites

During the first phase of microelectrode insertion, neuronal activity was amplified, filtered with band pass of 500Hz to 6 KHz, and actions potentials discriminated on the basis of their wave form using a preamplification and time-amplitude windows discriminator system (Bak Electronics, Germantown, MD, USA). The recording of neuronal activity served to control the depth of the electrode tip during its progression through the brain, with zero depth corresponding to the first encountered activity.

The analysis of the functional properties of the recorded neurons activity allowed the localization of the site of insertion in term of anatomical area. This identification was very useful to reach the regions of interest. From the depth of the first cortical region it was possible to estimate the position of the penetration sites with respect to the sulci. On the basis of this position, consultation of the brain atlas allowed an indicative localization of the site of stimulation, an estimation of the required depth to be traversed, and the number of cortex to pass through in order to reach the fronto-parietal operculum.

b. Localization of the of the microelectrodes by mean of ultrasound imaging

During the daily stimulation sessions, an ultrasound imaging system (Logiq 400 CL ProSeries, General Electric Medical system®) was employed in order to visually guide the advancement of the microelectrode through the brain and to localize each of the microstimulation sites into the studied regions. The ultrasound system had access to the monkey brain by means of an ultrasound transducer (endocavity GE-E721® probe) positioned in the chamber controlateral to the stimulation. During visualization, the brain was scanned in coronal-like sections in which bilateral sulci were easily discernable (particularly the cingulate, lateral and temporal sulci). Due to the artifacts of the recording chamber, the microelectrodes were visible starting from 8-10 mm of depth. Although the visualization of the gray matter derived from the refraction obtained by the sulci, thus with a minimal error margin in term of depth, ultrasound imaging represented a crucial instrument in the localization of the position of the microelectrode with respect to the stimulated region.

The figure B1 shows a typical coronal-like image obtained during an experimental session; left hemisphere is visible on the left side of the image, while the right hemisphere is visible on the right side. Below the probe situated in the top of the image, one can easily identify the cingulate sulcus, the ipsi- and controlateral Sylvian fissures, and the ipsi- and controlateral temporal sulci. Furthermore, the microelectrode is visible in the left hemisphere (contralateral to the probe) with the tip situated in the dorsal portion of the insula.

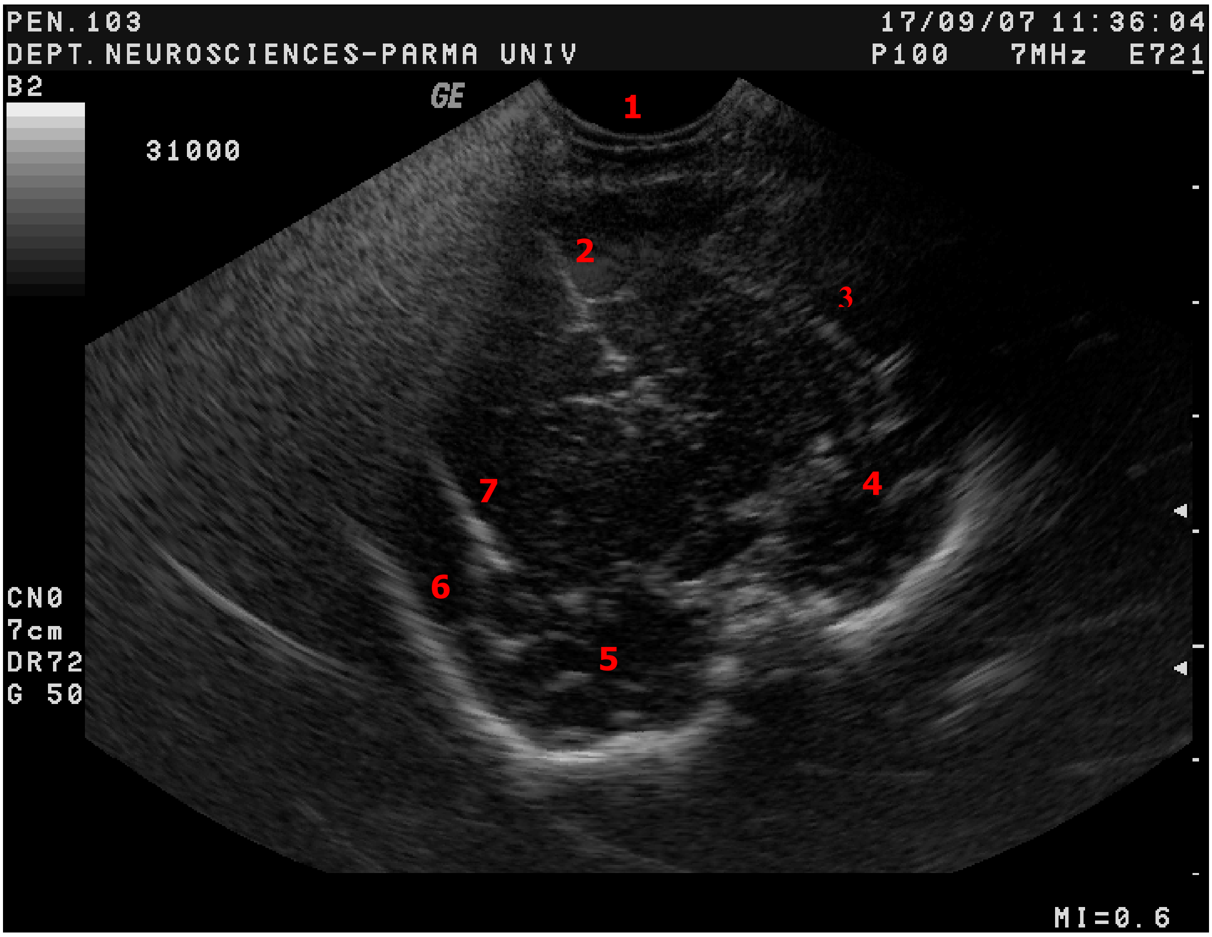


Figure B1: Coronal-like Ultrasound slice of the macaque monkey taken during an ICMS experimental session. 1: Location of the ultrasound probe, 2: Cingulate sulcus, 3: ipsi-lateral sylvian fissure, 4: ipsi-lateral superior temporal sulcus, 5: contralateral superior temporal sulcus, 6: contralateral sylvian fissure, and 7: the microelectrode trajectory and position.

c. Microstimulation experimental setup

Once the microelectrode reached the fronto-parietal operculum, recording of neuronal activity was interrupted and microstimulation procedure started.

The experimental microstimulation setup consisted of two stimulators ("stimulator 1": LACE electronica®, and "stimulator 2": Biphasic pulse generator, BAK instruments Inc.®) on which parameters of the frequency, train and characteristics of the stimulation wave were set, and an isolation unit (Stimulus Isolator, WPI®) on which the stimulation's intensity was established. The stimulation was triggered by means of an external button. Circuit closure gave rise to electrical stimulation current, to the illumination of a led in synchrony with the stimulation, used as event indication for the videos analysis, and an analogical signal acquired in order to synchronize the stimulation with the ECG signal (see below for technical details).

d. Estimation of Microstimulation parameters

The first experimental sessions were pilot sessions devoted to the determination of the adequate parameters to be employed during the experiment. The previous electrical stimulation studies on rhesus monkeys were performed with obsolete techniques where stimulation was always a voltage discharge and was generally delivered through bipolar electrodes or surface macroelectrodes without any indication of the impedances, making the conversion of stimulation parameters impossible for the use with our system. Furthermore, performing the experiments in acute conditions under anesthesia, they employed very long stimulation trains (up to 30 sec) without any consideration of the possible tissue damage that could be produced.

The first parameters tested were those employed in our Department during recording experiments in the motor and premotor cortex. These stimulations consisted in 0.2 msec monophasic pulses delivered at frequency of 333Hz, with train duration varied between 50/100/150msec and intensity varied up to 40μA. Such parameters resulted insufficient to evoke any response in the regions of interest. In a following phase, a series of stimulations were performed employing the stimulation parameters established by Graziano and colleagues (2002-2004-2005) during stimulation of premotor cortex: biphasic wave at 200Hz, up to 500msec of stimulation train, up to 100μA of intensity. Also in this case, no response could be evoked in the insula and opercular regions. In a last phase, new stimulation parameters were tested after consultation of the literature relative to the human electrical stimulation studies. Human electrical stimulation was generally performed at low frequency allowing an increasing of both current intensity and train duration. In the studies performed by the Lyon group, two stimulation types were employed (Ostrowsky et al., 2002):

- Low frequency stimulations: 1Hz, 1-3msec biphasic pulses, 1-5mA, and 5-10sec train duration.
- High frequency stimulations: 50Hz, 0.1-0.5msec biphasic pulses, 0.8-6mA, and 5sec train duration.

The authors reported that the low frequency stimulation rarely evoked responses and thus we opted to test the high frequency stimulation. In order to avoid any risk of tissue damage, low impedance (<150kΩ) microelectrodes were used. The possibility to increase the train duration opened the possibility to map the insular and opercular cortex comparing the responses of two different ICMS approaches: behavioral scale ICMS and short train ICMS. At the end of this phase, the following stimulation parameters were selected:

- Pulse width: biphasic pulses were used in this study in order to minimize the risk of tissue damage. Single pulse width was 0.2msec and was chosen on the basis of previous ICMS studies (Theovnick et al., 1996-2006).
- Frequency: the frequency of the stimulation was fixed at 50Hz. Responses could be evoked also at lower frequency down to 11Hz, but frequency threshold was not systematically tested.
- Intensity: the maximum intensity used in the experimental protocol was 4mA. Stimulations with higher current intensities were performed few times only in the first phase of parameters estimation, for safety measure. In any case, responses evoked by an intensity higher than 4mA were likely considered as the result of current diffusion. After standard intensity stimulations, stimulations with decreasing intensities were performed in order to determine the threshold able to evoke behavioral responses.
- Behavioral time scale stimulations (BTS-ICMS): Behavioral time scale stimulations (BTS) consisted in long train durations sufficient to evoke a complete behavioral response. A BTS experiment led to study the probable role of the context into which stimulations occurred. BTS stimulations were performed employing train durations of 3 sec. Four sec. trains were occasionally used. Train duration superior

to 4 sec. was never used because train duration of 3 sec. was generally sufficient to evoke the complete behavioral responses.

- Short train ICMS (ST-ICMS): short train stimulations were performed in order to evoke brief twitches of single effectors. Train duration in this case was obtained by decreasing the standard BTS train until the shortest train able to evoke behavioral responses.

e. ECG recording procedure and apparatus

During each experimental session, ECG signals were acquired in order to assess the effect of the ICMSs on the autonomic nervous system. For this purpose, before starting stimulation two adhesive surface electrodes (Medtronics®) were placed on the back of the monkey. ECG signal was amplified, filtered (CED 1902®) and acquired (CED Power 1401®; Spike2®) in parallel with the stimulation event. The Figure B2 shows two examples of ECG acquisition. The ECG tracing is visible on the top parts of both figures, while the stimulation train is visible at the bottom. The Figure B2-B shows the portion of the Figure B2-A a in which was occurred the stimulation. The components of ECG tracing (P, QRS, and T) are visible. Visualization of the deflection allowed evaluating if bradycardia was due to atrio-ventricular block or to the sinus node.

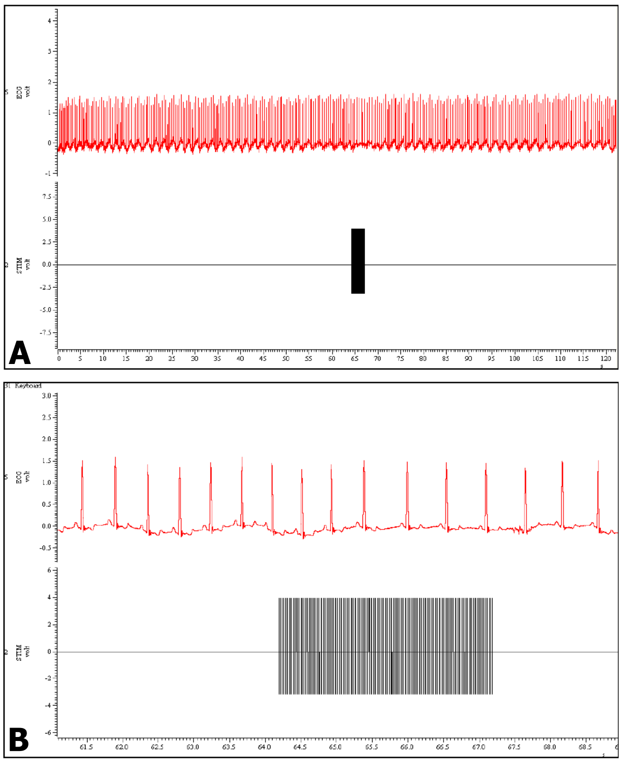


Figure B2: ECG tracing acquired during a ICMS trial. The ECG is represented by the red tracing and the occurred stimulation is represented by the black tracing.

6. Experimental protocol

Once the region of stimulation was reached and identified, ICMS experiment started in order to investigate the functional properties of the opercular and insular regions. During ICMS behavioral as well as cardiac responses were monitored.

An ICMS consist in the delivery of an electrical discharge with the established standard parameters (BTS parameters) i.e. 4mA biphasic pulses delivered at 50Hz for train of 3sec. In each stimulation site ICMS was repeated three times with a minimum delay of 30sec between stimulations. Two observers scanned the entire animal for evoked movements. Behavioral responses were considered only when they were clearly identified by two observers, observers agreed on the type of movements and they evoked at least on 50% of the trials. When a complex behavioral response was evoked, ICMSs were systematically performed under different contextual conditions depending on the evoked response.

In the case of reliable behavioral responses, thresholds were determined:

- The temporal threshold corresponding to the short train stimulation (ST-ICMS) consisting in the shortest train evoking behavioral responses. This threshold was determined by gradually decreasing train duration while maintaining the standard intensity.
- The intensity threshold consisted in the minimum intensity that could evoke behavioral responses. This threshold was determined gradually decreasing the intensity while maintaining the standard train duration.

When ICMS evoked behavioral responses that seemed to be motor, stimulation with classic parameters as well as Graziano's parameters were randomly performed. In no case these stimulations evoked apparent behavioral responses. Behavioral responses were filmed and described in details on the experimental protocol. ICMSs were performed at steps of 500µm in depth and ecograph images were taken at steps of 1mm in order to control the localization of the stimulation sites. At each stimulation site, ECG signal was acquired during one of the standard BTS-ICMSs, ST- ICMSs (temporal threshold) and intensity threshold. An ECG acquisition trial comprises three components:

- Pre-stimulus period consisting of 60sec of baseline preceding the stimulation period.
- Stimulus period, corresponding to train duration.
- Post-stimulus period consisting of 40sec of recovery period following the stimulation period.

ECG recording was performed under condition of tranquility of the monkey.

7. Data analysis

a. Regions of interest (ROIs)

The stimulated regions were subdivided in four regions of interest (Figure B3):

- The upper bank corresponding to the fronto-parietal operculum.
- The dorsal insula corresponding to the dorsal portion of the insular cortex
- The ventral insula corresponding to the ventral portion of the insular cortex
- The lower bank corresponding to temporal operculum

This subdivision was respected in the analysis of all collected data and in the presentation of both behavioral and cardiac results.

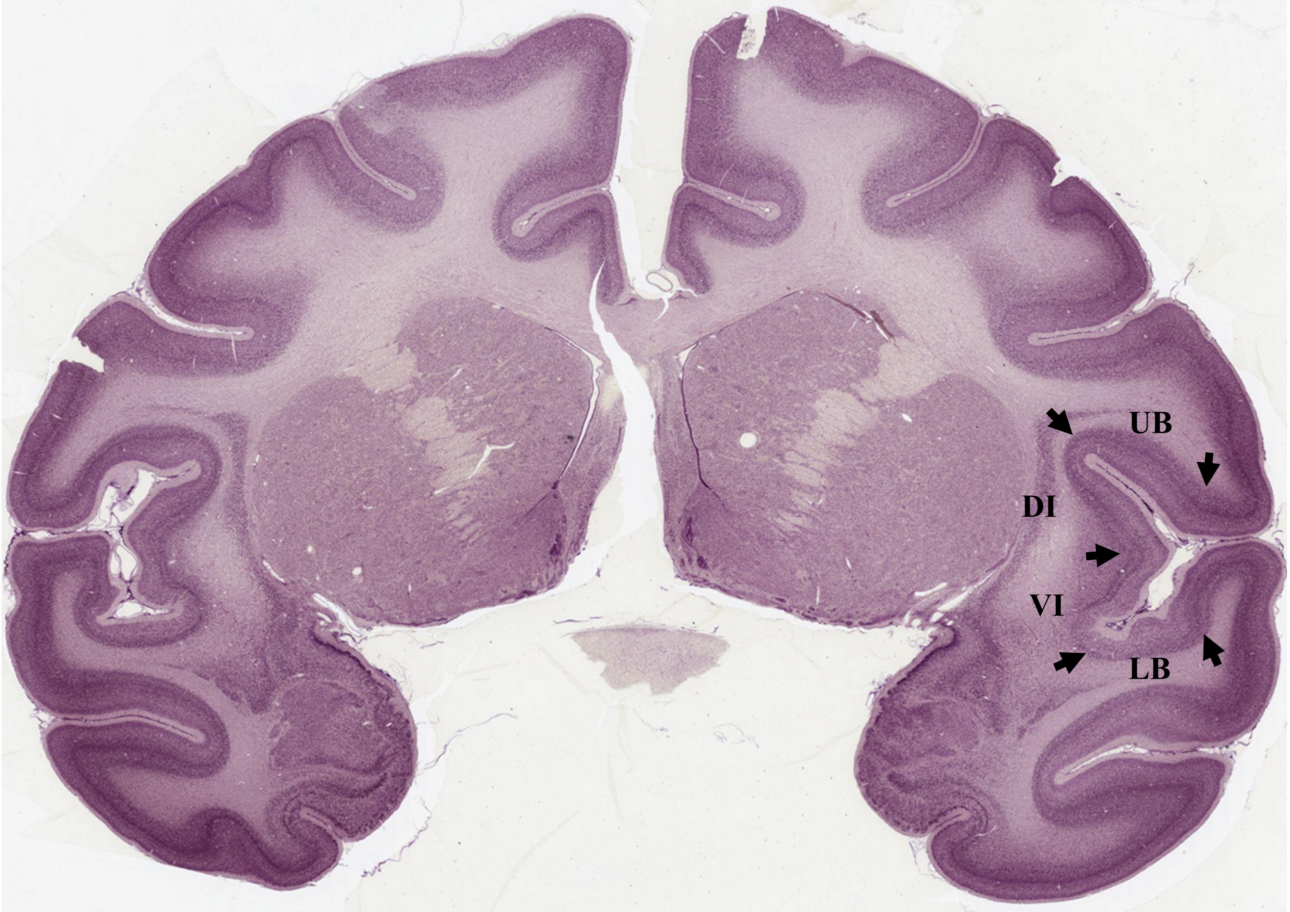


Figure B3: The four regions of interest in the ICMS experiment illustrated on a coronal section of the macaque monkey brain (from brainmaps.org) . The stimulated regions were subdivided in four regions of interest: UB: Upper bank (fronto-parietal operculum), DI: Dorsal insula, VI: Ventral insula, and LB: Lower bank (temporal operculum).

b. Analysis of behavioral data

b.1. Behavioral time scale analysis

The behavioral responses evoked by BTS-ICMSs were classified on the basis of the involved effectors, complexity/articulation of evoked behaviors, meaning of evoked responses, and naturalistic aspect of evoked responses. These responses were collected in five macro-categories: oro-alimentary behaviors, complex behaviors, simple sensory-motor responses, communicative responses and miscellaneous responses. Each of these macro-categories comprises different categories.

b.2. Short train stimulation analysis

The responses evoked by ST-ICMSs were classified on the basis of the involved effectors. These responses were used to build a somatotopic map of the studied regions.

The temporal and intensity thresholds were used to build temporal and intensity stimulability maps of the studied regions.

c. Analysis of ECG signals: Heart rate variability

c.1. Analysis of single stimulation

In a first phase of the heart rate variability analysis, ECG signals were singularly analysed. The raw signal was filtered, automatically analyzed for QRS complexes detection, and then manually corrected. Instantaneous heart rate (HR) was then calculated as the inverse temporal difference between every two consequent QRS's: $HR[t] = 1/(QRS[t] – QRS[t-1])$. The temporal trend of the effect of the instantaneous heart rate was constructed by means of resampling HR_{10s} at 0.5 sec.

Baseline HR was calculated as the mean HR in a one-minute period immediately preceding the stimulation. ICMS effect on the HR was searched in a 10sec period (HR_{10s}) starting at stimulation onset. Bradycardic and tachycardic effects (BC and TC) were calculated as the min-HR and max-HR in that period, subtracted and normalized with the baseline HR, i.e., $BC=(\min(HR_{10s})-HR_{baseline})/HR_{baseline}$ & $TC=(\max(HR_{10s})-HR_{baseline})/HR_{baseline}$.

c.2. Population analysis A: ROIs analysis of rostro-caudal penetration groups

The stimulations were grouped by AP position and cortical ROIs. In this analysis we analyzed the temporal profiles of the instantaneous heart rate during the stimulations and post-stimulus period and statistically investigated the consistency of stimulation effect in every sample time ***t***. For this purpose, we determined the average HR in every sample time ***t*** that was significantly different from zero (normalized baseline). The Figure B4 illustrates:

- The percentage of the variation of the heart rate with respect to the normalized baseline corresponding to the zero on the y-axis.
- The x-axis corresponding to the temporal axis, where the stimulation period corresponds to the portion prior to the dashed vertical line, while the post-stimulus period corresponds to the portion after the dashed line.
- The responses relative to the ROIs illustrated in different colours; dorsal bank (blue), dorsal insula (purple), ventral insula (red), and ventral bank (green).
- The statistical significance indicated by the dot size: small: p<0.05; medium: p<0.01; large: p<0.001.

This profile analysis was aligned with the stimulation onset.

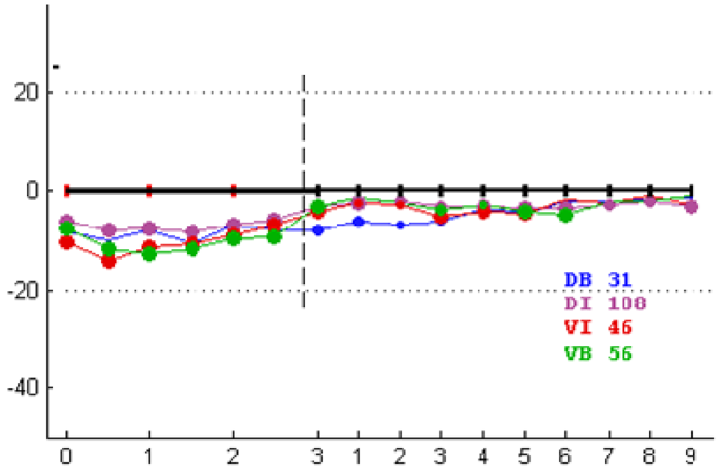


Figure B4: Illustration of the temporal profiles of the instantaneous cardiac frequency during and after the stimulation period.

Such type of analysis was performed in order to assess the heart rate variability in reference to the antero-posterior position, putting together the stimulations performed at the same antero-posterior position but at different medio-lateral position. This analysis allowed the examination of the temporal trend of the cardiac responses evoked at a given antero-posterior position in terms of velocity and modality of the responses and recovery. Furthermore, it was possible to compare the amplitude of cardiac responses occurring in the different ROIs at the same antero-posterior position as well as the cardiac responses occurring in the same ROI at different antero-posterior position.

c.3. Population analysis B: ROIs analysis of single penetration

All stimulations were grouped by A-P and M-L stereotaxic position, and cortical ROIs (dorsal bank, dorsal insula, ventral insula, ventral bank). Here we estimated the consistency of the bradycardic and tachycardic effects in each stimulation by applying a t-test and a threshold of 20% on the mean effect. The effect of a given stimulation was considered to produce, e.g., bradycardia, if its *mean* BC was lower than 0.20 and its BC was significantly different from zero ($p<0.05$, two-tail, uncorrected). If a given stimulation produced both bradycardia and tachycardia, it was classified as producing a biphasic effect.

This type of analysis allowed the localization of the single penetrations where ICMSs were able to evoke a cardiac response as well as the sign of this response. It was possible to evaluate the difference in the ICMSs effects along the antero-posterior, the medio-lateral, and the dorso-ventral axis.

c.4. Hemispheric lateralization of the cardiac responses

A population analysis was performed in order to assess the potential hemispheric lateralization of the cardiac responses evoked by the ICMSs. Two populations were confronted taking the same number of stimulations performed in the rostral part of the studied regions (AP 27-21). The four ROIs were grouped into a single group. Two ANOVAs were performed on the bradycardic and tachycardic responses.

c.5. Analysis of Behavior- HRV correlation

The correlation between behavioral and cardiac responses was investigated performing the population analysis. In this case the populations consisted in the grouping of the stimulations that evoked similar behavioral responses despite their different antero-posterior or medio-lateral position.

III- RESULTS

1- Short train stimulation

a. Somatotopic organization

The ST-ICMSs performed at the temporal threshold with minimum train of 50msec showed the presence of a coarse somatotopic organization (figure B5) distributed on well-defined portion of the studied regions. This somatotopic organization is located on the fronto-parietal operculum and the dorsal portion of the insular cortex where BTS-ICMSs evoked mainly motor-like responses (see Behavioral time scale paragraph), while such organization is apparently missing in the ventral portion of the insula and the ventral bank.

In the anterior portion of the dorsal insula and orbitofrontal operculum (OFO) ST-ICMSs showed a predominance of brief responses involving the mouth, such as twitch of the tongue, lips, and jaw. Ventrally to these responses, ST-ICMSs evoked brief reflexes consisting in a sternal leap up (shivering involving particularly the sternum), since that BTS-ICMSs evoked in the same sites visceral responses and vagal activation consisting in decrease of the heart rate, such responses were classified as potential visceral reflexes. A representation of the hand prevails in the region immediately posterior. ST-ICMSs in this region evoked twitches of the digits, thumb, and wrist. Between these two regions there is a transition region where ST-ICMSs evoked overlapped and/or simultaneous hand and mouth responses. We were not able to detect any precise localization of the single digits or a segregation of the thumb with respect to the other digits. In a medial portion of the fronto-parietal operculum ST-ICMSs evoked twitches of the hand together with eye-blinking. Ventrally to this region, ST-ICMSs evoked twitches of the hand or the foot. More caudally, ST-ICMSs evoked jointly hand and foot twitches. Finally, the most caudal portion of the parietal operculum and the dorsal insula showed responses consisting in simultaneous twitches of joints of the upper and lower limbs.

In summary, ST-ICMSs demonstrated that the evoked responses follow a corporeal map along the rostro-caudal axis in the fronto-parietal operculum and the dorsal insula with the mouth represented in the rostral part and the upper and lower extremities in the caudal part. Unlike the dorsal regions, the ventral insula and the ventral bank don’t show any somatotopic organization since 79.6% of the stimulated sites (77% & 85% in MK1 and MK2 respectively) in these two regions resulted non responsive while in the remaining sites, the evoked behavioral responses belonged to the miscellaneous category where no clear effectors were involved in the manifested behaviors.

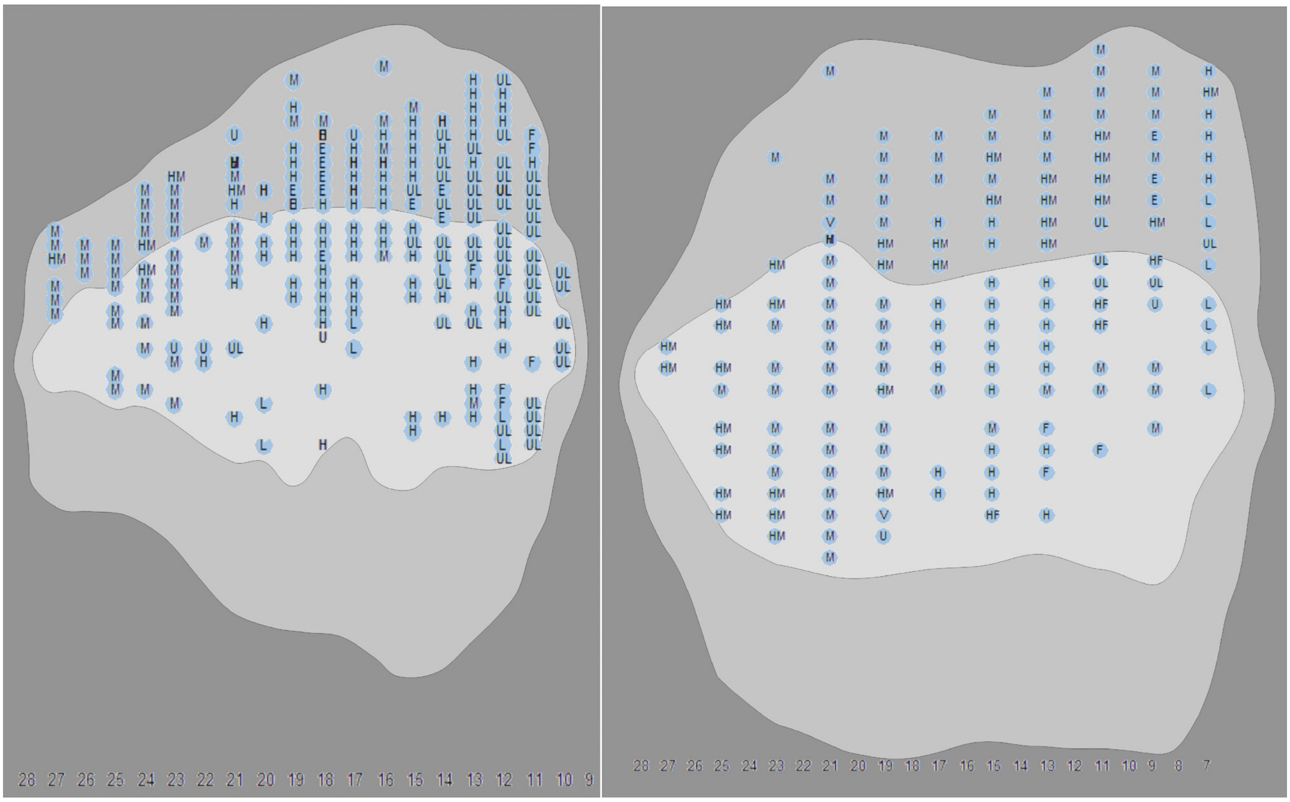


Figure B5: Drawing illustrating the map of the Somatotopic organization shown on an unfolding of the stimulated preisylvian regions of the left hemisphere of the macaque monkeys MK1 (left) and MK2 (right). In the lower line are indicated the antero-posterior stereotaxic coordinates. The upper and lower bank are in dark grey. The light grey indicates the insula. letters indicate the effectors involved in the ST-ICMS responses. E: eye; F: foot; H: hand; L: lower limb; M: mouth; T: tongue; U: upper limb; and V: visceral sites.

b- Train threshold distribution

The analysis of the temporal thresholds of the ICMSs evoking sensory-motor responses showed a decrease of the minimum temporal train along the rostro-caudal axis with a clear distinction between two complexes: the mid-anterior, and mid-posterior complex (Figure B5). The anterior complex where mouth responses prevailed showed threshold train varying from 0.1 msec to 1 sec, while in the middle complex, where hand responses prevailed, and the posterior complex, where upper and lower extremities prevailed, showed a decreasing of the threshold up to 50msec.

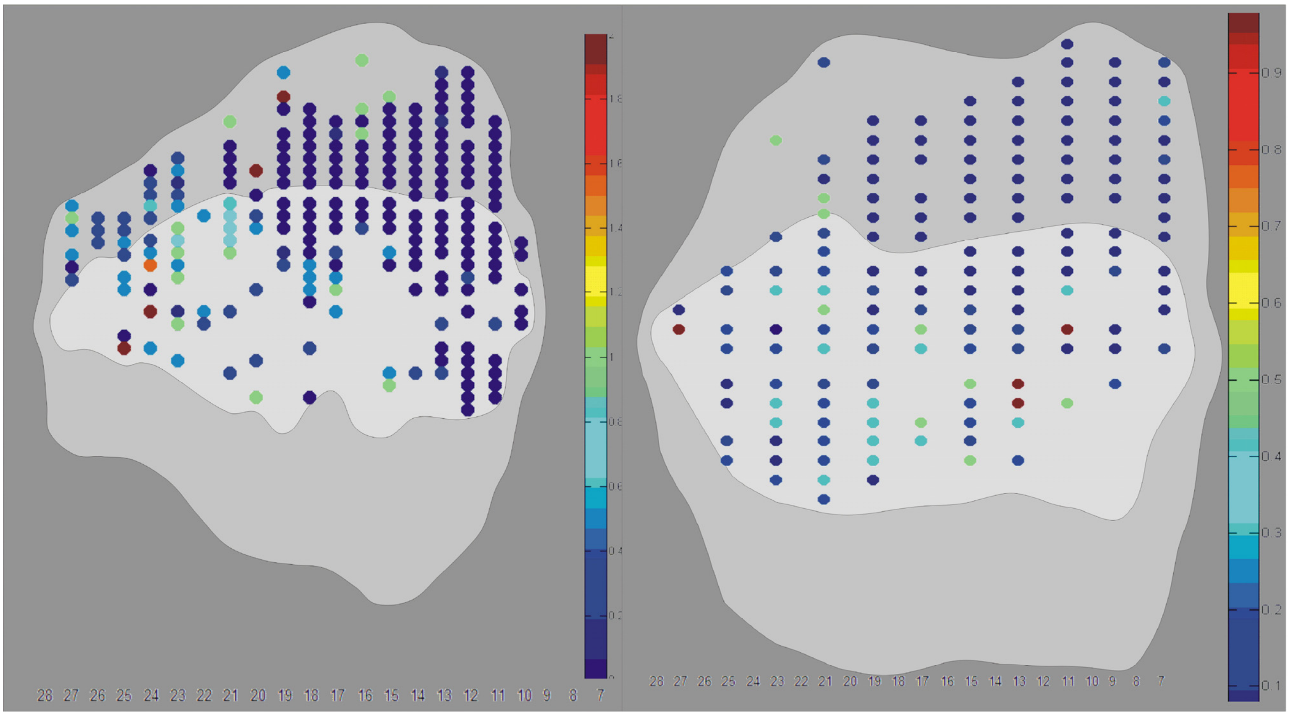


Figure B5: Distribution of the temporal threshold shown on an unfolding of the perisylvian regions of the left hemisphere of the macaque monkeys MK1 (left) and MK2 (right). The antero-posterior stereotaxic coordinates are shown in the lower part of the figure. The color scale beside each map indicates the duration of the ICMSs thresholds represented in the colored filled circles. On the unfolding, light grey indicates the insula territory and dark grey indicates upper and lower banks of the Sylvian fissure.

2. Behavioral time scale outcomes

Intracortical microstimulations (ICMSs) were applied in 4171 sites (2999 in MK1; 1772 in MK2) of the inner perisylvian region. Behavioural responses were evoked from 74.8% of sites (74.4% & 75.5%). The whole area was subdivided in four regions of interest (ROIs): upper bank of the Sylvian fissure, lower bank of the Sylvian fissure, dorsal insula, and ventral insula. Behavioral outcome (Figure B11) was classified in five macro-categories: oro-alimentary responses, complex behaviors, simple sensory-motor responses, communicative responses and miscellaneous responses (Figure B6).

a- Oro-Alimentary responses (OM)

Oro-alimentary responses (Figure B6; Figure B7; Figure B12) ranged from feeding behaviors to viscero-motor responses, and represented the 17.3% (13.3%; 24.2%) of the stimulated sites. The totality of these responses were evoked by ICMS of the upper bank (34% in both monkeys; 42% in MK1; 26% in MK2) and insula (66%; 58% and 74% in both monkeys, MK1 and MK2 respectively). These responses were evoked mainly in the rostral sectors (Figure B-12). Interestingly, such responses were totally absent in the lower bank of the Sylvian fissure.

The most represented oro-alimentary response consisted of a natural feeding behavior, constituted by chewing, sucking and mouthing movements, followed by swallowing. This response was mainly represented in the upper bank and in the dorsal insula (79.2%; 95.7% and 65.4% in monkeys MK1, and MK2 respectively), and constituted the 62.2% (58.8% in MK1; 65.3% in MK2) of the oro-alimentary responses; in the great majority of cases, ICMS evoked the three movements together. While chewing is most of the time strongly similar to a natural spontaneous behavior, in some cases the chewing rhythm looks more artificial, that is, a more slow and monotone mandibles movements than in the natural rhythm. In these cases, when ICMS starts during natural chewing, a shift from the natural rhythm to ICMS induced rhythm is observed.

In the most rostral part (Figure B12), 23.7% (20.1%; 27%) of the oroalimentary responses consisted of evoked feeding behaviors accompanied by a sudden inhibition of every ongoing arm movement spontaneously started before the stimulation. This response was evoked mostly in the upper bank and the dorsal insula (82.1%; 95% and 73.3% in both monkeys, MK1 and Mk2 respectively).

Stimulations produced an alteration of feeding behavior (Figure B12) in 3.7% (1.8%; 5.6%) of sites evoking oro-alimentary behavior. The most typical behavioral alteration was general refusal of food intake. This response was always tested in two experimental contexts; stimulations were performed in two phases of the feeding behavior, that is, during the bringing to the mouth or when the food was already inside the mouth (chewing). In the former, stimulation induced the monkey to refuse the food by throwing it away, while in the latter the animal spat it. A normal food intake behavior was resumed in all trials following ICMS. This suggests a lack of any conditional association. Other kinds of alteration of the alimentary behavior consisted of peeling fruit with teeth before eating it, something that we never observed in trials prior to or after ICMS.

Beyond refusal of food intake, stimulation of the anterior ventral insula (Figure B12) evoked other disgust-related behaviors such as grimace of disgust. This response represented 6.4% of the oro-alimentary responses, while in 4% of the oro-alimentary responses, ICMS of the anterior region evoked vomiting impulses. These two latter responses were more consistent in the first monkey than in the second one since the disgust grimace was evoked in 7.5 % of the oro-alimentary responses in the first monkey against the 0.7% in the second monkey and the vomiting impulses were evoked in 11.8% of the total oro-alimentary responses in the first monkey against the 1.4% of the second monkey. Although the inter-subject differences, the two responses were clearly identified and documented in same rostral sector in the two monkeys. In the first monkey, these two responses were predominantly presented in the in the ventral insula (95.7% and 93.3% for the disgut grimace and the vomiting impulses, respectively).

b- Complex behaviors

Complex behaviors involving upper limbs (Figure B6; Figure B8; Figure b13) were evoked from the central part of the dorsal complex (Figure B13) in 6.2% (3.8% in MK1; 10.3% in MK2) of stimulation sites. The vast majority of these responses were evoked in the upper bank and dorsal insula (92.2%; 100% and 89% in monkeys MK1 and MK2, respectively). While such responses were observed in the ventral insula on one of two monkeys (11% in MK2), they were completely absent in the lower bank in both monkeys. Complex behaviors evoked by stimulation included (Figure B8; Figure B13): (a) grasping movements; (b) bringing to the mouth followed by feeding behavior; (c) taking away from the mouth; (d) movements toward body parts; (e) visual exploration of the hand.

Grasping movements represented 13.1% (22.8% in MK1; 7.1% in MK2) of the stimulated sites. They were performed by closing the fingers and rotating the wrist, as if the hand was grasping something. 92.3% of these responses (88.5% and 100% in MK1 and MK2 respectively) were evoked by stimulation of the upper bank, predominantly from the caudal portion where complex behaviors were also evoked (Figure B13). While in the more caudal sites the evoked response was limited to grasping movements, in the more rostral sites (Figure B13) ICMS evoked a more complex response, that is, a whole bringing to the mouth movement followed by oro-alimentary movements. This response represented the 19.2% (25.4% in MK1; 15.3% in MK2) of the evoked complex behaviors. Like in the case of evoked grasping movements, this response was predominantly evoked by ICMSs performed in the upper bank (96.5%; 93.1% and 100% in both monkeys, MK1 and MK2 respectively). Both grasping movements and grasping, bringing to the mouth and feeding behaviour responses were absent in both ventral insula and lower bank.

In the second monkey, it has also been observed a response during which the monkey repetitively attempted to take away something from the mouth in a compulsive way, even if nothing was present in the mouth. This response represented the 10.4% of the evoked complex behaviors and was mainly evoked in the upper bank (63.2%) and with lesser amount in the dorsal insula (26.3%) and the ventral insula (10.5%).

In 28.3% (30.7% in MK1; 26.8% in MK2) of the total complex responses, Movements toward body parts, like the chest or the face without any further consequences could be evoked by ICMSs. In the first monkey, this response was evoked almost exclusively in the upper bank (97%) while in the second monkey the same response was observed approximately equally in the upper bank and dorsal insula with respectively the 53.1% and 46.9%. furthermore this response was predominantly presented in the medio-caudal sector of the upper bank (Figure B13).

In 33% (21.1% and 40.4% in MK1 and MK2 respectively), the ICMSs produced complex exploratory behavior of the hand. After ICMS starts the monkey repetitively raised the hand in front of his face and observed it. Since the movement was executed in a real natural way it was not possible to disambiguate between a behavior evoked directly by the stimulation or, otherwise, as being the consequence of a perceived sensation on the hand evoked by ICMS. While in the first monkey, this responses was almost equally distributed between the upper bank and the dorsal insula with respectively the 54.2% and the 45.8% of the responses, in the second monkey this behavior remaining most presented in the upper bank and dorsal insula (74.3%) was also observed in the ventral insula in the 25.7% of the cases. Moreover, this response was mostly evoked in a central sector of the dorsal bank (Figure B13).

c- Communicative responses

Communicative responses (Figure B6; Figure B14) were found in 3.3% (2.8% and 4.1% in MK1 and MK2 respectively) of the whole stimulated sites. This response was mainly evoked after stimulation of the middle part of the most ventral region of the insula (77.7%;69.4% and 87.5% in both monkeys, MK1 and MK2 respectively), at the border with the lower bank of the Sylvian fissure. The evoked behavior is “lip-smacking”, that is an affiliative facial expression consisting in a fast opening and closing of the lips. In some stimulation lip smacking was alternated with the so-called “silent bared teeth”, that is an exposition of the teeth usually employed to show the pacific intention of the exhibitor. Following primatologists, the alternation of lip-smacking and silent bared teeth in the rhesus monkey constitutes a particular facial expression called “teeth chattering”, usually attributed to subordinates toward dominant members of the group. Once The teeth chattering was evoked by the ICMS, the response was studied in different experimental contexts; when stimulation started while the monkey is exhibiting spontaneous emotional expressions, ICMS induced the monkey to interrupt the spontaneous behavior and replaced it with the communicative behavior; more interestingly, the original behavior was resumed again right after the end of the stimulation.

The social aspect of the evoked behavior was stressed by the fact that the response was evocable only when ICMS occurred during eye contact with the experimenter, while the simulation was totally ineffective when nobody was facing the monkey. This was particularly interesting since it suggested that the way the stimulation acts could be a decreasing of the threshold of a natural emotional response, elicited by simple eye contact.

d- Simple sensory-motor responses

Simple sensory-motor responses (Figure B6; Figure B9; Figure B15) could be evoked by ICMS of the upper bank of the Sylvian fissure and insula in 21.5% (21.8% and 20.8% in MK1 and MK2, respectively) of the stimulated sites. The vast majority of this macro-category responses was evoked from the dorsal bank and the dorsal insula (94.4%; 93% and 97% in both monkeys, MK1 and MK2 respectively). The remained responses were obtained by the stimulation of the ventral insula while these responses lacked completely in the ventral bank. The Simple sensory-motor responses involved: (a) mouth; (b) hand; (c) upper limb; (d) lower limb; (e) upper and lower limbs; (f) eyes.

Responses involving the mouth represented the 7.5% (4.6% and 12.7% in MK1 and MK2 respectively) of the simple sensory-motor responses; these responses were predominantly found in the upper bank (93.5%; 90% and 95.7% in both monkeys, MK1 and MK2 respectively) mainly from the most outer part of the upper bank of the investigated region of the (Figure B15). These responses could not be classified as feeding behavior since no oro-alimentary movement was observed, but simple responses of opening or closing of the mouth lasting all the duration of the stimulation.

Responses involving the hand represented 45% (54.5% in MK1 and 29.3% in MK2) of the simple sensory-motor responses; these responses were evoked from a wide region of the upper bank and dorsal insula (Figure B15). The most typical responses evoked by ICMS in this region are simple flexion or extension of fingers and wrist of the controlateral hand or loss of muscular tone (atonia).

Responses involving the upper limb were evoked in 10.2% (2.3% and 24.1% in MK1 and MK2 respectively) of the simple sensory-motor responses; these responses were evoked from the mid caudal part of the studied regions (Figure B15) The most typical upper limb movements evoked by ICMS are simple abduction or adduction of the controlateral arm.

Responses involving the lower limb represented movements are mainly represented in the caudal part of the insula and evoked in the 6.7% (5.2% and 9.5% in MK1 and MK2 respectively) of the sites evoking simple movements. This response typically consisted of simple abduction or adduction of the controlateral hidlimb.

Simple combined movements of upper and lower limbs were evoked in 17.8% (21.4% and 11.4% in MK1 and MK2 respectively) mainly in the caudal dorsal complex (Figure B15). They consisted of simultaneous flexion or extension (abduction or adduction) of the joints of the controlateral upper and lower limbs.

Stimulation evoking eye-blinking represented 12.4% (12.1% and 13.0% in MK1 and MK2 respectively) of the simple sensory-motor responses. These responses have been mainly found after stimulation of the middle sector of the upper bank (Figure B15). Eye blinking lasted during all stimulation time; generally, eye blinking was accompanied by upper limb movements or by upper and lower limb movements.

e- Miscellaneous responses

Many stimulations of the whole perisylvian region evoked behaviors that are uneasy to understand since the overt evoked behavior was most likely due to particular reactions to the effect of the stimulation, and not to the stimulation itself. However, since responses included in this class showed a strong repeatability, they were presented in the map as miscellaneous responses.

Miscellaneous responses (Figure B6; Figure B10; Figure B16) were found in the 26.5% (32.7% and 16% in MK1 and MK2 respectively) of the stimulations, and they were mainly found in the ventral insula (41%; 44% and 29% in both monkeys, MK1 and MK2 respectively) and lower bank (32%; 29% and 41% in both monkeys, MK1 and MK2 respectively).

It is worth to note that the vast majority (73%; 73% and 70% in both monkeys, MK1 and MK2 respectively) of miscellaneous responses are evoked by stimulations of the ventral complex; this data is interesting since it suggests that the functional domain in which these regions could be involved are distant from the sensory-motor functional domain.

Tremors of the limbs or of the whole body are mainly observed during stimulation of the posterior region (Figure B16) in , both in 38.4% (40.7% and 30.3% in MK1 and MK2, respectively). This response was mainly evoked from the upper bank (31.1%; 29.8% and 37.2% in both monkeys, MK1 and MK2 respectively) and in the dorsal insula (36.1%; 31.1% and 62.8% in both monkeys, MK1 and MK2 respectively). These responses did not seem to show a somatotopical arrangement, and the position of the limbs at the beginning of the stimulation did not affect the result; moreover, the effect of the stimulation did not last beyond stimulation time, and the monkey did not produce any facial expression suggesting pain or discomfort.

Stimulations evoking discomfort reactions were found in different portion of the stimulated region in 7.8% (5.5% and 15.8% in MK1 and MK2 respectively) of the miscellaneous responses. These reactions, involving mostly the hand or foot singularly or simultaneously, gave the impression of being a reaction to discomforting sensations due to the electrical stimulations. These reactions were almost exclusively evoked from the ventral complex (96%; 92.6% and 100% in both monkeys, MK1 and MK2 respectively)

In 9.3% (9.3% IN MK1 and 9.2% in MK2) of the miscellaneous responses ICMSs evoked leap up of the trunk followed by general psychomotor agitation. This response was exclusively observed in the ventral complex and completely absent in the dorsal one (Figure B16; Figure B10-left panel).

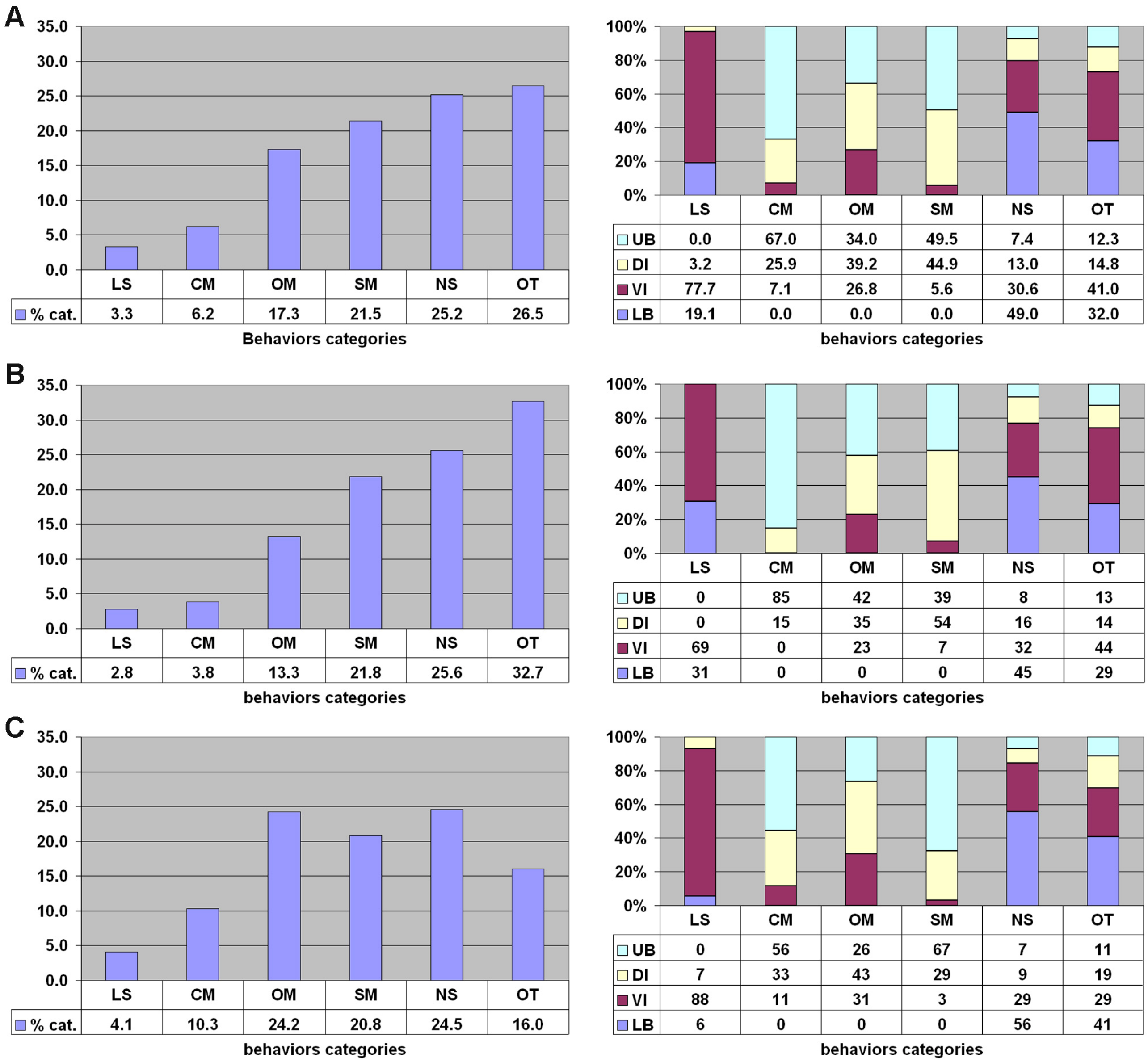
In 4.9% (5.7% and 2.1% in MK1 and MK2 respectively) of the miscellaneous responses the monkeys manifested discomfort grimaces followed by psychomotor agitation. The discomfort reaction was mainly evoked from the lower bank (66.1%; 62.5% and 100% in both monkeys, MK1 and MK2 respectively).

In 7.5% (3.8% and 20.4% in MK1 and MK2 respectively) of the miscellaneous responses ICMSs evoked controlateral displacement of the trunk and eyes. This response was totally restricted to the lower bank (Figure B16; Figure B10-left panel).

ICMSs of the ventral complex evoked postural adjustment movements (25%; 26.8% and 20.1% in both monkeys, MK1 and MK2 respectively). This response was observed only in the ventral insula (Figure B16; Figure B10-left panel) or simple twitch of the trunk without any subsequent reaction (6.8%; 8.2% and 2.11% in both monkeys, MK1 and MK2 respectively). Also in this case the response was observed only in the ventral complex (Figure B16; Figure B10-left panel).

d- Unresponsive sites

The stimulation of 25.2% (25.6% and 24.5% in MK1 and MK2 respectively) of the sites resulted unsuccessful to evoke any detectable behavioral response. Unresponsive sites were found in the whole stimulated regions but the vast majority of them were concentrated in the lower bank (49%; 45% and 56% in monkeys MK1 and MK2, respectively) and in the ventral insula (30.6%; 32% and 29% in monkeys MK1 and MK2, respectively). In these two regions unresponsive sites did not show any segregation, since they were distributed along all of the stimulated region (Figure B17).



A

	LS	CM	OM	SM	NS	OT
% cat.	3.3	6.2	17.3	21.5	25.2	26.5

	LS	CM	OM	SM	NS	OT
UB	0.0	67.0	34.0	49.5	7.4	12.3
DI	3.2	25.9	39.2	44.9	13.0	14.8
VI	77.7	7.1	26.8	5.6	30.6	41.0
LB	19.1	0.0	0.0	0.0	49.0	32.0

B

	LS	CM	OM	SM	NS	OT
% cat.	2.8	3.8	13.3	21.8	25.6	32.7

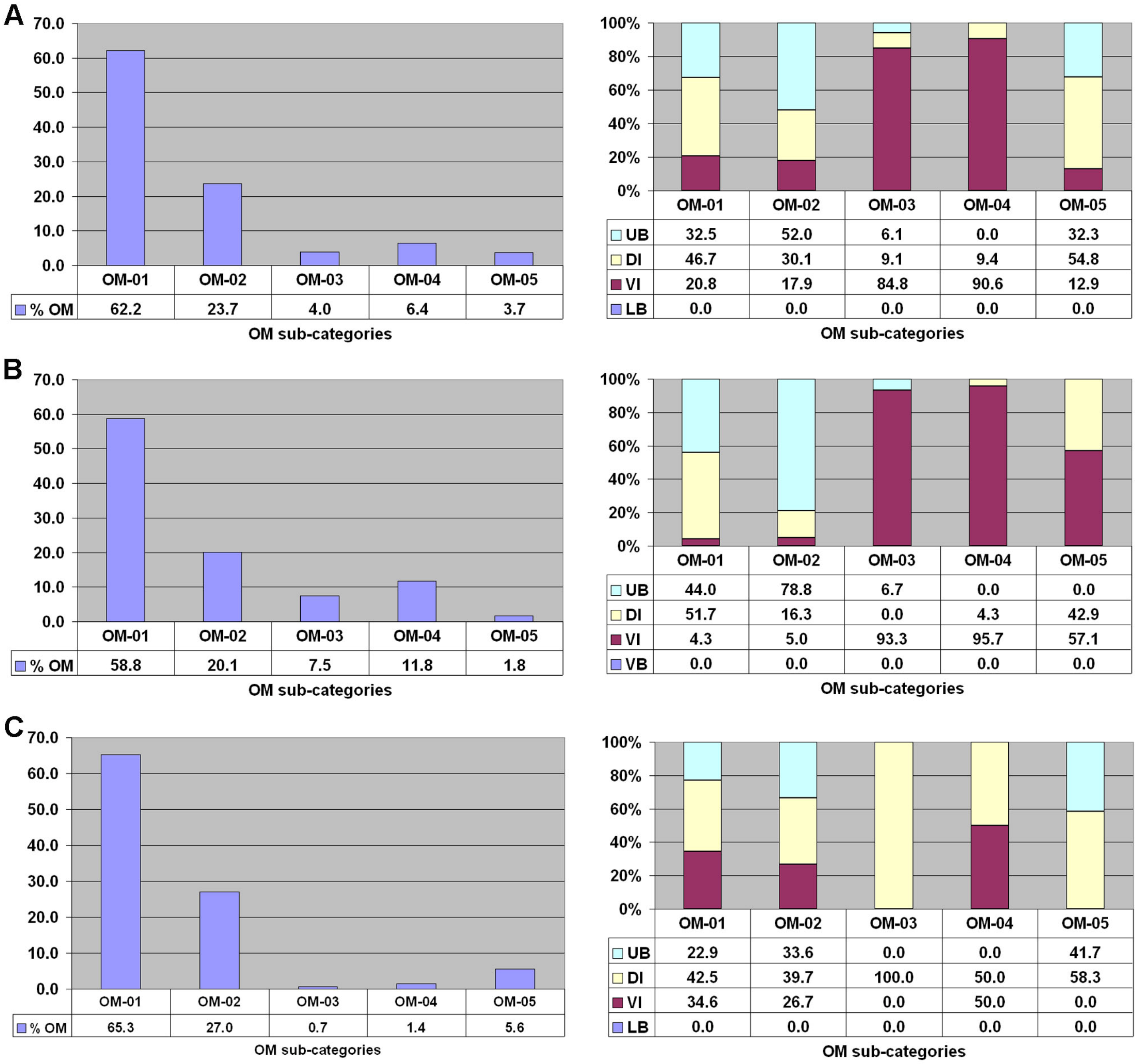
	LS	CM	OM	SM	NS	OT
UB	0	85	42	39	8	13
DI	0	15	35	54	16	14
VI	69	0	23	7	32	44
LB	31	0	0	0	45	29

C

	LS	CM	OM	SM	NS	OT
% cat.	4.1	10.3	24.2	20.8	24.5	16.0

	LS	CM	OM	SM	NS	OT
UB	0	56	26	67	7	11
DI	7	33	43	29	9	19
VI	88	11	31	3	29	29
LB	6	0	0	0	56	41

Figure B6: Percentage of the macro-categories on the total of the stimulated sites (left panels) and distribution of each macro-category responses within the four ROIs (right panels) in (A) both monkeys, (B) MK1 and (C) MK2. LS: lip smacking; CM: Complex movements; OM: oro-alimentary movements; SM: simple sensory-motor movements; OT: other (miscellaneous) responses; NS: unresponsive sites; UB: upper bank; DI: dorsal insula; VI: ventral insula and LB: lower bank.



A

	OM-01	OM-02	OM-03	OM-04	OM-05
% OM	62.2	23.7	4.0	6.4	3.7

	OM-01	OM-02	OM-03	OM-04	OM-05
UB	32.5	52.0	6.1	0.0	32.3
DI	46.7	30.1	9.1	9.4	54.8
VI	20.8	17.9	84.8	90.6	12.9
LB	0.0	0.0	0.0	0.0	0.0

B

	OM-01	OM-02	OM-03	OM-04	OM-05
% OM	58.8	20.1	7.5	11.8	1.8

	OM-01	OM-02	OM-03	OM-04	OM-05
UB	44.0	78.8	6.7	0.0	0.0
DI	51.7	16.3	0.0	4.3	42.9
VI	4.3	5.0	93.3	95.7	57.1
VB	0.0	0.0	0.0	0.0	0.0

C

	OM-01	OM-02	OM-03	OM-04	OM-05
% OM	65.3	27.0	0.7	1.4	5.6

	OM-01	OM-02	OM-03	OM-04	OM-05
UB	22.9	33.6	0.0	0.0	41.7
DI	42.5	39.7	100.0	50.0	58.3
VI	34.6	26.7	0.0	50.0	0.0
LB	0.0	0.0	0.0	0.0	0.0

Figure B7: Percentage of the oro-alimentary sub-categories responses, calculated on the total of the oro-alimentary responses (left Panel) and distribution of each sub-category responses within the four ROIs (left panel) in (A) both monkeys, (B) MK1 and (C) MK2. OM-01: feeding behaviors; OM-02: feeding behaviors + inhibition of arm movements; OM-03: vomiting impulses; OM-04: grimace of disgust; OM-05: alteration of the feeding behavior. Other abbreviations as in Figure B6.

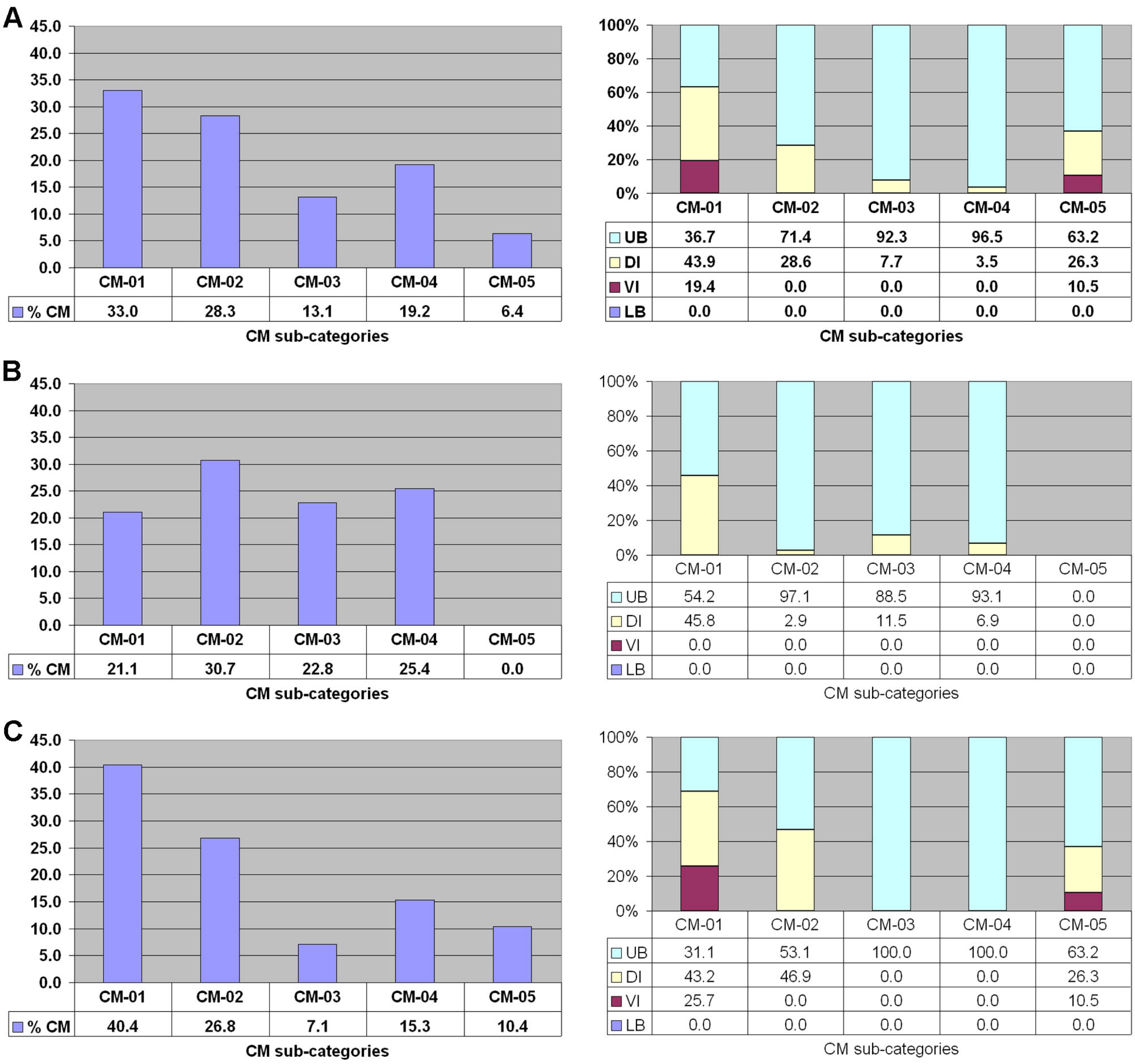
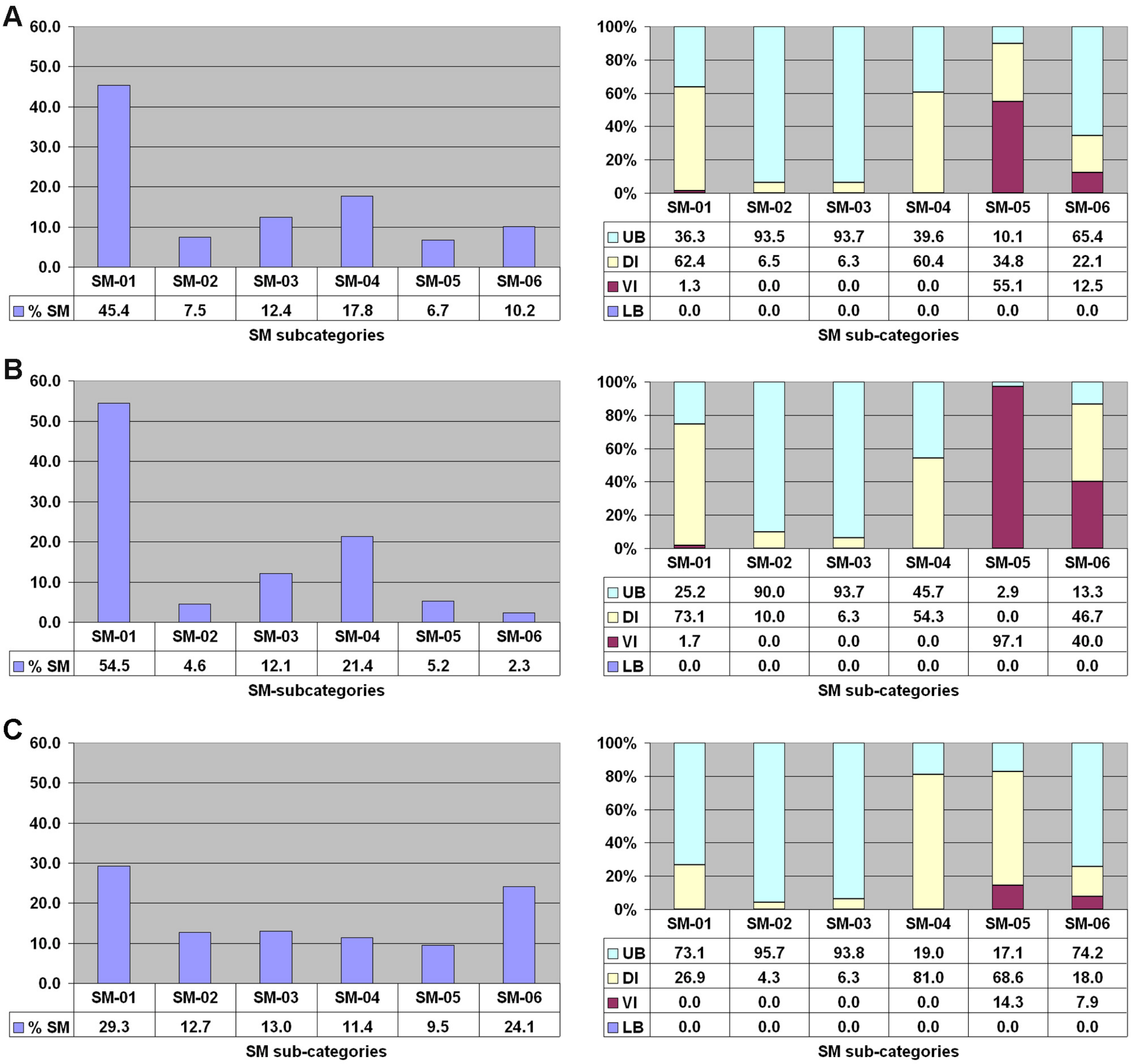


Figure B8: Percentage of the complex behaviors sub-categories responses calculated on the total of the complex behaviors responses (left Panel) and distribution of each sub-category responses within the four ROIs (left panel) in (A) both monkeys, (B) MK1 and (C) MK2. CM-01: the monkey visually explore the hand; CM-02: the monkey makes movements toward body parts; CM-03: grasping movements; CM-04: the monkey makes a grasping and bringing to the mouth followed by chewing and swallowing movements; CM-05: the monkey repetitively take away form the mouth. Other abbreviations as in Figure B6.



A

	SM-01	SM-02	SM-03	SM-04	SM-05	SM-06
% SM	45.4	7.5	12.4	17.8	6.7	10.2

	SM-01	SM-02	SM-03	SM-04	SM-05	SM-06
UB	36.3	93.5	93.7	39.6	10.1	65.4
DI	62.4	6.5	6.3	60.4	34.8	22.1
VI	1.3	0.0	0.0	0.0	55.1	12.5
LB	0.0	0.0	0.0	0.0	0.0	0.0

B

	SM-01	SM-02	SM-03	SM-04	SM-05	SM-06
% SM	54.5	4.6	12.1	21.4	5.2	2.3

	SM-01	SM-02	SM-03	SM-04	SM-05	SM-06
UB	25.2	90.0	93.7	45.7	2.9	13.3
DI	73.1	10.0	6.3	54.3	0.0	46.7
VI	1.7	0.0	0.0	0.0	97.1	40.0
LB	0.0	0.0	0.0	0.0	0.0	0.0

C

	SM-01	SM-02	SM-03	SM-04	SM-05	SM-06
% SM	29.3	12.7	13.0	11.4	9.5	24.1

	SM-01	SM-02	SM-03	SM-04	SM-05	SM-06
UB	73.1	95.7	93.8	19.0	17.1	74.2
DI	26.9	4.3	6.3	81.0	68.6	18.0
VI	0.0	0.0	0.0	0.0	14.3	7.9
LB	0.0	0.0	0.0	0.0	0.0	0.0

Figure B9: Percentage of the simple sensory-motor movements sub-categories responses calculated on the sensory-motor movements responses (left Panel) and distribution of each sub-category responses within the four ROIs (left panel) in (A) both monkeys, (B) MK1 and (C) MK2. SM-01: hand simple movements; SM-02: mouth simple movements; SM-03: eye blinking; SM-04: upper and lower limbs simple movements; SM-05: lower limb simple movements; SM-06: upper limb simple movements. Other abbreviations as in Figure B6.

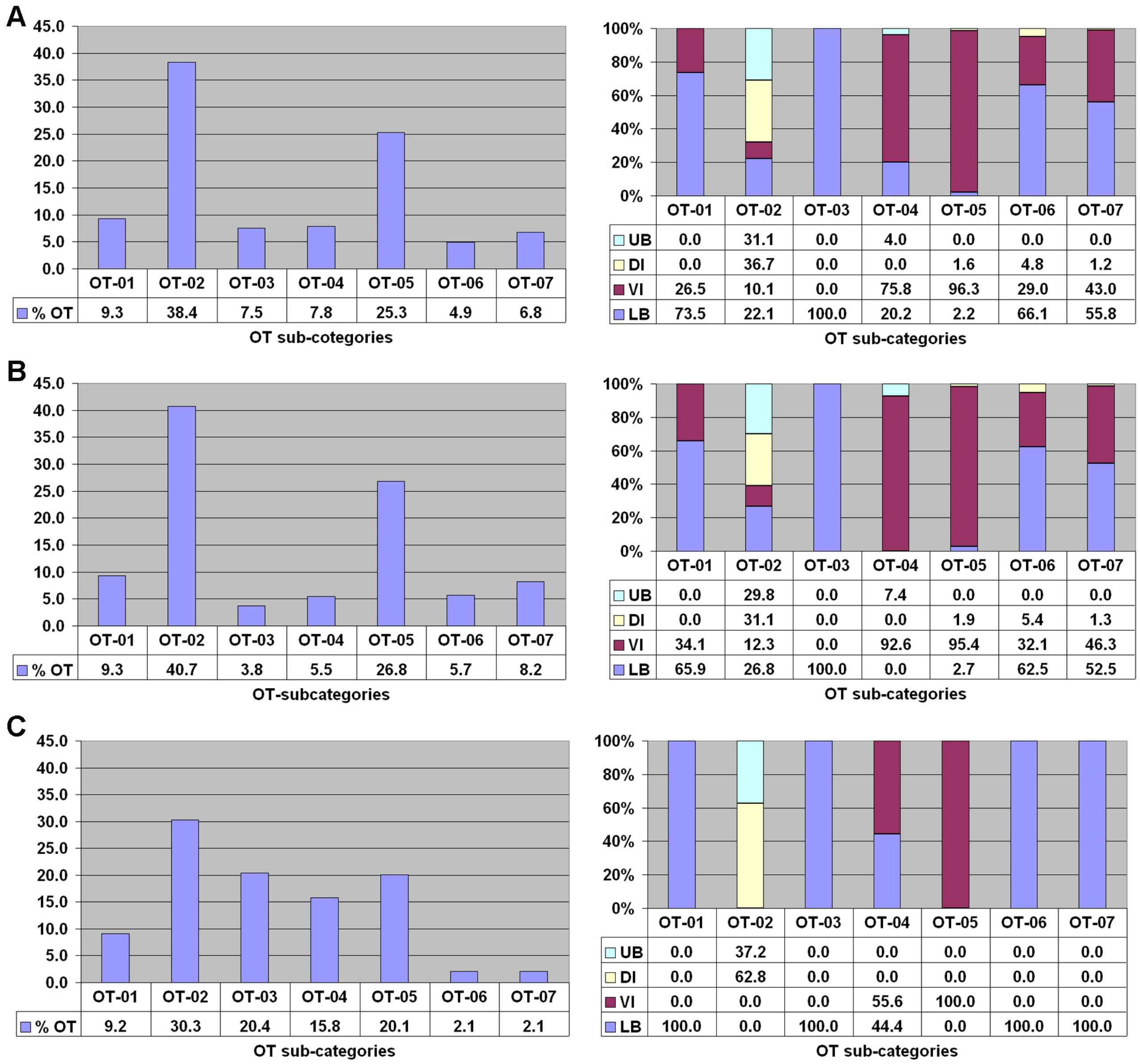


Figure B10: Percentage of the other (miscellaneous) sub-categories responses calculated on the total of the other (miscellaneous) macro-category responses (left Panel) and distribution of each sub-category responses within the four ROIs (left panel) in (A) both monkeys, (B) MK1 and (C) MK2. OT-01: twitch followed by psychomotor agitation; OT-02: general tremors; OT-03: controlateral displacement of trunk and eyes; OT-04: discomfort reaction; OT-05: postural adjustment; OT-06: discomforting grimace followed by general agitation; OT-07: twitch of the trunk. Other abbreviations as in Figure B6.

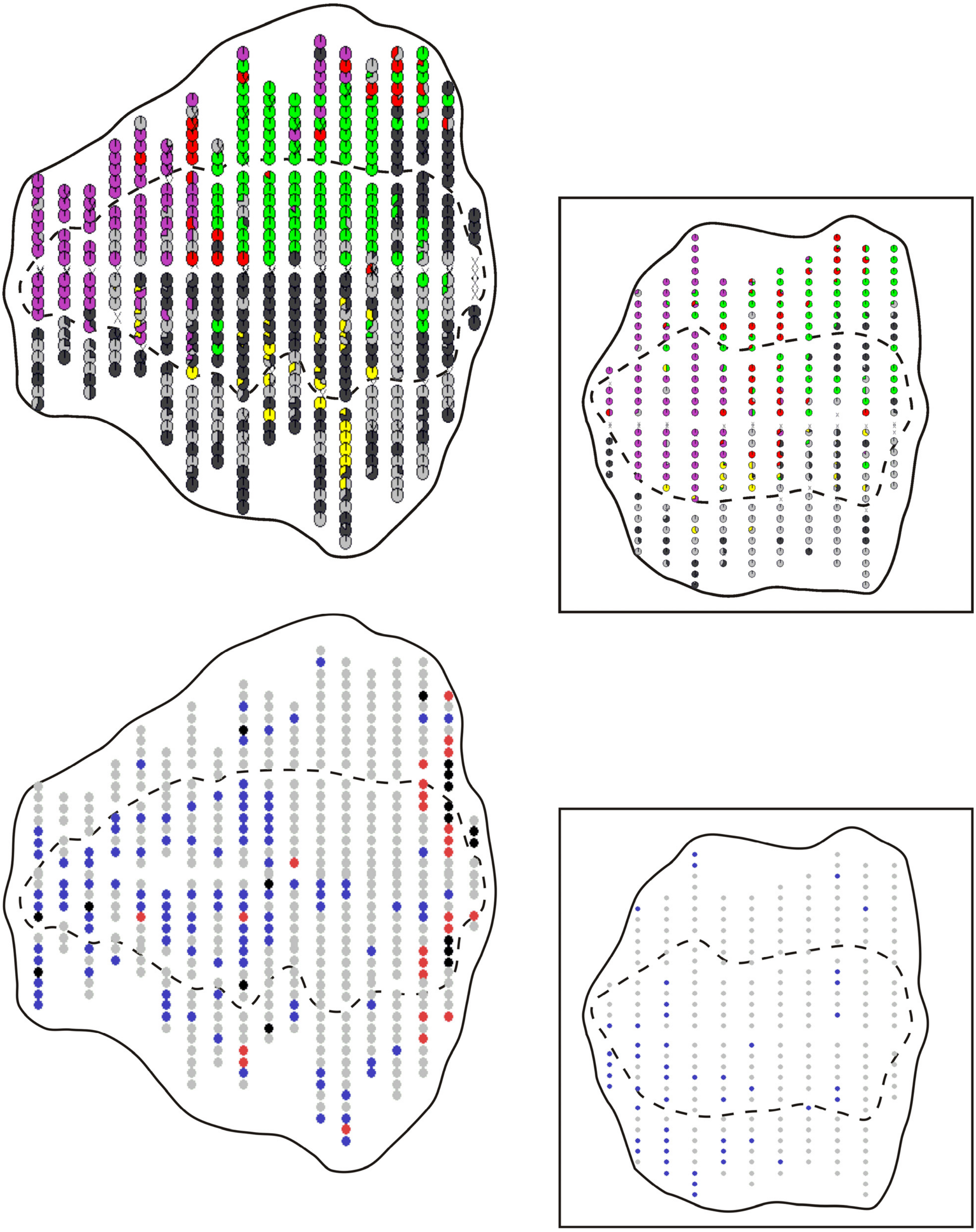


Figure B11: Distribution of behavioral macro-categories (upper row) and cardiac responses (lower row) shown on an unfolding of the perisylvian regions of the left hemisphere of macaque monkey MK1 (left) and MK2 (right). The dashed line indicates the limits of the insula. Each circle indicates the position of one penetration. Colors indicate the type of responses evoked by ICMS. Behavioral map. Purple: oro-alimentary responses; red: complex behaviors; yellow: lip-smacking; green: simple movements; dark grey: miscellaneous responses; light grey: non responsive sites. Cardiac responses map. blue: bradycardia; red: tachycardia; black: biphasic; light grey: non responsive sites.

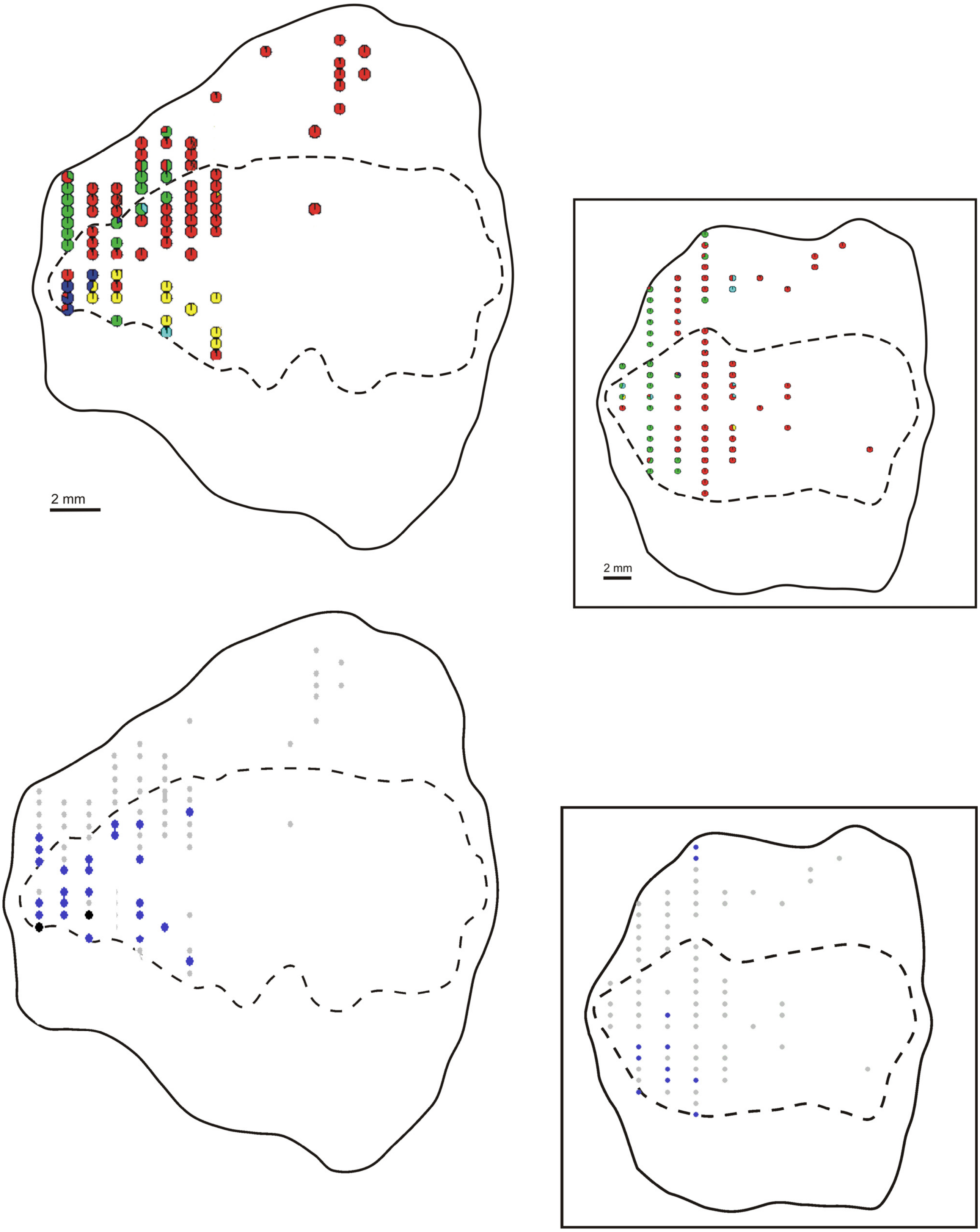


Figure B12: Distribution of oro-alimentary sub-categories (upper row) and cardiac responses evoked in the same sites (lower row) shown on an unfolding of the perisylvian regions of the left hemisphere of macaque monkey MK1 (left) and MK2 (right). Behavioral map. Red: feeding behaviors; green: feeding behaviors + inhibition of arm movements; dark blue: vomiting impulses; yellow: grimace of disgust; light blue: alteration of the feeding behavior. Other conventions as in Figure B11.

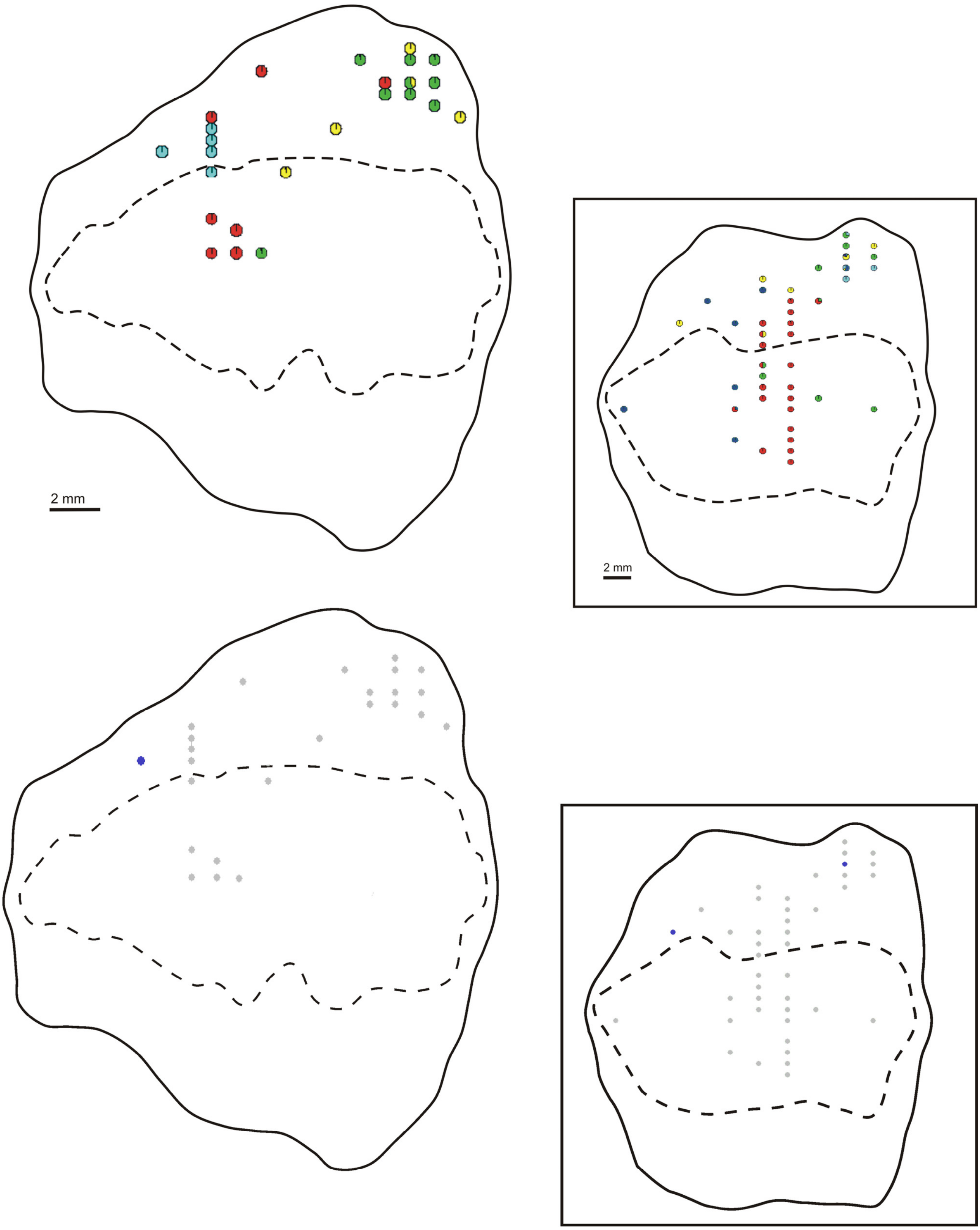


Figure B13: Distribution of complex behaviors sub-categories (upper row) and cardiac responses evoked in the same sites (lower row) shown on an unfolding of the perisylvian regions of the left hemisphere of macaque monkey MK1 (left) and MK2 (right). Behavioral map. Red: visual exploration of the hand; green: movements toward body parts; dark blue: grasping movements; yellow: movements toward the mouth followed by feeding behavior; light blue: movements toward the mouth followed by taking away from the mouth. Other conventions as in Figure B11.

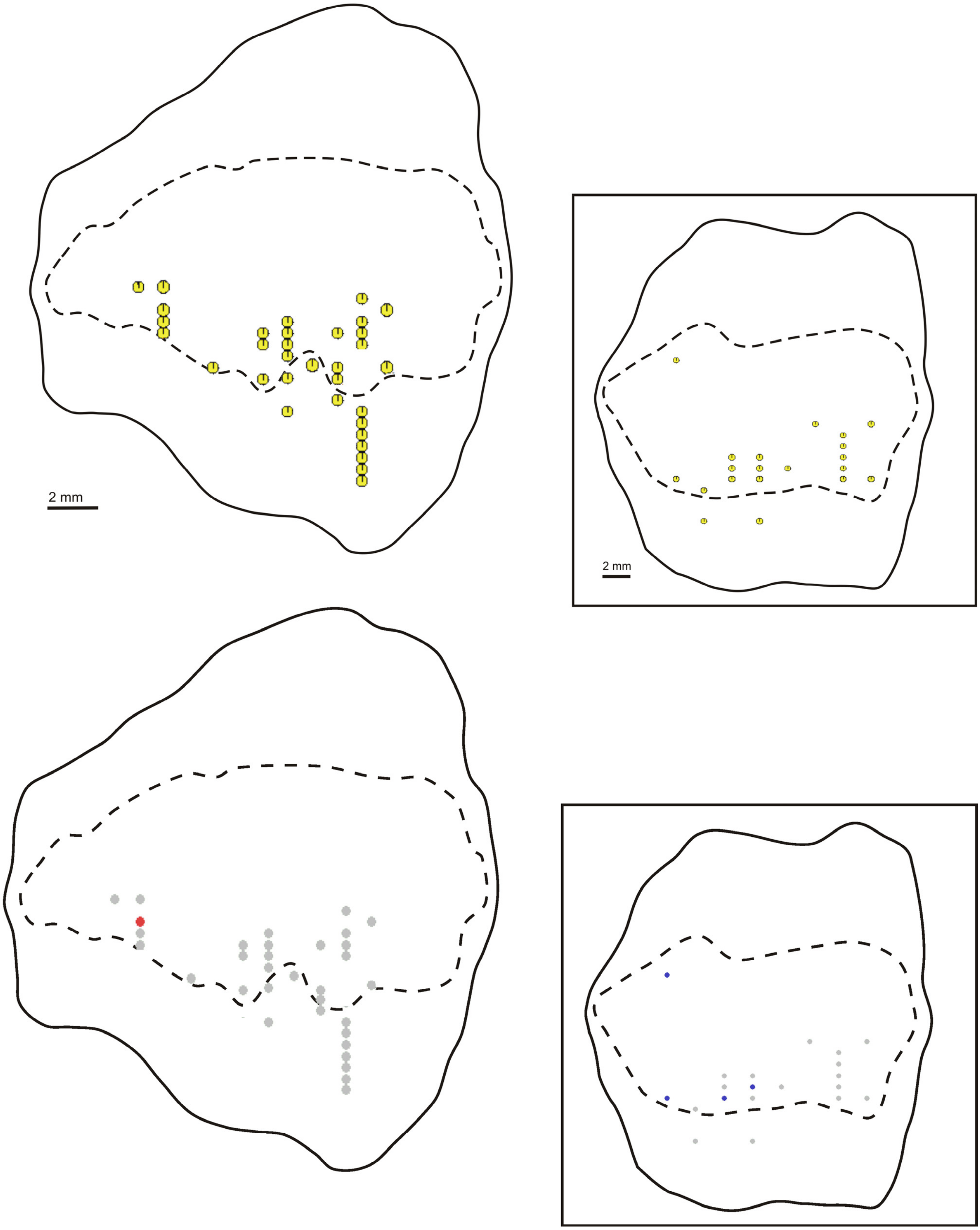


Figure B14: Distribution of communicative responses (upper row) and cardiac responses evoked in the same sites (lower row) shown on an unfolding of the perisylvian regions of the left hemisphere of macaque monkey MK1 (left) and MK2 (right). Conventions as in Figure B11.

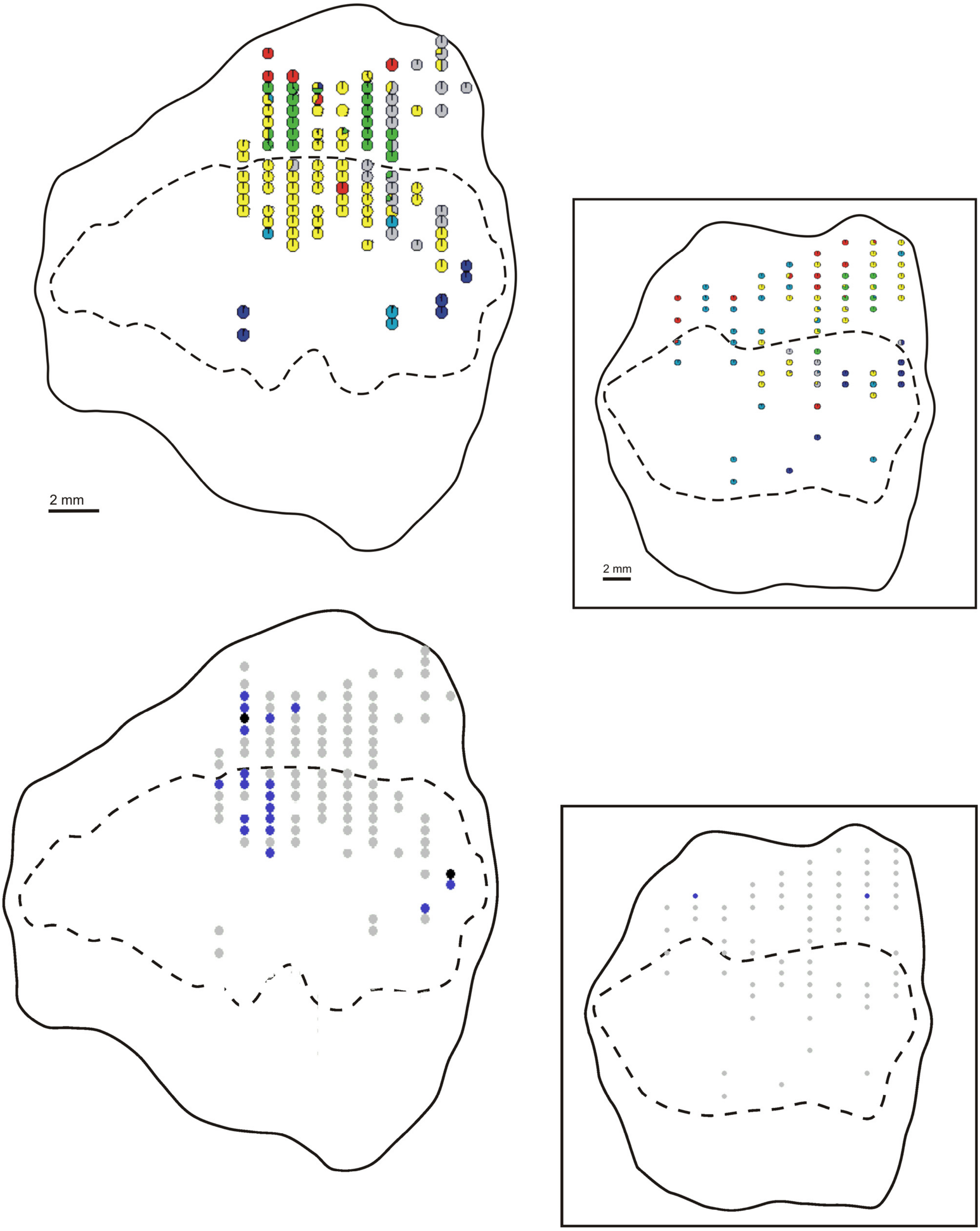


Figure B15: Distribution of simple movements sub-categories (upper row) and cardiac responses evoked in the same sites (lower row) shown on an unfolding of the perisylvian regions of the left hemisphere of macaque monkey MK1 (left) and MK2 (right). Behavioral map. Red: mouth movements; green: eye blinking; dark blue: lower limb movements; yellow: hand movements; light blue: upper limb movements; grey: upper and lower limbs movements. Other conventions as in Figure B11.

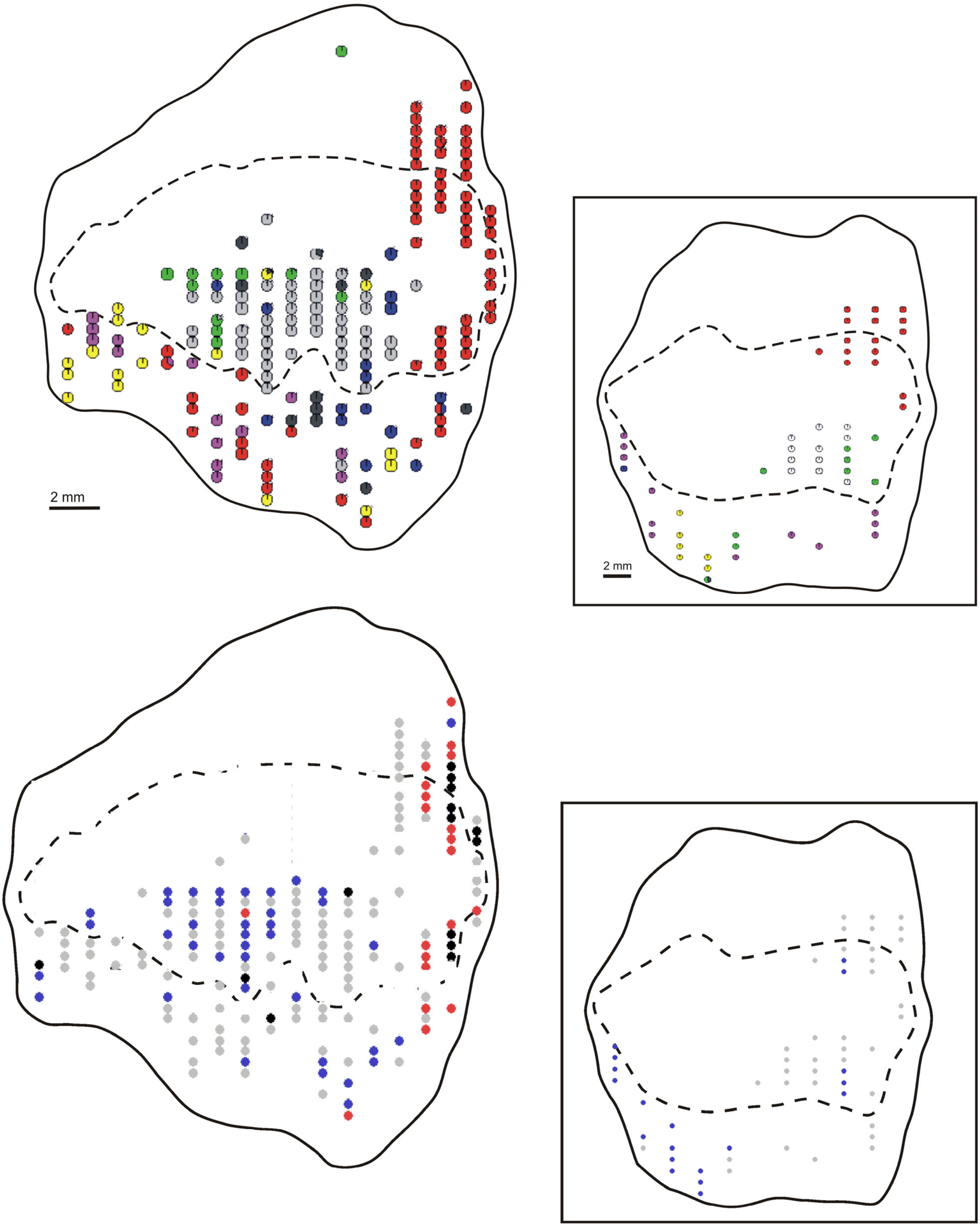


Figure B16: Distribution of the other (miscellaneous) sub-categories (upper row) and cardiac responses evoked in the same sites (lower row) shown on an unfolding of the perisylvian regions of the left hemisphere of macaque monkey MK1 (left) and MK2 (right). Behavioral map. Red: general tremors; green: discomfort reaction; dark blue: twitch of the trunk; yellow: twitch followed by psychomotor agitation; light grey: postural adjustment; dark grey: grimace of discomfort followed by psychomotor agitation; purple: controlateral displacement of trunk and eyes. Other conventions as in Figure B11.

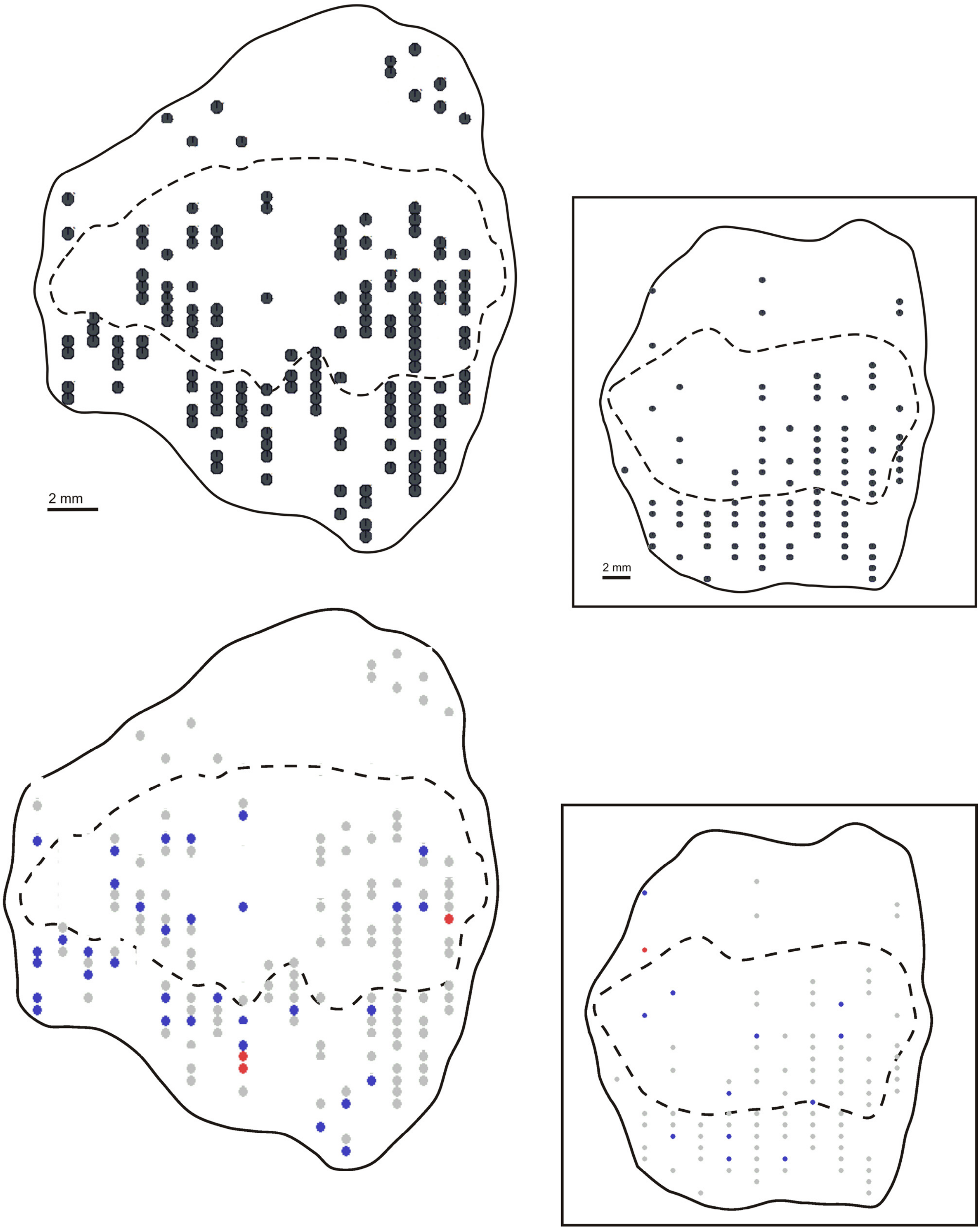


Figure B17: Distribution of unresponsive sites (upper panel) and cardiac responses (lower panel) shown on the unfolding of the perisylvian regions of the left hemisphere of macaque monkey MK1 (left) and MK2 (right). On the behavioral maps: dark grey dots: unresponsive sites. Other conventions as in Figure B11.

3- Heart rate variability (HRV)

The analysis of heart rate variability in the temporal domain (instantaneous frequency) demonstrated that intracortical microstimulations of the insula and perisylvian regions were able to evoke different types of modulation of the autonomic nervous system. The variety of the evoked responses was assessed by means of single stimulation analysis, while the results of the population analysis showed the distribution of these responses into the studied regions, the amplitude of the cardiac variation, the temporal trend of the cardiac responses and the correlation of these variations to specific behavioral responses. Furthermore, the comparison between data form two hemispheres of the same monkey showed the results relative to the hypothesis of a lateralization of the ANS control.

a- Single stimulation: types and profiles of cardiac responses

The single stimulation analysis demonstrated three types of cardiac responses: bradycardic, tachycardic and biphasic responses. Moreover, in numerous sites ICMSs did not evoke any change of heart frequency. In view of the fact that in literature there are no studies on macaque monkeys that indicated standard criteria regarding the variation considered as cardiac response, the percentage of the evoked cardiac responses on the total of the stimulated sites are calculated and presented in the Figure B18 at different thresholds of heart rate variation.

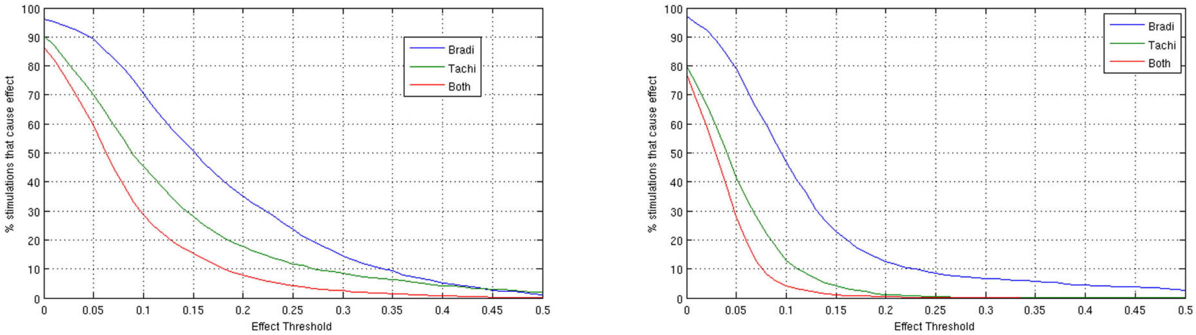


Figure B18: percentage of the cardiac responses at different thresholds. Blue line: Bradycardia; Green line: Tachycardia; red line: Biphasic responses

a.1- Bradycardia:

Bradycardic responses (Figure B19) consisted in a fast drop of the instantaneous heart frequency due to an increase of the R-R interval. This phenomenon takes place at the first or second couple of the QRS complex following the start of the stimulation. Bradycardia was never due to atrioventricular (AV) block. All evoked bradycardic responses showed an increase of the interval between the successive QRS complexes, maintaining invariant the P-Q interval (sinus bradycardia). In few cases bradycardic responses produced fusion beats (Figure B20). A fusion beat occurs when an atrial stimulation is applied in synchrony with the discharge of the ventricular focus. The aspect of the QRS is intermediate between that of normal complex and that occurring during a ventricular tachycardia and in some cases the P wave is visible (in this case, the P wave is closer to the QRS complex than in normal cases). The presence of fusion beats during the stimulations is due to the delay evoked on the atrial stimulus, consequently it represents an indirect confirmation of the fact that evoked bradycardia is of sinus origin and not due to AV block. Bradycardic responses are characterized by a fast recovery of the normal frequency.

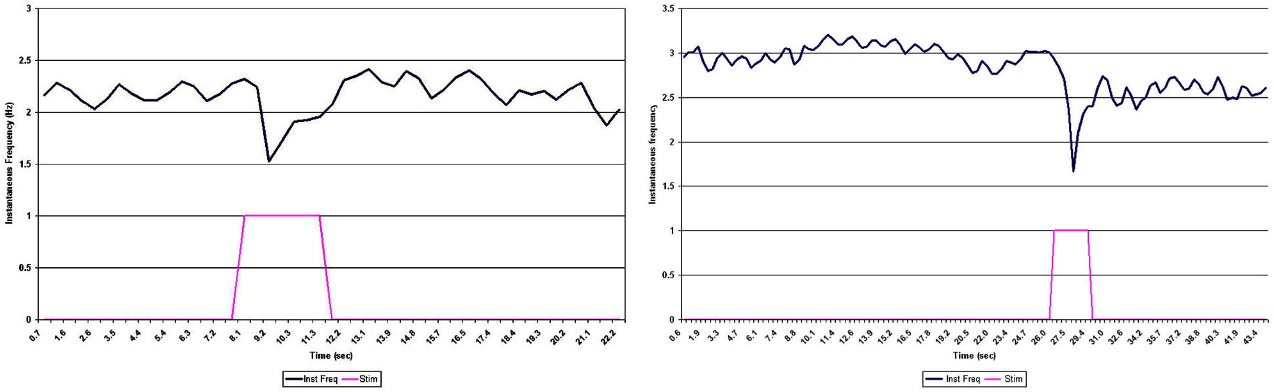


Figure B19: Examples of bradycardia evoked during ICMS. The dark blue trace indicates the temporal profile of the instantaneous heart frequency before, during and after ICMS trial. The purple line indicates ICMS delivery. The horizontal axis indicates the time in sec and the vertical axis indicates the instantaneous heart frequency in Hz.

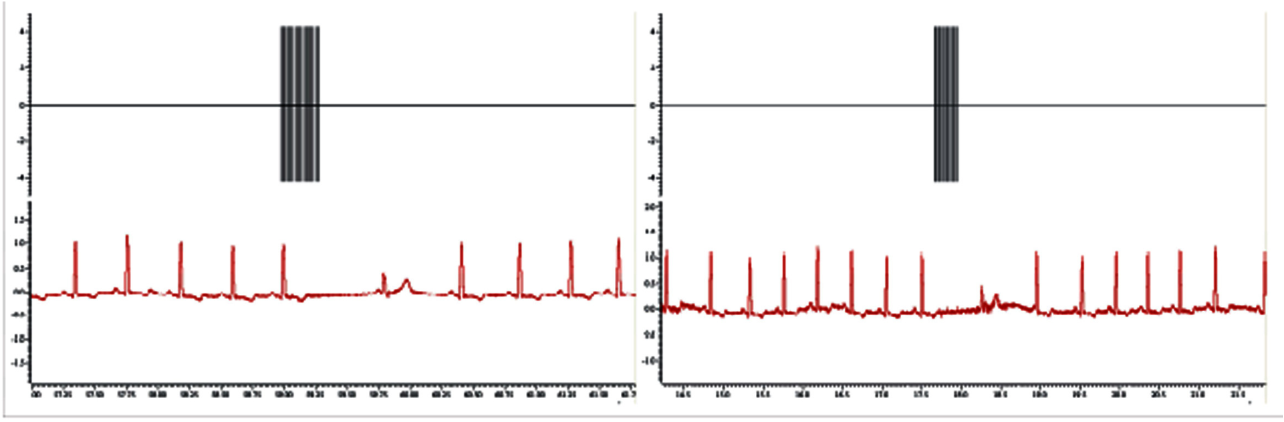


Figure B20: Examples of fusion beats. ICMS delivery is indicated in black. The ECG trace is indicated in red.

a.2- Tachycardia:

Tachycardic responses (Figure B21) consisted in the increasing of instantaneous frequency with respect to baseline frequency. The phenomenon starts with a latency of about 1sec from the beginning of the stimulation and reaches its peak between the third and fifth second afterward.

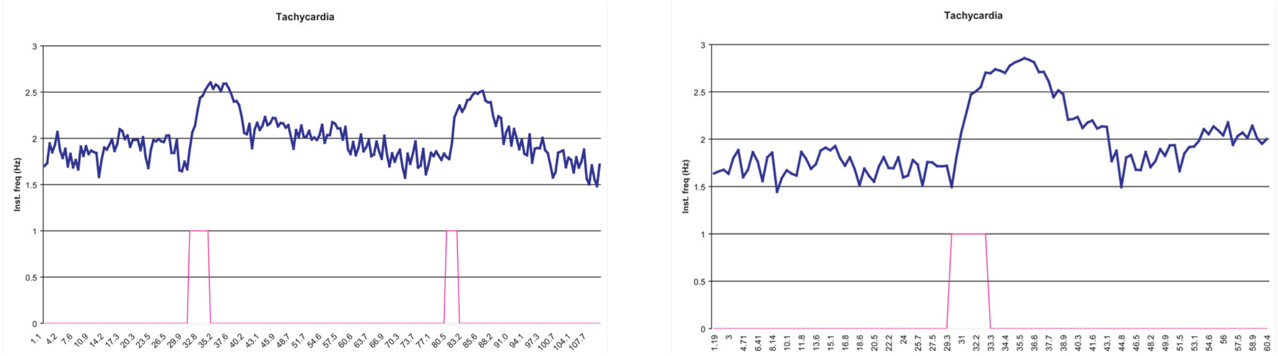


Figure B21: Examples of tachycardic responses evoked during ICMS trials. Conventions as in Figure B18.

a.3-Biphasic responses:

In few cases ICMS evoked biphasic responses (Figure B22). Such responses are characterized by a first phase of bradycardia (with the same characteristics of a typical bradycardic response described above) followed by a tachycardic phase (similar to the described tachycardic responses). The hypothesis that biphasic responses could be due to a physiological compensation after bradycardia is not plausible because of the presence of bradycardic responses with higher amplitude without being followed by tachycardic phase. Biphasic responses could be genuine responses due to synchronic sympathetic-parasympathetic activation.

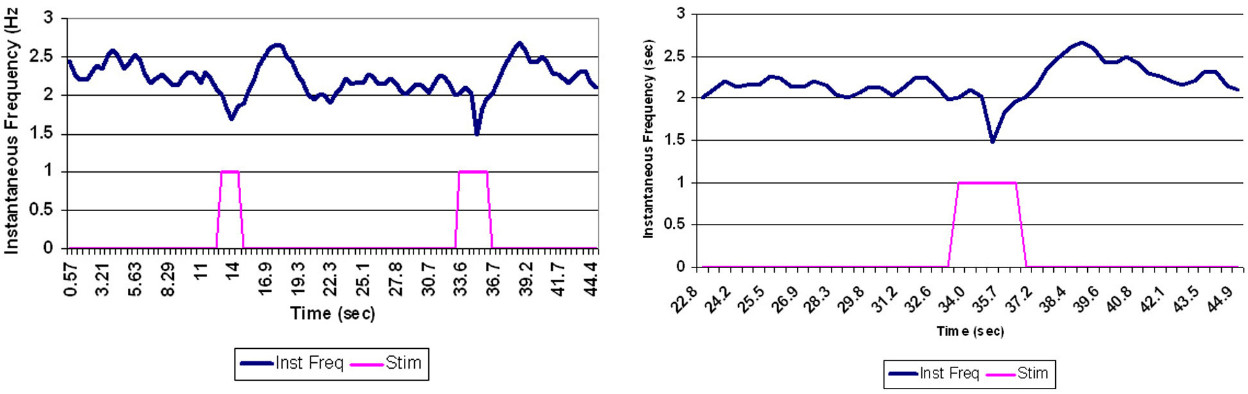


Figure B22: Examples of biphasic effect evoked during ICMS trials. Conventions as in Figure B18.

b- Population analysis

b.1-Population analysis A: ROIs analysis of rostro-caudal penetration groups

The population analysis with the antero-posterior variable performed on three temporal epochs (rest activity, stimulation period, post-stimulation period) showed the temporal trend and the average of the responses amplitude in relation to the antero-posterior position. The Figure B23 and Figure B24 illustrate the results of the analysis of each antero-posterior position for the four ROIs (fronto-parietal operculum in blue, dorsal insula in purple, ventral insula in red, and temporal operculum in green; the antero-posterior position is indicated for each position).

The results showed a clear predominance of the bradycardic effects in the large portion along the antero-posterior axis. Furthermore the results showed a clear distribution of the bradycardic responses in relation to the effects amplitude; the greatest amplitudes of the cardiac effects were evoked in the anterior portion (AP27-AP22 in MK1; AP27-AP23 in MK2) of the stimulated regions with tendency to biphasic responses in the most anterior positions. The results showed a gradual decreasing of the effects amplitude in the middle portion until disappearance of the evoked effect in the posterior portion (AP19-AP-13 in MK1; AP17-AP7 in MK2). Tachycardic and biphasic responses were evoked only in one of two monkeys. These effects were evoked in the most posterior part of the stimulated regions (AP12-AP10 in MK1). One possible explanation for the lack of these effects in MK2 may be due to the fact that ICMSs didn’t reach the most posterior portion of the interested regions, due to the recording chamber position.

Moreover, the results showed a clear distinction of the effect amplitudes in relation to the regions of interest. The greatest effect amplitudes were observed in the ventral regions (ventral insula & lower bank). In the first monkey the ventral insula showed the greatest amplitude of the evoked modulations while in the second monkey the ventral bank is the region whose stimulation produced the greatest amplitudes of the evoked modulations.

Finally, an inter-individual difference could be noted. The results showed a difference in the amplitudes of the evoked effects between the two monkeys. Despite ICMs evoked the same cardiac changes in both monkeys, the first monkey showed greater effect amplitudes.

The population analysis with the AP variable confirmed the temporal trend evoked by the single stimulation: bradycardic responses have a brief latency and fast recovery, thus suggesting a direct path to the ANS modulation rather than a secondary effect of the stimulation. Tachycardic responses had greater latency and slow recovery. Although such features normally characterize physiological sympathetic responses, it is not possible to discriminate if the cardiac effect is directly due to the stimulation or is a consequence of the behavioral response evoked by the stimulation. The biggest modulation amplitudes were evoked at the extremities of the stimulated regions (for both signs in the first monkey and for the bradycardic effect in the second monkey).

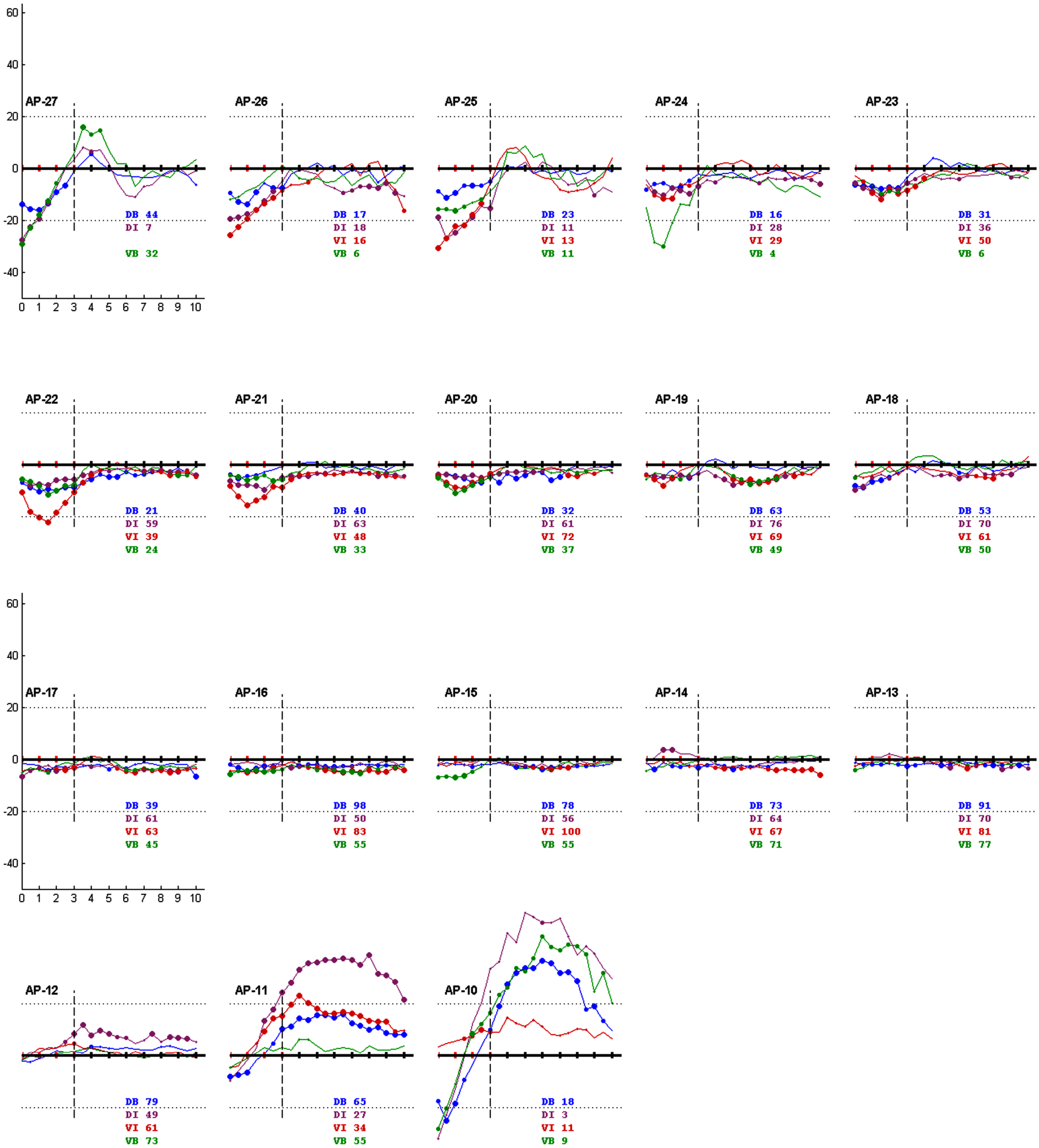


Figure B23: Illustration of the temporal profiles of the instantaneous cardiac frequency during and after the stimulation period in MK1 of each antero-posterior position for the four ROIs illustrated in different colors; dorsal bank (blue), dorsal insula (purple), ventral insula (red), and ventral bank (green). The antero-posterior position is indicated for each position. The y-axis indicates the percentage of the variation of the heart rate with respect to the normalized baseline corresponding to the zero on the y-axis. The x-axis corresponds to the temporal axis, where the stimulation period corresponds to the portion prior to the dashed vertical line, while the post-stimulus period corresponds to the portion after the dashed line. Statistical significance is indicated by the dot size: small: p<0.05; medium: p<0.01; large: p<0.001. This profile is aligned with the stimulation onset.

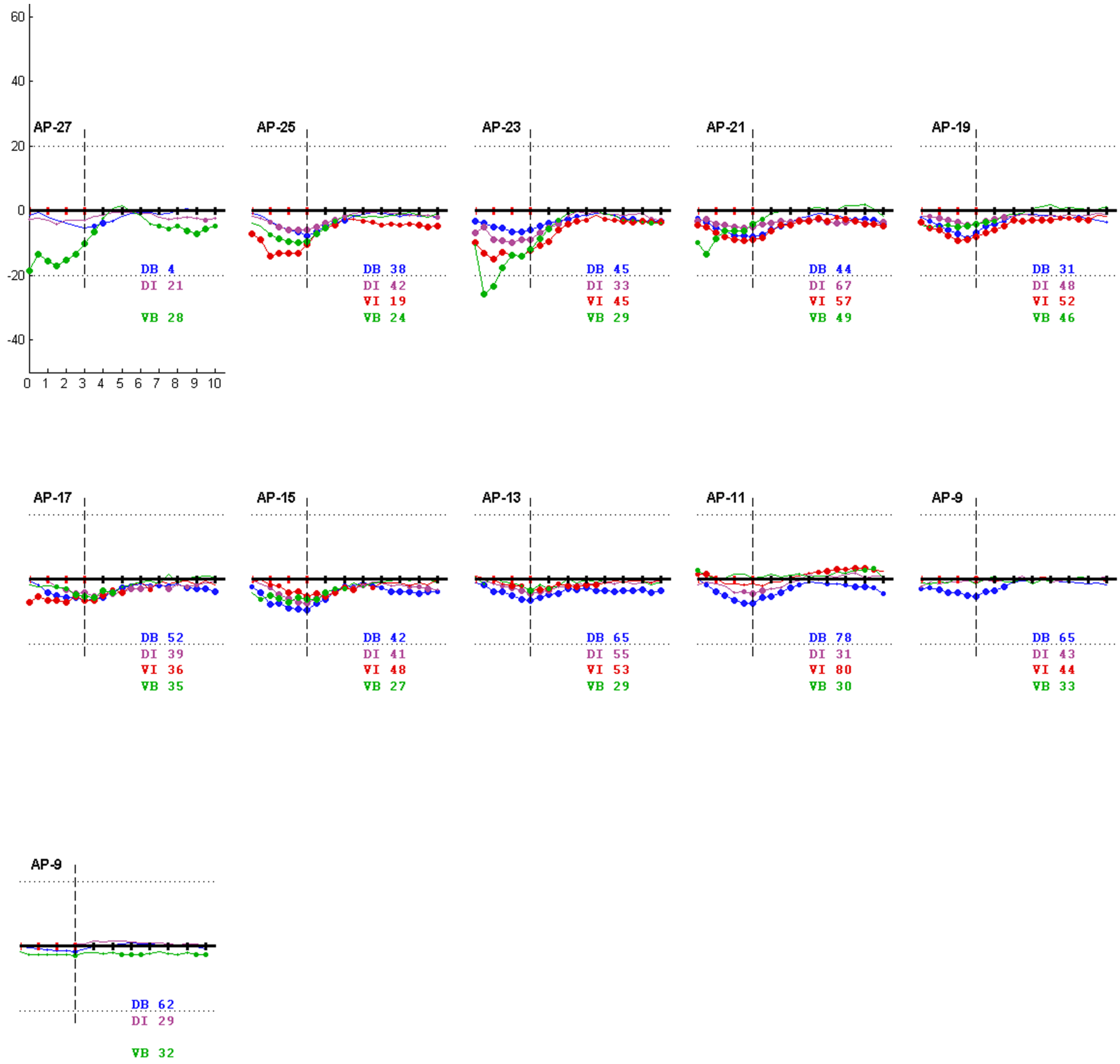


Figure B24: Illustration of the temporal profiles of the instantaneous cardiac frequency during and after the stimulation period in MK2 of each antero-posterior position for the four ROIs. Conventions are as in figure B22.

3.b- Population analysis B: ROIs analysis of single penetration

The Figure B11-lower row shows the maps of the heart rate changes evoked by the ICMSs on an unfolding of the perisylvian regions of the left hemispheres of two monkeys. Blue filled circles indicate the penetration sites where the electrical stimulations evoked bradycardic effects, red filled circles indicate tachycardic responses, and black filled circles indicate biphasic responses. In view of the fact that the ICMSs evoked almost the same cardiac effects on both monkeys but with difference in the amplitudes of these evoked effects and that in literature there are no studies on macaque monkeys that indicated standard criteria regarding the variation considered as cardiac response, only the variations that exceed 20% in MK1 and 15% in MK2 with respect to the average of the rest activity were represented on the maps . The population analysis with variables AP and ML illustrated in the Figure B11 showed the spatial distribution of the evoked cardiac variations within the single penetration sites and over the four ROIs. The results confirmed those of the population analysis with the variable AP that is the bradycardic effect is by far the prevailing evoked modulation except the most posterior portion where ICMSs evoked tachycardic and biphasic responses. On the basis of the modulation threshold chosen in this analysis, bradycardic responses are predominantly presented in the ventral insula in MK1 and in the ventral insula and the ventral bank in MK2, confirming that ventral regions are those whose stimulation produces the highest effects amplitudes.

4- Behavior-HRV correlation

The synchronous recording of the behavioral responses evoked by BTS-ICMSs and the ANS modulations by way of the heart rate variability analysis allowed the integration of both data in order to better understand the functional aspects of the studied regions. A correlation analysis between the behaviors and the ANS modulation was performed. The lower row in the Figure B12, B13, B14, B15, B16, and B17, shows the sign of the heart rate variation during to the behavioral responses, for each category of response. Figure B25 and Figure B26 illustrate the temporal profiles of the instantaneous frequency relative to each behavioral sub-category.

The results of the single penetration analysis showed that modulation of the instantaneous frequency is mainly obtained from sites whose stimulations evokes oroalimentary responses and miscellaneous responses. Moreover, modulation of instantaneous frequency was also obtained from behaviorally unresponsive sites, this suggesting that the heart rate modulation is a genuine viscero-motor response, and can not be considered to be the consequence of a subjective sensation evoked by the stimulation.

While the oro-alimentary sites were predominantly bradycardic, the miscellaneous responses sites showed more variability.

The results of the temporal profile analysis showed: a) similar trend to what was showed by the single penetration analysis, that is the cardiac changes were mostly evoked by the ICMSs evoking at the same time oro-alimentary and miscellaneous responses. b) within the oro-alimentary and miscellaneous macro-categories, the single sub-categories showed similar profiles in both monkeys. The feeding behavior (OM-01), the feeding behavior accompanied by arm movement inhibition (OM-02, the grimace of disgust (OM-04) and the alteration of the feeding behavior (OM-05) showed in both monkeys bradycardic effects, while the vomiting impulse (OM-03) responsive sites showed biphasic trend. Within the miscellaneous responses macro-category, both the twitch followed by psychomotor agitation (OT-01) and discomfort grimace followed by psychomotor agitation reaction (OT-06) showed strong fall of the instantaneous heart rate with biphasic tendency in the first monkey. The discomfort reaction (OT-04) showed biphasic effect in both monkeys with more intense bradycardic effect in the first monkey. The controlateral trunk and eyes displacement (OT-03) showed bradycardic profile in both monkeys. The postural adjustment (OT-05) showed poor effects in both monkeys. general tremors (OT-02) and the twitch of the trunk (OT-07) showed different effects between the two monkeys.

The other behavioral sub categories showed poor heart rate modulation (no effects or a slight bradycardic effect) in both monkeys.

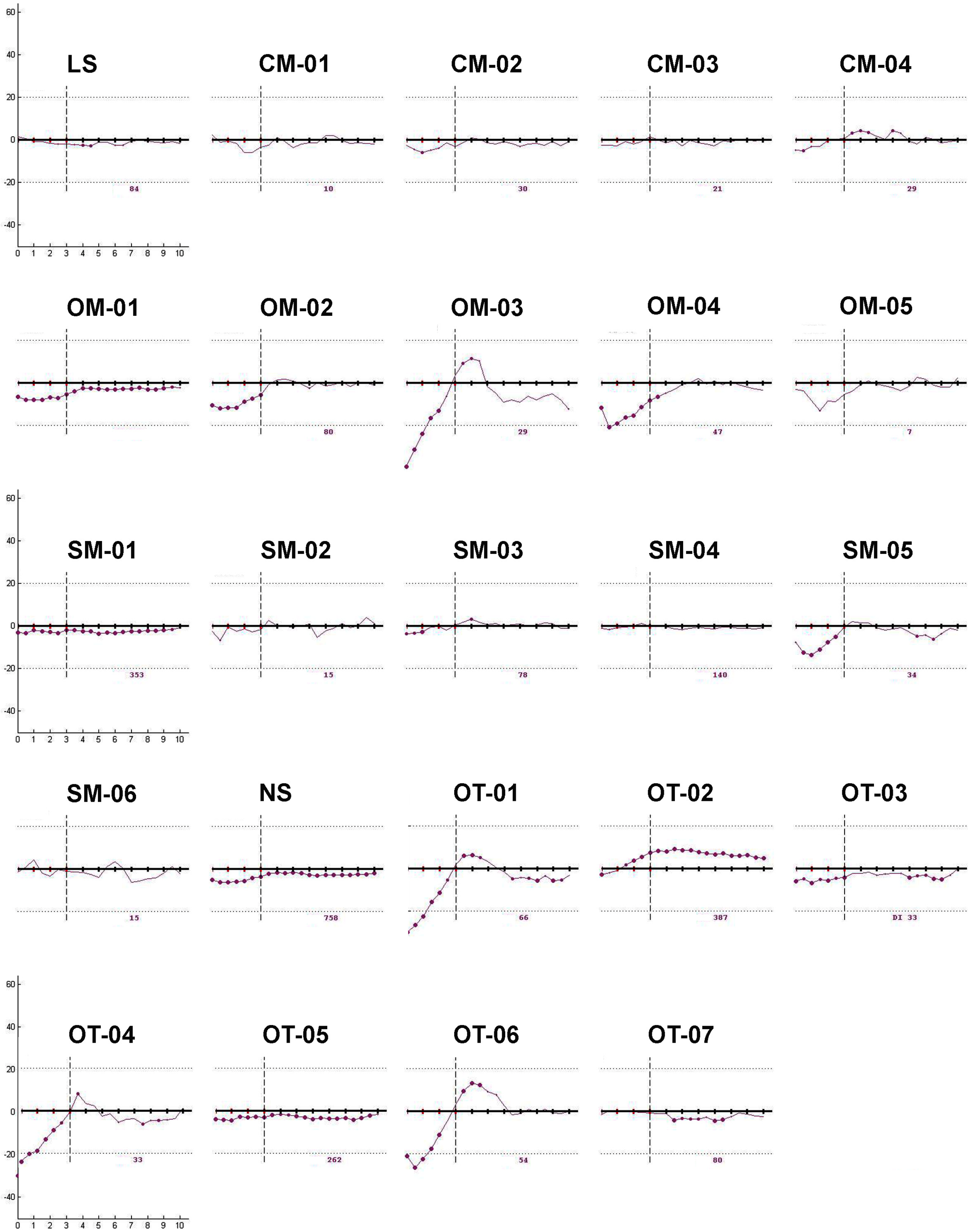


Figure B25: Behaviors-HRV correlation in MK1. Illustration of the temporal profiles of the instantaneous heart rate recorded during ICMSs evoking the same behavioral sub-category responses. Abbreviations as in Figures B7, B8, B9 and B10. Conventions as in Figure B22.

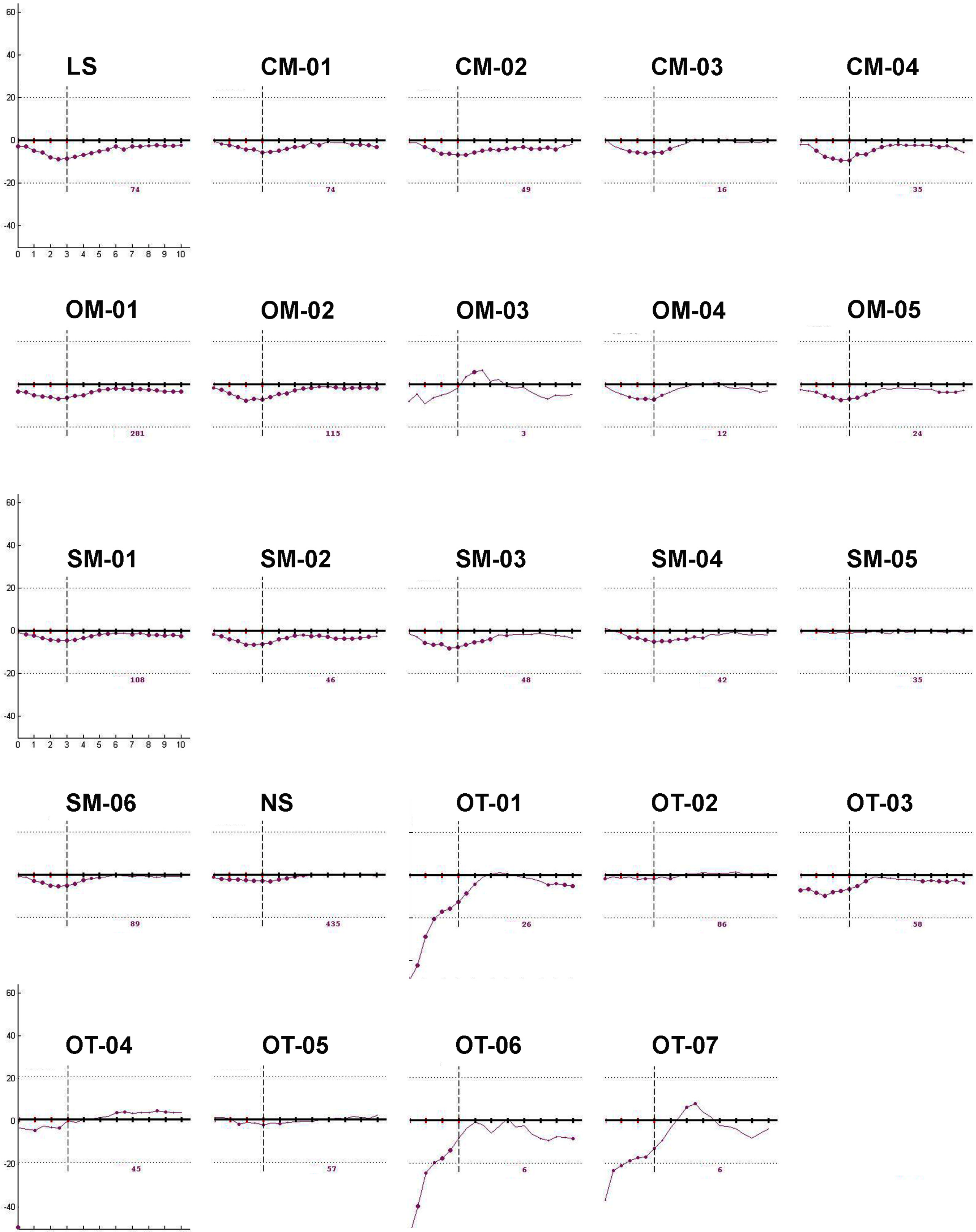


Figure B26: Behaviors-HRV correlation in MK2. Illustration of the temporal profiles of the instantaneous heart rate recorded during ICMSs evoking the same behavioral sub-category responses. Abbreviations as in Figures B7, B8, B9 and B10. Conventions as in Figure B22.

5- Hemispheric lateralization of the cardiac responses

The results of the analysis performed on the bradycardic and tachycardic responses in the anterior perisylvian regions of both monkeys (AP 27-21) demonstrated the lack of lateralization of the modulation sign (bradycardia vs. tachycardia) but a lateralization of the effects amplitudes, that is, response amplitudes are greater in the right hemisphere than in the left hemisphere.

ICMSs evoked tachycardia and bradycardia significantly different from baseline ($p<0.001$) in both hemispheres (Figure B27). This result shows lack of lateralization of the modulation signs. Furthermore, both bradycardic and tachycardic effects evoked by electrical stimulation are significantly higher in the right hemisphere than in the left one ($p<0.001$).

These results suggest that the hypothesis of sympathetic-parasympathetic lateralization is not plausible in the case of monkeys, since both hemispheres demonstrated parasympathetic dominance, although both effects were evocable in both hemispheres. On the other hand, there is an apparent lateralization of the amplitudes of the evoked effects. ICMSs in the anterior perisylvian regions have greater effect on the ANS in the right hemisphere than in the left hemisphere.

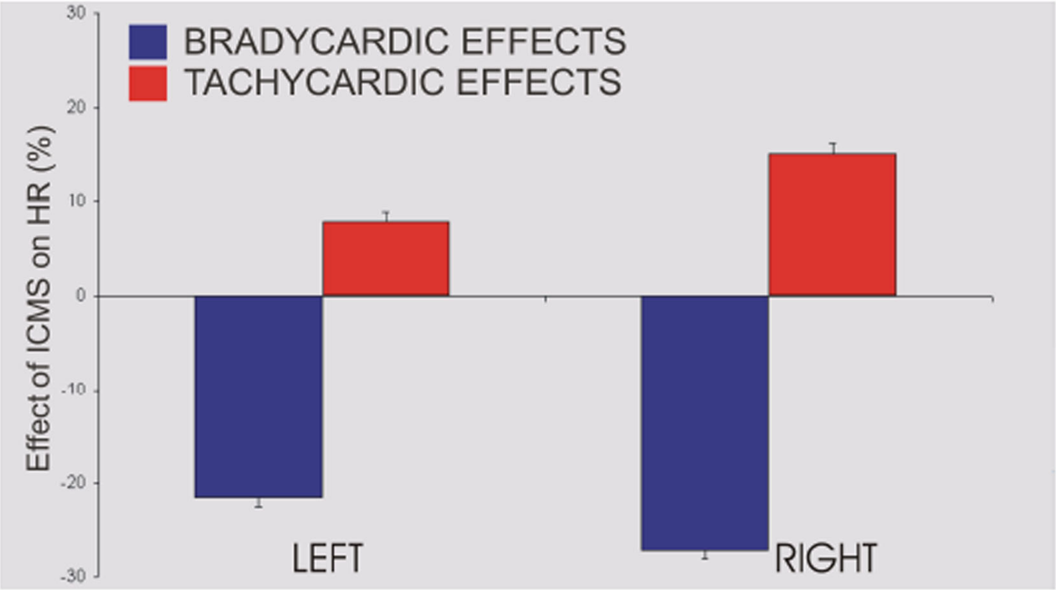


Figure B27: Hemispheric lateralization of the HRV in MK1.

IV- DISCUSSION

As it was showed in the Introduction, the insula of Reil and the perisylvian regions were demonstrated to be involved in a wide range of functions. In this study we tried to answer the question about these supposed functions by means of an approach integrating the monitoring of behavioral and cardiac responses during intracortical microstimulations performed both with short trains and behavioral time scale.

Our findings showed that the insula and the inner perisylvian regions were involved in a wide range of behaviors such as oro-alimentary, sensory-motor and communicative behaviors as well as a miscellaneous of behaviors. Furthermore ST-ICMS revealed a coarse somatotopic organization of the involved effectors in the insula and the parieto-frontal operculum. In this study there was evidence of the role of the studied regions in the control of the ANS with lateralization of the effects amplitudes.

1. Short-term stimulation (ST-ICMS)

a- Somatotopic organization

In the present study short-term stimulations (ST-ICMSs) evoked brief responses such as simple twitches of a given body part in the case of very short trains (minimum 50msec) or brief movements comparable to that evoked by BTS-ICMS in the case of longer trains (>200msec). These results allowed us to assess the hypothesis of a potential somatotopic organization of the involved effectors inside the stimulated regions. ST-ICMS experiments are usually employed in the cortical mapping studies, particularly in the mapping of motor areas.

The hypothesis of somatotopic organization in the perisylvian regions was raised by earlier electrical stimulation studies. Frontera (1956) described a coarse somatotopic organization only in the ventral portion of the insula, where face and upper extremities were represented in the rostral part and lower extremities represented in the caudal part. However, Frontera reported lack of systematic evoked responses, thus discarding the hypothesis of a secondary extrapyramidal motor cortex. Showers and Lauer (1961) described a coarse somatotopic distribution in both dorsal and ventral portions of the insula where movements involving the face were evoked in the whole insula, while upper and lower extremities were represented in the anterior and posterior portions, respectively.

Our findings revealed a significant functional difference between the dorsal (frontoparietal operculum and dorsal insula) and ventral (ventral insula and temporal operculum) complex of the perisylvian regions. That is in accordance with the functional differentiation suggested by earlier studies. Furthermore, recent architectonic studies are in line with such segregation, suggesting an anatomical continuity between the fronto-parietal operculum and the dorsal insula (Morel et al., 2008) and a major differentiation between the dorsal and ventral portions of the insula.

Our study demonstrated a coarse somatotopic organization within the fronto-parietal operculum and the dorsal insula, where the mouth was represented in the rostral portion, followed by the representation of the hand, and a caudal portion where upper and lower limbs are represented. In the present study, unlike the earlier studies, a somatotopic representation of the body was very limited in the ventral insula to its rostral portion and totally lacking in the ventral bank. The lack of such somatotopic organization was due to the nature of the evoked responses and demonstrated the functional gap of these regions with the sensory-motor system. The hodological studies support the results of the present study demonstrating that connections with sensory and motor areas prevail in the dorsal complex of the perisylvian regions. The dorsal complex resulted to be connected with different areas of the frontal, prefrontal and parietal areas involved in the control of the sensory-motor modalities. The frontal operculum (OFO and PrCO) has connections with the prefrontal area 46v and with the lateral region of the ventral premotor cortex (PMv). This latter is robustly connected with the dorsal insula (Cippolloni & Pandya, '99) and is involved in the control of mouth movements. The identification of a mouth representation in the anterior dorsal complex is in agreement with the hodological data. Caudally, the parietal operculum has connections with the prefrontal area 46v and with premotor and motor areas such as the posterior bank of the arcuate sulcus (F5a & F5p), the convexity of the ventral premotor area (F5c & F4) and the lateral motor cortex (Cippolloni & Pandya, '99; Belmalih et al., 2007). Furthermore, the parietal operculum has connections with the primary somatosensory area, the inferior parietal lobule and the inferior bank of the intraparietal sulcus. Connections studies seem to confirm the functional similarity between the dorsal perisylvian regions and the sensory-motor areas. In contrast, no particular connection between the ventral complex of the perisylvian regions and sensory-motor areas has been detected. The connections of the temporal operculum are predominantly with the orbitofrontal and medial prefrontal regions, and to a lesser extent with the lateral area 10 and anterior cingulate gyrus. These connections are in agreement with the scarce presence of somatotopic representation found by ST-ICMSs.

In dorsal perisylvian regions, the mouth has a dual pattern representation; anteriorly, the mouth representation covers a wide field in the dorsal insula and the frontal operculum, while more caudally the mouth representation covers the most lateral portion of the dorsal bank. BTS-ICMSs of the anterior regions evoked complex oro-alimentary behaviors, while BTS-ICMSs of the more caudal field evoked simple movements like mouth opening and/or closure, suggesting different functional roles of the two stimulated fields. The caudal field likely overlaps with the mouth region identified by means of single cell recording (Robinson and Burton, 1970). According to that study, trigeminal receptive fields were situated in the anterior portion, while hand and digits receptive fields were predominant in the posterior portion of the map; the hand and digits zone is followed by the representation of the arm, upper and lower portion of the trunk, and lower limbs. The somatotopic organization described by Robinson & Burton agrees with the present study, even if the somatotopic organization in our study derived from responses evoked by electrical stimulation.

b- Dorso-ventral somatopic organization of the anterior perisylvian regions

ST-ICMS of the ventral anterior insula evoked in several occasions brief reflexes consisting of shivering of the chest. BTS-ICMS of the same sites evoked visceral responses such as vomiting impulses associated with violent fall of instantaneous heart frequency. These responses could be the observable counterpart of potential visceral reflexes. According to this hypothesis, a zone of visceral representation was located immediately ventral to the mouth representation field. This suggested continuity in term of somatotopic organization. It seems likely to speculate that the topographic organization of the *Simiusculus* of the precentral surface (Woolsey, 1958) where lower limbs are represented dorsally followed ventrally by the representation of the upper limb and mouth continues inside the sylvian fissure. The representation of the mouth in the dorsal perisylvian regions is implicated with complex goal-related oro-alimentary behaviors with visceral interface (BTS-ICMSs evoked oro-alimentary responses together with vagal activation) and continues in the ventral insula with an explicit visceral representation, since ICMS evoked presumable visceral responses together with vagal activation.

c- Train threshold distribution

The results of this study showed that observable responses were evoked in the regions of the parietal operculum and the posterior dorsal insula at low temporal threshold (50ms), while the threshold required to evoke intelligible responses in the frontal operculum and the anterior dorsal insula showed more variability, where thresholds ranged from 50 msec (where simple twitches were evoked) up to 1 sec (where the evoked responses were comparable to those evoked by BTS-ICMS). This data is very interesting since train duration of temporal threshold of the anterior complex is comparable to the train duration of behavioral time scale stimulation in the motor areas (Graziano et al, 2002-2004-2005) and, in principle, one would expect the frontal operculum to be more excitable. The fact that ICMS of the anterior complex evoked oro-alimentary responses suggests that this region is involved in a neural network controlling the feeding behaviors. The fact that in this region ST-ICMS needs longer trains than the posterior complex in order to evoke a behavioral response suggests that the role of the anterior perisylvian complex within this neural network, even if related to the motor programs of feeding behavior, is not only/strictly involved in the control of their execution. It seems likely that this region is more involved in triggering feeding behavior rather than fine-tuning its execution. The relatively lower thresholds required to evoke behavioral responses in the posterior complex suggest that this region occupies, within the neural networks, a position closer to the executive levels.

This functional view is supported by the anatomical connections. The anterior complex has connections with the orbital and fronto-opercular areas, with the ventral premotor cortex (F5), the opercular portion of SI and the anterior cingulate cortex. While the connectivity pattern of the posterior complex seems to be more somato-motor dependent. This sector has connections with parietal lobe areas, with the ventral premotor cortex (F5), with the supplementary motor area (F3), with the primary motor cortex and with the posterior cingulate cortex.

2- Functional properties of the INSULA of Reil at behavioral time scale stimulation

a- Oro-alimentary responses

Oro-alimentary responses were evoked in the anterior portion of the stimulated regions and ranged from normal feeding behavior like chewing, mouthing and swallowing to an alteration of such behavior such as food refusal and more visceral aspects such as vomiting. Classically, the anterior insula and the anterior frontal operculum were anatomically described as gustatory cortex (Sanides et al., 1968) and on the basis of this description neurophysiological studies mainly investigated the gustatory modality (Yaxley et al., 1990) or somatosensory modality (Ogawa et al., 1989; Verhagen et al., 2004). Nevertheless, the percentage of the responsive neurons to such stimuli was always very low. Generally, a percentage lower than 10% of the recorded neurons was responsive to taste stimuli, while about 20% of recorded neurons was responsive to somatosensory and motor stimuli (Augustine, 1996; Smith-Swintosky et al.,1991; and Plata-Salaman et al.,1992). Even though a great percentage of neurons recorded in the insula and the frontal operculum were uncharacterized, the label of gustatory area is still largely attributed to the anterior insula and the anterior frontal operculum. The gustative sensorial processing could not be the prevailing function of the anterior complex but should be one of various functions in relation with feeding behavior; according to Augustine (1996) such behavior could involve the integration of visceral responses, and somesthesic sensations suggesting the presence of multisensorial coding. In this study we demonstrated that BTS-ICMS evoked more or less complete motor feeding program where the monkeys manifested feeding behavior including one or more of chewing movements, swallowing, or mouthing and finished by the swallowing. These behaviors were evoked with empty mouth. Since the decreasing of the stimulation intensity evoked decreasing in the responses dynamicity, and since ST-ICMS evoked brief twitches of the tongue and lips, it is improbable that such behaviors can be due to a sort of gustative hallucination evoked by the ICMS. Furthermore, when the stimulation was delivered during spontaneous feeding behavior, an alteration of the chewing rhythm occurred, and this is incompatible with the gustative hallucination hypothesis. Consequently, ICMS of this area supports the hypothesis that processes of motor, sensorial, and visceromotor integration form a part of the functional aspects of the dorsal anterior perisylvian regions. This hypothesis is further corroborated by the results of heart rate variability analysis showing bradycardic effects evoked jointly to feeding behaviors, indicating vagal activation and thus visceromotor responses. Furthermore, the hypothesis of a genuine response directly induced by electrical stimulation is supported by the clinical cases where oro-alimentary movements were described during epileptic insular seizures (Isnard et al., 2004).

Visceral responses were evoked in the anterior complex mostly in the ventral insula. The distribution of the sensory and viscero-motor responses in the anterior perisylvian regions suggest a topographic organization of these responses with the sensory-motor aspects of oro-alimentary responses represented in the dorsal portion while the more visceral aspects represented in the ventral portion. BTS-ICMSs of the ventral portion of the anterior complex evoked explicit visceral behaviors such as vomiting impulses, or more complex visceral behavior such as food aversion behavior consisting in refusal during food intake or food spitting from the mouth. Similar vomiting behavior was recently described in epileptic patients during seizures involving the anterior insula (Catenoix et al., 2008). An interesting data is the potent bradycardic effects jointly evoked with the visceral responses. The evoked food aversion behaviors suggest the hypothesis of an evoked decrease of disgust threshold of the monkeys, while the hypothesis of conditioning should be discarded since the refused or spitted food during the stimulation was accepted and eaten before and after the stimulation.

b- Complex and Simple movement responses

BTS-ICMSs along the mid-posterior dorsal perisylvian regions evoked motor behavioral responses. These responses include complex goal-related movements such as grasping, bringing to the mouth, taking away from the mouth, movements toward the body, visual exploration and more simple movements. The finding of motor responses is of particular interest since the hypothesis of a secondary extrapyramidal motor area was discarded by earlier neurophysiologists (Frontera, 1956) in favor of a somatosensory functional view. However, evoking such behaviors is not entirely inexplicable, given that hodological studies showed connections of these regions with ventral premotor cortex, and with the supplementary motor areas (Mesulam & Mufson, 1983; Luppino et al., 1993; Belmalih et al., 2007). PET studies demonstrated that repetitive movements of the hand and arm increase the cerebral blood flow in the posterior region of the insula (dysgranular and granular), that is, the regions investigated in the present study whose ICMS evoked motor behaviors (Colebatch et al., 1991). Several studies performed on hospitalized patients after strokes that involve the motor areas showed that the phase of the functional recovery is characterized by increasing activity of specific cerebral regions including the insula (Augustine, 1996). Furthermore, clinical observations reported that during the later phases of the epileptic seizures involving the posterior dorsal insula the patients manifested upper and lower limbs movements (Isnard et al., 2004). Some of the evoked goal-related behavior such as visual exploration of the hand or taking away from the mouth seems to be triggered by sensations evoked by the stimulation, inducing a voluntary-like program relative to these sensations. This hypothesis is supported by clinical observations reporting that during epileptic seizures the patients reported painful and non-painful sensations and manifested in few cases complex behaviors such as rubbing of the face (Isnard et al., 2004).

c- Communicative responses

The rhesus monkey is a social animal. Inside the group the hierarchy and social interactions among group members are established by means of facial expressions. There are specific facial expressions for menace, submission, and affiliation; such expressions have at the same time communicative and emotional valence. A very important element in the social interaction is the eye. Direct eye contact is indispensable in order to start the interaction between two individuals and is generally considered as an aggressive message during conflict situations. Furthermore, the eyes are the most scanned body part during observation of facial expressions (Gothard et al., 2004).

In this study we showed that BTS-ICMSs evoked communicative responses in the most ventral part of the mid-posterior insula. These responses mostly consisted in lip smacking (considered as an affiliative gesture) alternated in some cases with teeth chattering (a submission expression) and were evoked only in case of eye contact between the experimenter and the monkey. In case of lack of eye contact, lip smacking was interrupted and ICMS remained without effect. Furthermore, when ICMS occurred during a spontaneous avoidance grimace, the monkey shifted to the lip smacking and again to his spontaneous expression at the end of the stimulation. The necessity of a contextual condition to evoke lip smacking allowed us to discard the hypothesis of a genuine activation of the motor program of communicative expressions in favor to a role of the insula in the neural circuit controlling the socio-emotional interactions. Where one of roles of these interactions is to establish the of the social hierarchic positions and ranks within a given social group. In monkeys, clarifying these positions is mostly done through emotional communicative facial expressions. Recently Craig (2002-2009) highlighted the key role of the insular cortex in the introception and thus the re-representation of the "sense of the physiological condition of the body" providing the bases of subjective feelings, self awareness and consciousness of emotional experiences. Furthermore it was suggested (Frith et al., 1999; Lewis, 2008) that awareness of any stimulus requires three mental processes: first, a mental representation of one self; second, a mental representation of that stimulus; and, third, a mental representation of the salient interrelationship between oneself and that stimulus at the moment of the occurring interaction. Damasio suggested that in front of an emotionally significant stimulus, the re-representation of the state of the body allows one to judge and predict the effect of the stimulus on the body, thus enabling to react in the appropriate mode.

On the basis of these observations, it seems likely that electrical stimulation of the insular cortex evoked a re-mapping of the internal body states. In absence of the electrical stimulation, the direct eye contact between the monkey and the experimenter evoked no-reaction or a mouth-opened threat or avoidance reaction. This new re-representation of the internal body state evoked by the electrical stimulation led to a re-consideration of the stimulus (the eye contact with the experimenter) calling new emotional experiences and thus inducing a new appropriate reaction (lip smacking) to the new significance of the stimulus.

d- Miscellaneous responses

In this study we showed that ICMS of the ventral perisylvian regions and the most posterior complex evoked observable behavioral responses that were repetitive and stereotyped but non decipherable in terms of goal-relatedness or simple movement responses. Nevertheless, some of these responses, interpreted and classified as painful, discomfort and agitation responses, were clearly manifested. It is likely to hypothesize that the manifested responses were the visible reaction of the monkey to some sensations evoked by the stimulations, but it is not equally easy to determine which kind of sensation could be evoked by the stimulations as it was not possible to attribute a determined sensation to each one of the manifested reactions. The ventral perisylvian regions are considered to be associative areas or to be involved in sensorial processing. Moreover electrical stimulation studies on humans reported painful and thermic sensations evoked by stimulation of the posterior dorsal insula (Ostrowsky et al., 2002; Mazzola et al., 2006-2009; Afif et al., 2008), although in our study the great part of the discomforting and agitation responses were evoked from larger fields in the mid-posterior portion of the ventral insula. It is possible that these responses have different causes according to the different stimulated sites. Responses evoked in the anterior portion could be related to visceral sensations evoked by the stimulation (e.g., Abdominal pain), while the more posterior responses, evoked ventrally to the sensory-motor responses of hand and limbs, could be due to painful paresthesiae of hand and limbs. In some cases, the discomforting and agitation responses were accompanied by biphasic cardiac responses. This indicates that such behavioral responses go together with dual activation of the sympathetic-parasympathetic system. Recently Paton and colleagues (2005) reported that besides the reciprocal relation between the sympathetic and parasympathetic nervous system control (sympathetic activation vs. parasympathetic inactivation and vice versa) as in the baroreflex case, sympathetic-parasympathetic co-activation can occur during a variety of behavioral and non behavioral responses such as the startle reflex and somatic nociception. The authors suggested that the the dual activation seems to be particularly related to protective reflexes (nociceptive reflexes, orientation, defensive, etc…) and not to regulation or homeostatic reflexes. In line with the present analysis, the convergence between biphasic cardiac responses and behavioral responses of likely nociceptive origin is in accord with the literature.

Stimulation of the temporal operculum evoked axial rotation towards the controlateral side generally accompanied by gaze shifting to the same side. It is interesting to note that electrical stimulation of the outer portion of the temporal operculum was performed very early (Ferrier, 1887) where stimulation evoked eyes and neck controlateral torsion. Furthermore, clinical observation reported that during epileptic seizures involving the posterior inferior quadrant of the insula and extending to the superior temporal gyrus, head rotation occurred among other behaviors during the last phases of the seizure, normally started with auditory hallucination (Isnard et al., 2004). Since the monkeys were head-restrained, with neck rotation physically impossible, the stimulation effect could represent the axial displacement related to the attempted head rotation.

3- Heart rate variability

The role of the insula and inner perisylvian regions in the control of the ANS modulation was investigated by means of heart rate variability performing time domain analysis. The time domain analysis is a type of analysis investigating the variations of the instantaneous frequency of any tracing, and calculated in function of temporal distance between a given event *t* and the precedent event *t-1.* Such analysis allows the dynamic representation of the signals aligning on the temporal axis the instantaneous frequency of a tracing with an external event. The heart rate variability analysis in the time domain allows to visualize the temporal trend of the instantaneous cardiac frequency along the temporal axis and thus allows to visualize the potential changes of heart frequency during and immediately after the electrical stimulation, enabling to investigate the effect of the stimulation of a given site on the modulation of the ANS.

a- Profiles of cardiac responses

The recording of cardiac activity during electrical stimulations showed three different effects of the stimulations: bradycardic effect characterized by fast drop of the frequency followed by fast recovery to the normal activity, tachycardic effect characterized by an increase of the frequency occurred with longer latency and followed by slow recovery, and biphasic effect were bradycardia is followed by tachycardia before the recovery of the normal frequency.

The fast recovery observed during the bradycardic responses is largely in accordance with the mechanism of the vagal activation described in the literature. One of the reasons in favor of the fast effect of vagal stimulation with respect to the sympathetic one resides in the anatomy of the ANS towards the heart. The preganglionic fibers of the ANS are fast myelinic while the post-ganglionic fibers are slow amyelinic. The parasympathetic ganglions are located in proximity of the target organs while the sympathetic ganglions are situated far form target organs. Consequently, parasympathetic stimuli reach the heart mostly by means of myelinic fibers reducing the transport time with respect to the sympathetic stimuli. Furthermore, the temporal trend of sympathetic and parasympathetic responses can be explained on the basis of the properties of the different neurotransmitters. The parasympathetic system has Ach as neurotransmitter. Ach directly activates the K+ channels in the myocardic cells, allowing a fast effect of vagal activation. Moreover, the Ach is rapidly hydrolyzed by the cholinesterase present in the SA and AV nodes, allowing a fast wash-out of the vagal effect. The slow trend of the tachycardic responses is due to the fact that the release of noradrenalin is relatively slow and the activation of myocardic cells occurs by means of second messengers. The released noradrenalin is re-uptake by the axonal terminals of the sympathetic fibers, a much slower process in comparison with the enzymatic hydrolyzation of Ach.

b-Distribution of cardiac responses

The analysis of the distribution of cardiac responses showed that in the two monkeys the prevalent response is bradycardia. Along the dorso-ventral axis (with respect to the ROIs), the bigger effect amplitudes were evoked in the ventral insula and the ventral bank. Along the antero-posterior axis, the anterior perisylvian regions showed the major bradycardic effect. Moving caudally, an effect decrease was observed until the disappearance of the bradycardic effect in the mid-posterior regions. In one of the two monkeys the most posterior stimulated regions showed a tendency to inversion with predominance of tachycardic and biphasic responses. As we showed previously, the cardiac responses converge with the behavioral responses evoked in the same sites. The oro-alimentary and visceral responses were accompanied with vagal activation, while biphasic and tachycardic responses accompanied discomfort and tremor responses. The antero-posterior distribution of the cardiac responses is in line with the afferent connections of the ANS. The anterior insula has robust connections with the thalamic nucleus VPMpc that receives visceral information from the nucleus tractus solitarius (NTS). The NTS receives afferent parasympathetic connections from the cranial nerves relative to taste and olfaction and involved in the regulation of different autonomic reflexes. In contrast, the thalamic connections of the posterior insula are mostly with VPI that receives sympathetic afferent connections relative to homeostatic aspects such as temperature, nociception, hormonal and immunological activities from the lamina I of the spinal cord. Our study demonstrated convergence between the results emerged from analysis of recorded cardiac activity and hodological studies relative to the connections with the central ANS afferences.

c- Lateralization of cardiac responses

The hypothesis of possible lateralization of the ANS control in the perisylvian regions, and particularly in the insular cortex, was raised by Oppenheimer and colleagues

(1992). In electrical stimulation study on the insula of epileptic patients before temporal lobotomy these authors reported that in the left hemisphere the cardiac responses were mostly bradycardic, while in the right hemisphere the cardiac responses were mostly tachycardic. Oppenheimer and colleagues advanced the hypothesis of hemispheric lateralization, with vagal dominance in the left insula and sympathetic dominance in the right one. Furthermore, another consideration emerges from the results presented in Oppenheimer’s work, that is, the difference in the amplitudes of the responses both between left and right hemisphere and between anterior and posterior portions of the same insula. Even if such data was not sufficiently investigated by the authors, it suggests that hemispheric lateralization also concerns the amplitude of the cardiac responses.

In spite of the clinical importance with respect to the precautionary measures during neurosurgical and neurological practices and with respect to the hypothesis of the sudden death, the hypothesis of Oppenheimer and colleagues remained without any confirmation or rejection. In this study, even if the analysis was performed on stimulation sites limited to the anterior portion of the perisylvin regions, we demonstrated that ICMSs evoked significant cardiac responses in both hemispheres. The possibility of evoking both bradycardic and tachycardic responses in both hemispheres disproves the hypothesis of sympathetic dominance in the right hemisphere and parasympathetic dominance in the left hemisphere. Consequently our results discard the hypothesis of hemispheric sympathetic-parasympathetic lateralization. Furthermore, such hypothesis not only is incongruent with our data but also with the literature. The anterior perisylvian regions are involved in gustatory and olfactory modalities; such modalities are in connection with the parasympathetic system. The hypothesis of sympathetic dominance in the right insula means that the anterior insula should be involved in functions other than gustatory and olfactory or, alternatively, that oro-alimentary responses should be evoked jointly with sympathetic activation. Both of these hypotheses are falsified.

The present study showed stronger response amplitudes of both modalities (bradycardia and tachycardia) in the right hemisphere than in the left hemisphere. These data confirm the hypothesis of the lateralization of the response amplitudes. The cardiovascular complications reported by clinical observations are more critical in cases of stroke including the right insula; such consequences are generally associated with hyperactivity of the sympathetic system (Christenssen et al., 2005). As shown in the present study we can advance an alternative interpretation, that is, cardiovascular consequences due to sympathetic hyperactivity could follow stroke of both right and left insula, but in the first case the intensity of the effect is stronger, thus leading with higher probability to more severe dysfunctions. This would mean that with equal brain damage, lesion of the right insula is mostly candidate to more severe cardiovascular consequences.

C- Functional connection study

I. INTRODUCTION

Previous hodological studies supplied precious information on the afferent and efferent connections of the insula and perisylvian regions shedding light on their functions together with the functional data provided by ICMS and cell recording studies. Nevertheless, these connection studies have never been performed on the same experimental subjects where the functional properties of the injected sites were investigated by means of neurophysiological techniques. The hodological studies concerning the insula were performed on "naïve" experimental subjects, consequently the functional interpretation of the results needs the integration of the functional data obtained from physiological studies performed on other experimental subjects despite the fact that there are inter-subjects differences (even if minimal).

The present hodological study is performed on one of the two monkeys of the ICMS study (MK1). We used physiological data obtained by the ICMS study to guide the locations of tracer injections in order to assess:

- The connectivity patterns of the injected sites.
- The consistency with previous hodological studies.
- The convergence between the connectivity patterns and the physiological functions of the injected sites reported by the ICMS study.

II. Materials and methods

1. Subject and surgical procedures

The experiment was performed on one of the two rhesus monkeys (*macaca mulatta*) used in the ICMS study. Animal handling as well as the surgical and experimental procedures complied with the European guidelines and Italian laws on the human care and use of laboratory animals, and were approved by the Veterinarian Animal Care and Use Committee of the University of Parma and authorized by the Italian Health Ministry.

Tracer injections were performed under aseptic conditions and general anesthesia, with the animal placed in the stereotactic apparatus. No particular surgery was needed since the bone overlying the regions of interest was removed in the first surgery in which the recording chambers were implanted. Removal of connective tissue was performed in order to expose the dura mater and a small incision on the dura was performed when needed. For details concerning anesthesia, vital parameters monitoring, and post-surgery treatment, see materials and methods section in ICMS study.

2. Selection of the injection sites

The choice of the injection sites was based on the functional data obtained by the electrical stimulations performed in the functional mapping study of the insula and the perisylvian regions. In the present study we will present the results of three injections; one in the left hemisphere and two in the right hemisphere. The injection sites were chosen as representative sites of the most interesting functions elicited by the electrical stimulations, consequently the stereotactic coordinates of the injection sites were known from the previous ICMS study:

- The first injection was performed in the most anterior part of the anterior complex where ICMSs evoked oro-alimentary behaviors (mostly visceral) jointly to potent bradycardia.
- The second injection was performed in the ventral middle insula where ICMSs evoked communicative responses.
- The third injection was performed in the dorsal posterior complex where ICMSs evoked responses mostly involving the lower extremity in both simple movements and miscellaneous responses categories.

3. Tracer Injections and Histological Procedures

Once the appropriate sites were chosen, the retrograde tracers Fast Blue (FB, 3% in distilled water, Dr Illing Plastics GmbH, Breuberg, Germany), Diamidino Yellow (DY, 2% in 0.2 M phosphate buffer at pH 7.2, Dr Illing Plastics), and the retro-anterograde tracer-Dextran conjugated with tetramethylrhodamine (10 000 MW, Fluoro-Ruby, FR, 10% 0.1 M phosphate buffer, pH 7.4; Invitrogen--Molecular Probes), were slowly pressure-injected through a glass micropipette (tip diameter: 50--100 lm) attached to a 1- or 5-µL Hamilton microsyringe (Reno, NV). The desirable depths of the injection sites were determined on the basis of the data of the ICMS study; in addition, these sites were reached under visual guidance by means of the ultra-sound imaging (see materials and methods of ICMS study). Table 1 summarizes the locations of injections, the injected tracers, and their amounts. The tracer injections were performed with respect to the appropriate survival periods following the injections (28 days for FR, 12--14 days for FB, and DY). After these periods the animal was deeply anesthetized with an overdose of sodium thiopental and perfused consecutively with saline, 3.5-4% paraformaldehyde, and 5% glycerol, prepared in 0.1 M phosphate buffer and pH 7.4, through the left cardiac ventricle. The brain was then blocked coronally on a stereotaxic apparatus, removed from the skull, photographed, and placed in 10% buffered glycerol for 3 days and 20% buffered glycerol for 4 days. Finally, it was cut frozen into coronal sections of 60-μm thickness.

Monkey	Hemisphere	Area	Tracer	Amount
MK1	L	Ial	FR 10%	1 * 1 μl
MK1	R	Idg	FB 3%	1 * 0.2 μl
MK1	R	SII/Ig	DY 2%	1 * 0.2 μl

Table C1: Localization of the cortical injection sites, tracers and amount employed in the experiment.

For the visualization of fluorescent tracers FB, or DY, every fifth section was mounted, air-dried, and quickly coverslipped for fluorescent microscopy.

One series of each fifth section was processed to visualize antero-retrograde tracer FR, using the following protocol: After rinsing in PBS, the sections were incubated for 72 h at 4 C° in a primary antibody solution of rabbit anti-FR (1:3000; Invitrogen) in 0.3% Triton, 5% normal goat serum in PBS, and, after rinsing in PBS, were incubated in biotinylated secondary antibody (1:200, Vector) in 0.3% Triton, 5% normal goat serum in PBS. Subsequently, after rinsing in PBS, FR labeling was visualized using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and the Vector SG peroxidase substrate kit (SK-4700, Vector) as a chromogen. In all cases, one series of each fifth section was stained by Nissl method (0.1% thionin in 0.1 M acetate buffer, pH 3.7).

III. Data Analysis

1. Injection Sites and Distribution of Retrogradely Labeled Neurons

The fluorescent material was examined at 400X, with a Nikon Universal epi-fluorescence microscope equipped with a narrow band excitation filter (BP 365/11), a dichroic mirror (FT 395), and a barrier filter (LP 395). The FR material was analyzed at 200X, with a Zeiss Axioscop 2 microscope, using a white light.

Two concentric zones were defined for each tracer injection: a central zone, defined as the core and a surrounding zone, defined as the halo.

The core of the FR injection site was defined by a very heavily stained area surrounding the point of tracer administration, of about 1 mm in diameter for injections of 1 µL. A region of dense, nonspecific-cell and background staining that, though with a decrease in intensity, extended for several millimeters, surrounded the injection site.

The core of the fluorescent tracers injection sites (FB & DY) was considered to include an inner zone (zone I), sharply delineated around the needle track, which appeared necrotic and intensely fluorescent and a second zone (zone II), less sharply delineated and less brilliantly fluorescent, in which almost all of the neurons and many glial cells showed bright fluorescence. The halo was defined as the region, which contained some background tissue fluorescence, and in which almost all neurons and some glial cells were labelled. Zones I and II should correspond to the effective area of uptake and transport of fluorescent tracers.

The comparison between cytoarchitectonic characteristics of the Nissl-stained sections adjacent to the injections sites and the cytoarchitectonic criteria of the areas of the perisylvian regions, described by Mesulam and Mufson (1982) and Carmichael and Price (1994) was made in order to attribute the exact locations of the injections sites.

Retrogradely-labeled neurons were analyzed in sections every 300 µm and only plotted in representative sections. The fluorescent neurons were identified as follows: FB-labeled neurons by a sky-blue fluorescence in the cytoplasm; DY labeled neurons by a yellow-green fluorescent nucleus; The FR labeled neurons and terminals were identified by a dark-blue coloration in the cytoplasm and synaptic buttons, respectively. In each examined section the outer and inner cortical borders and the location of each labeled neuron were plotted with the aid of inductive displacement transducers mounted on the X and Y axes of the microscope stage. The transducer signals were digitized and stored by using software developed in our laboratory that allows the visualization of section outlines, of grey-white matter borders, and of labeled cells.

2. Areal Attribution of the Labeling

The attribution of the labeling to the Fronto-parietal operculum areas was made according to the architectonic subdivision by Roberts & Akert (1965); Jones and Burton (1976); Krubitzer et al. (1995); and Cippolloni & Pandya (1999). The insula was subdivided according to Mesulam & Mufson (1982), while the orbito-frontal cortex and the anteriormost part of the agranular cortex were subdivided according to Carmichael & Price (1994). The Temporal areas of the STS and inferior temporal gyrus were subdivided according to Boussaoud et al. (1990) and Saleem & Tanaka (1996) ; Saleem et al. (2000, 2008) respectively. The labeling in the agranular frontal areas was attributed according to the subdivision made by Matelli et al.(1985, 1991) and Belmalih et al. (2009). In the amygdala it was adopted the subdivision of Pitkanen and Amaral (1998). For the labeling in the cingulate cortex we used the architectonic subdivisions made by Morecraft et al. (2004-2007).

IV. RESULTS

1. Injection Sites

All the injection sites illustrated in Figutre C1, but one, were completely restricted to the grey cortical matter involving the entire cortical thickness.

In the anterior perisylvian complex the injection site (FR) was located within the caudo-lateral agranular orbital cortex (OFa-p) recognized by Carmichael & Price (1994) as the lateral agranular insula area (Ial) (Figure C1-A). The halo of the injection site spread to the other agranular insular areas in the caudal orbital cortex and area 12o (OFdg) in the lateral orbital cortex. The ICMS study showed that the area of the injection site is involved in visceral aspects of oro-alimentary behavior and in the vagal activation of the autonomic nervous system. In this case, there is a narrow strip of the tracer in area 46v/8r above the injection site. Consequently, on the basis of what we know in the literature on the anatomical connections (Gerbella et al.; 2010) of both injected and contaminated area, we excluded the labeling due presumably to the tracer in the contaminated area.

In the middle perisylvian complex, the tracer (FB) was injected in the ventral insula (Figure C1-B). The injection site was strictly limited to the grey matter of the dysgranular insula. Electrical stimulation of the injected zone evoked communicative responses where the monkey produced lip-smacking only in case of eye contact with the experimenter.

The third tracer (DY) was injected in the posterior perisylvian complex (Figure C1-C). The injection site was located in the most medial portion of the parietal operculum (SII) bordering the granular insula. The electrical stimulation of the area covered by the tracer injection evoked mainly hindlimb responses.

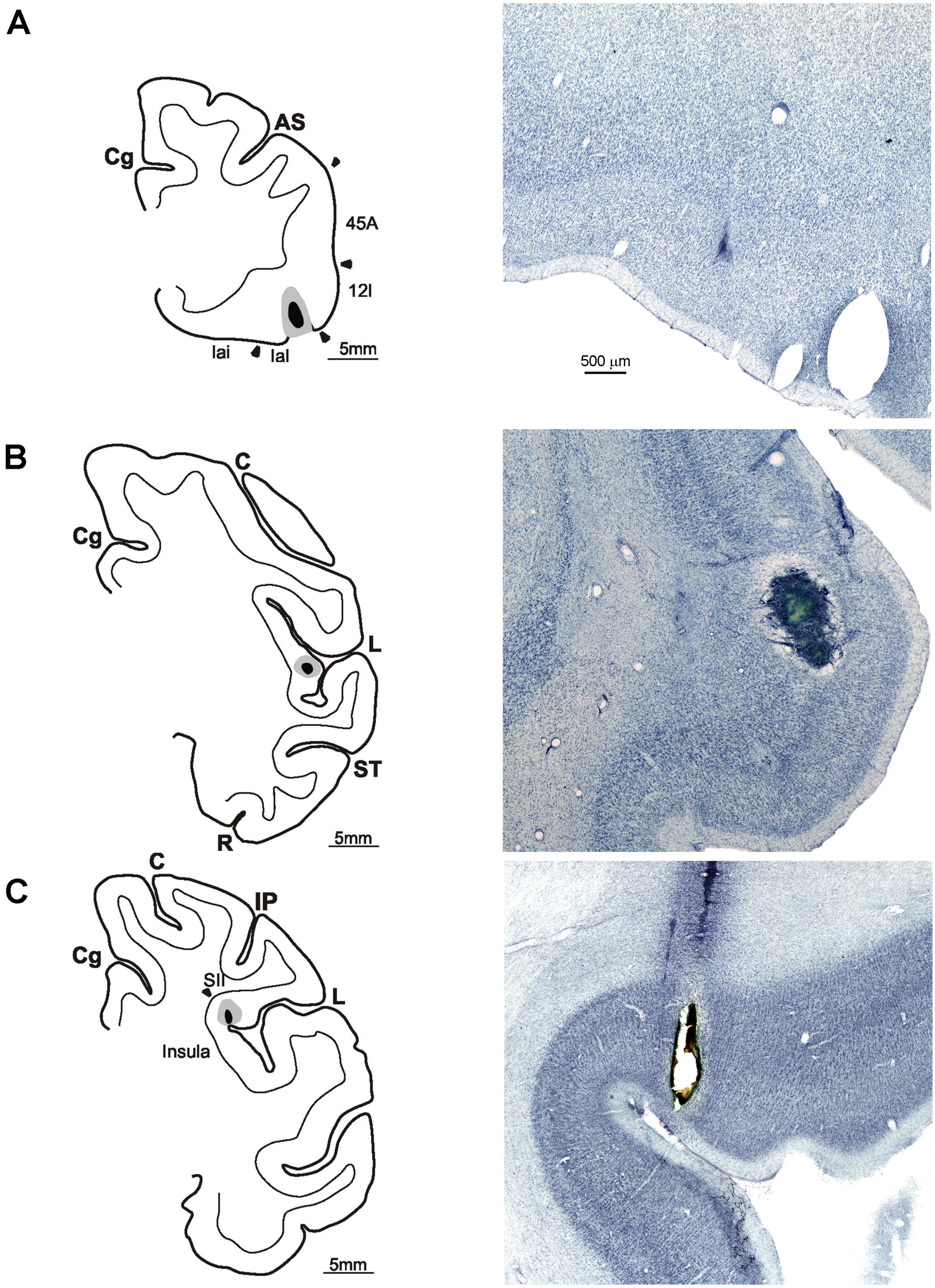


Figure C1: Locations of the injection sites shown in coronal sections (in the left side) through the core (black) and the halo (light gray), and in photomicrographs of Nissl-stained sections (in the right side). A: the FR injection site in Ial, B: the FB injection site in ventral Idg, and C: DY injection sites in SII/Ig. AS: superior arcuate sulcus; C: central sulcus; Cg: cingulate sulcus, IP: inferior parietal sulcus, L: lateral fissure, R: rhinal sulcus, ST. superior temporal sulcus.

2. Connections of the anterior perisylvian complex

a. Cortico-cortical connections

Figure C2 shows the distribution of the retrograde (red) and the anterograde (blue) labeling observed following the FR injection.

In the frontal lobe the labeling (Figure C3-A) was very dense in the prefrontal areas 12l/12o (OFg/OFdg) , 45A and 8 (Figure C2, sections e-h). Strong connections were also observed in areas 12r and 46v (Figure C2, sections a-g). In the dorso-lateral prefrontal cortex, the labeling was found also in areas 9 , 8b, 8r (Figure C2, sections a-f) and the dorso-mesial area 6 (F6/F7) (Figure C2, sections f-g). Relatively dense labeling was observed in the orbital areas 13m (OFdg), 13b (OFdg), 11 and 14 (Figure C2, sections a-g). Two other sectors of the frontal lobe were labeled: area 44, in the fundus of the inferior arcuate sulcus, was strongly labeled while more weak labeling was observed in the ventral premotor area F5.

In the orbito-frontal operculum (OFO) and in the precentral cortex (PrCO) (Figure C2, sections i-k), relatively dense labeling was found; this labeling extends more weakly in the adjacent the dysgranular opercular area (DO) (Figure C2, sections j-k).

In the medial wall, relatively thick connections were (Figure C3-B) observed in the anterior cingulate areas 32, 24b/c, 24a and 24b (Figure C2, sections c-k).

In the temporal cortex, dense labeling (Figure C3-C) was found in the temporal gyrus (TG) (Figure C2, sections i-j) and in the rostral part of the area STP (Figure C2, sectionK). While relatively lower density of labeling was observed in a more caudal sector of area STP, in area IPa of the STS fundus, and in the areas TEam, TEa/d of the inferior temporal gyrus (Figure C2, sections k-m). Labeling was also found in the entorhinal cortex (ECO) (Figure C2, sections j-m).

b. Amygdaloid connections

Figure C4-A and Figure C2 show the distribution of the retrograde (red) and the anterograde (blue) labeling in the amygdala.

Dense retrograde labeling was found in the basolateral nucleus of the amygdala (Figure C3-D; Figure C2, sections i-m), mostly in its parvicellular division, while anterograde labeling was seen in its intermediate and magnocellular divisions. Relatively weak labeling was found in both lateral and basal medial nuclei of the amygdala.

c. Brain stem connections

Figure C5 shows the labeling in the brain stem. Brain stem connections were found with the caudo-lateral portion of the hypothalamus as well as with the ventral tegmental area (VTA).

3. Connections of the middle dysgranular insula

a. Cortico-cortical connections

Figure C6 shows the distribution of the retrograde (blue) labeling observed following the FB injection in the ventral middle dysgranular insula (Idg).

Within the insula, very dense labeling was found in the adjacency of the injection site, conversely to the relatively weak labeling observed in the anterior agranular and the posterior granular portions.

In the frontal lobe, dense labeling was found in the orbito-frontal areas 12m, 11, 13l, and 13m (Figure C6, sections b-c) and albeit more weakly in the ventrolateral prefrontal areas 12r and 46v/45A (Figure C6, sections a-c). Outside of the prefrontal cortex, relatively robust connections were found in the area 44 (Figure C6, section d) of the fundus of the inferior arcuate sulcus, in the frontal operculum area PrCO (Figure C6, section f) and in the newly architectonic defined granular frontal area (GrFr) (Figure C6, section d); in the premotor cortex, relatively weak labeling was observed only in the convexity sector of area F5 (F5c) (Figure C6, section e).

In the medial wall, labeling was found in the cingulate areas 24b/c (Figure C6, sections e-h).

In the parietal operculum, dense labeled cells were found in the second somatosensory area (SII) (Figure C6, sections i-k).

In the temporal cortex, relatively dense labeling extends from the rostral temporal pole (TG) (Figure C6, sections f-g) throughout the areas IPa and TEa/m of the STS and the area TE of the inferior temporal gyrus (Figure C6, sections h-k) to the entorhinal cortex (ECO) (Figure C6, sections g-j).

b. Amygdaloid connections

Figure C4-B and Figure C6, sections i-j show the distribution of the retrograde (blue) labeling found in the amygdala following the FB injection in the ventral middle dysgranular insula.

Dense labeled cells was found in the ventral portion (parvicellular division) of the basolateral nucleus of the amygdala, while more weak labeling was observed in the dorsal portion (intermediate and magnocellular divisions) of the basolateral nucleus as well as in the lateral and basal medial nuclei.

4. Connections of the posterior perysylvian complex (SII/Ig)

Figure C7 shows the distribution of the retrograde (green) labeling observed following the DY injection. The injection site was located in the posterior portion of the perisylvian regions in a position between the most medial part of the parietal operculum and the most lateral part of the granular insula.

Dense labeling was observed in the opercular and insular territories surrounding the injection site (Figure C7, sections f-g). This labeling extends caudally throughout the retroinsular area (Ri) (Figure C7, section h) and the opercular portions of the areas PF and PG (PFop & PGop) to the postauditory cortex (PA) and the temporoparietal area Tpt (Figure C7, sections h-j).

Relatively week labeling was found in the caudal portion of the mesial premotor area (F3) and the rostral part of the mesial sector of the primary motor cortex (F1) (Figure C7, sections c-e), while this labeling become more consistent in a more posterior section of F1 (Figure C7, sections f-h).

Dense labeling was observed in the mesial and lateral portions of the primary somatosensory cortex (SI) as well as in PE , PEc and MIP areas in the posterior parietal cortex (Figure C7, sections g-j).

In the inferior parietal lobule, relatively weak labeling was also found in the area AIP of the ventral bank of the IPS (Figure C7, section h).

In the medial wall, dense labeling was observed in the mid-posterior cingulate areas 24c/d and 23 (Figure C7, sections a-g).

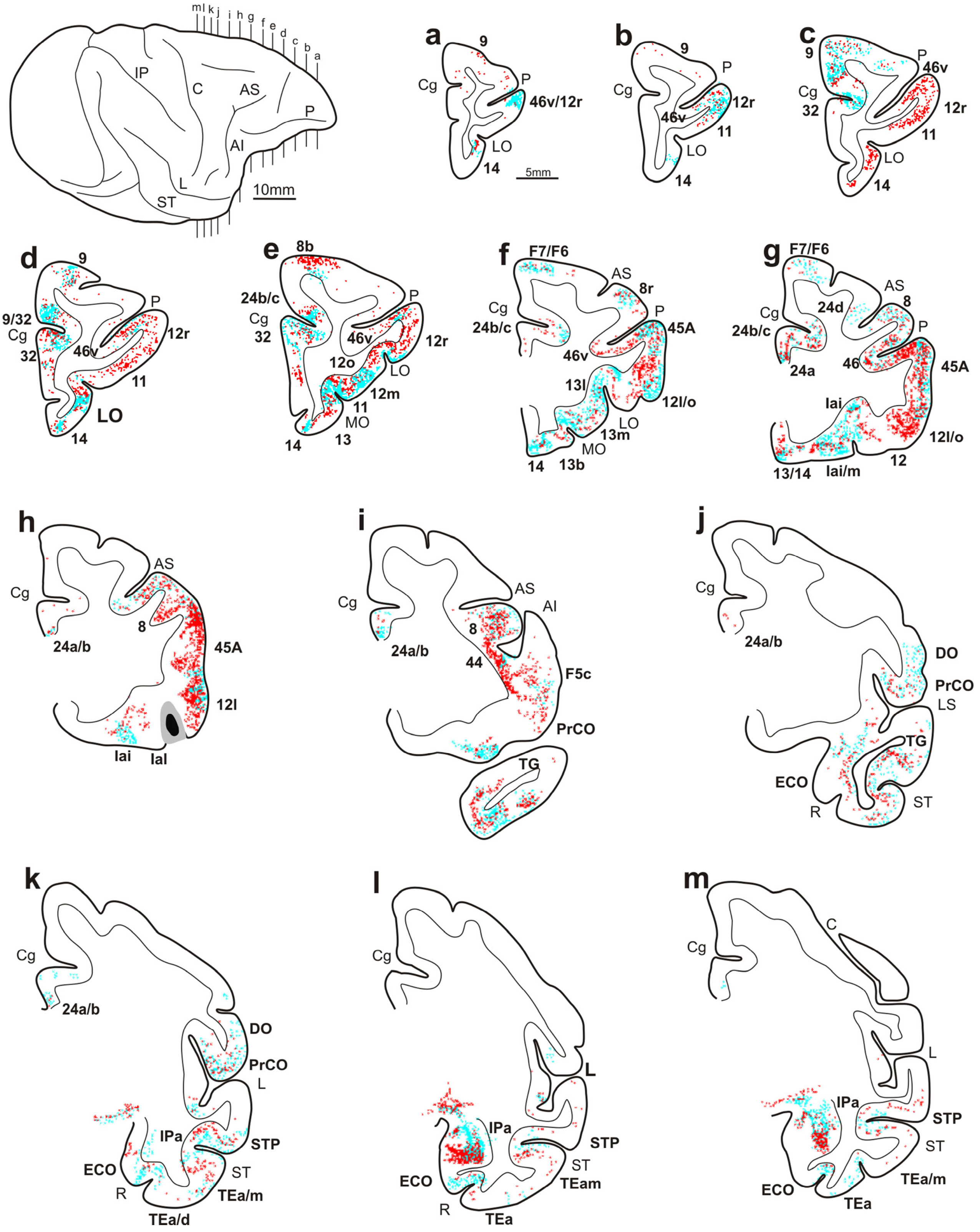


Figure C2: Distribution of the retrograde (red) and anterograde (blue) labeling observed in the case of FR injection, shown in drawings of coronal sections. Sections are shown in a rostral to caudal order (a-m). The dorsolateral view of the injected hemisphere in the upper left part of the figure shows the level at which the sections were taken. AI: inferior arcuate sulcus; LO: lateral orbital sulcus, P: principal sulcus, other abbreviations as in figure C1.

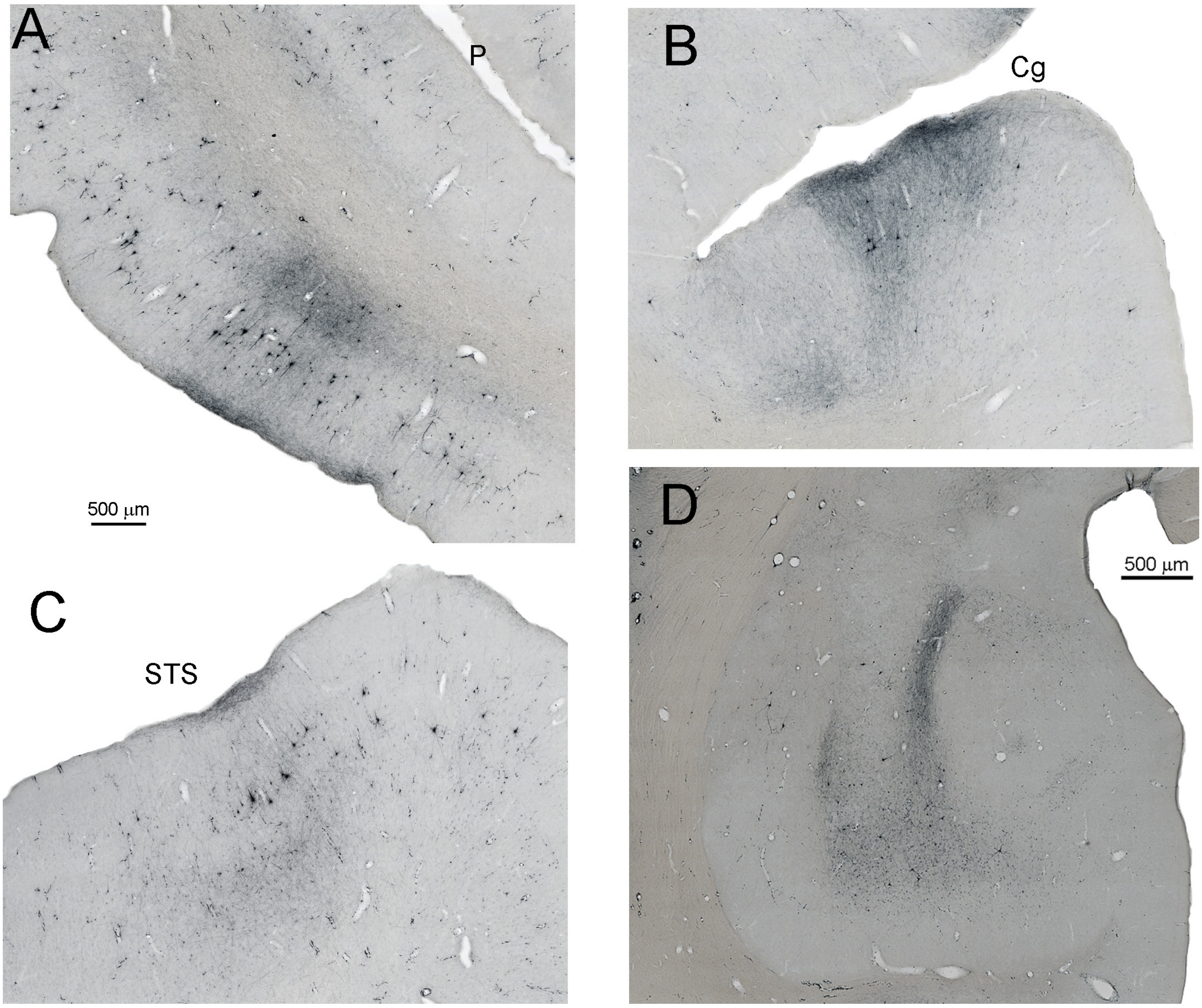


Figure C3: Photomicrographs showing examples of the retrograde and anterograde labeling observed following FR injection in Ial. A: area 11; B: 24b/c; C: TG; D: amygdala.

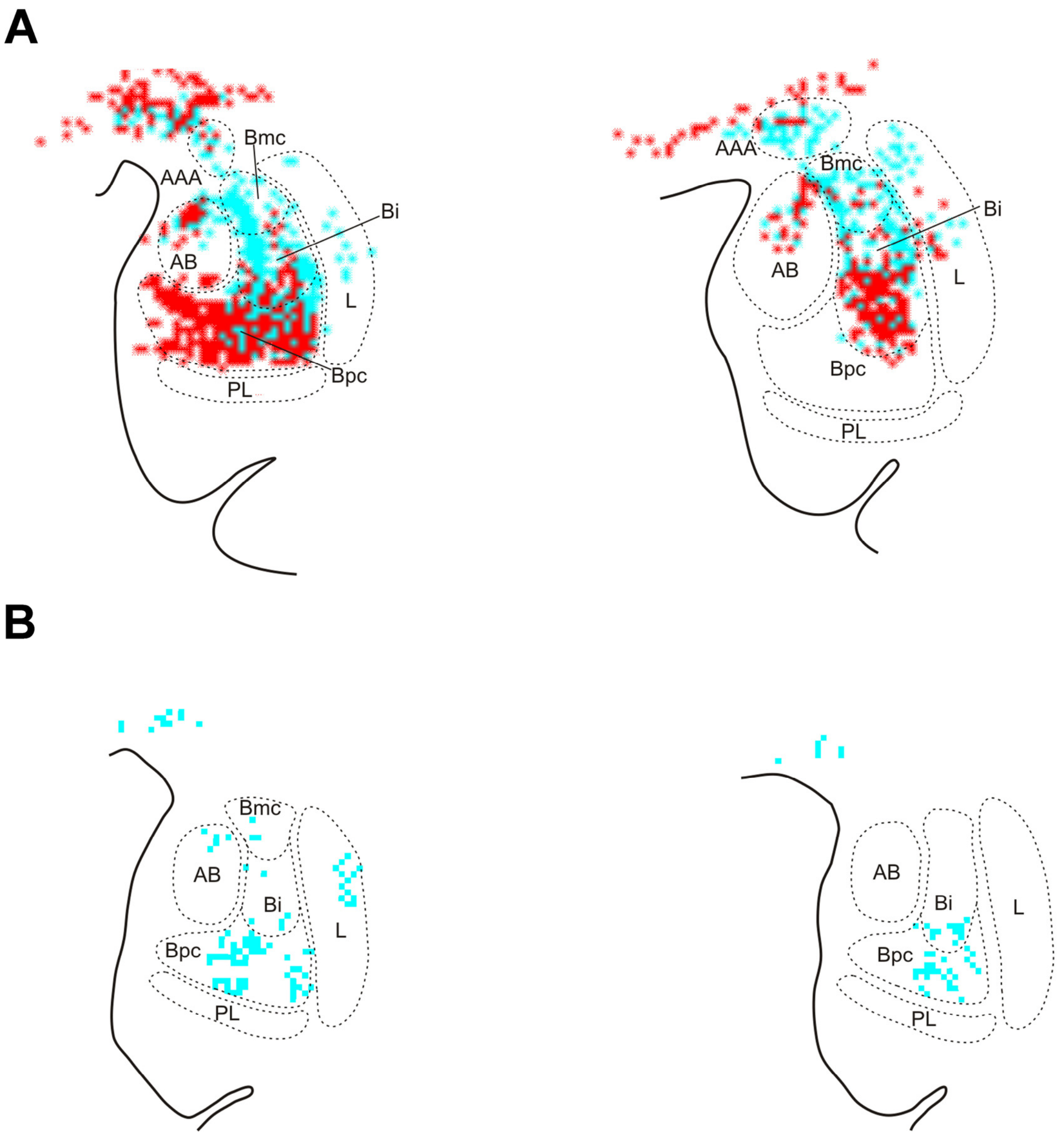


Figure C4: A: Distribution of the retrograde (red) and anterograde (light blue) labeling observed in the amygdala following the FR injection. B: Distribution of the retrograde labeling observed in the amygdala following the FB injection. AAA: anterior amygdaloid area; Bi: basal nucleus, intermediate division; Bmc: basal nucleus, magnocellular division; Bpc: bsal nucleus, parvicellular division; L: Lateral nucleus of the amygdala; PL: paralaminar nucleus of the amygdala.

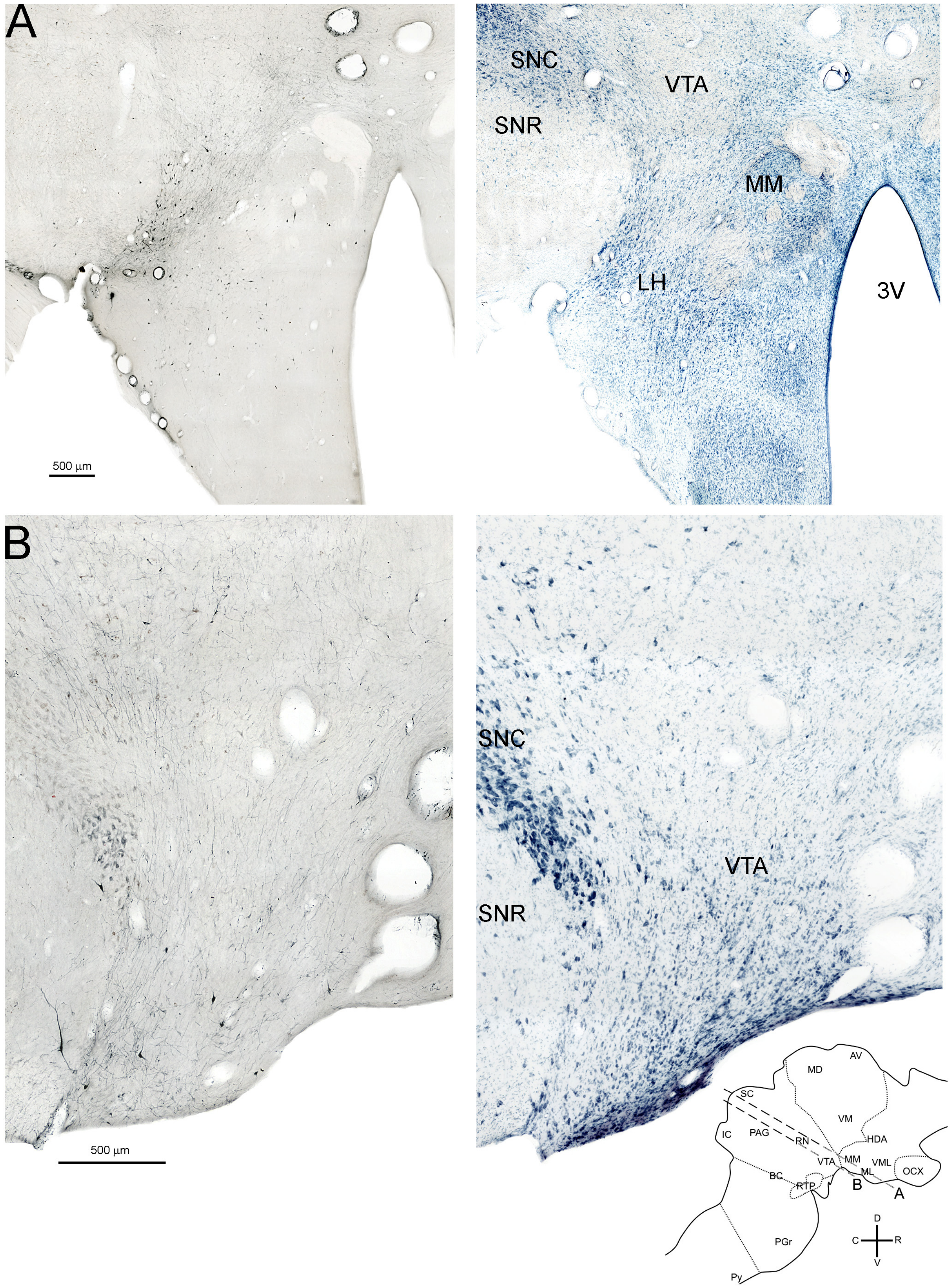


Figure C5: Distribution of retrograde and anterograde labeling observed in the brain stem regions following the FR injection, shown in photomicrpgraphs of FR-stained sections (left). In the nissl-stained photomicrographs (right), are indicated the areal subdivision of the section. The lateral view of the brain stem in the lower right part of the figure shows the levels at which the the sections were taken. 3V: third ventricle; LH: lateral hypothalamus; MM: medial mammillary nucleus; SNC: substantia nigra compacta; SNR: substantia nigra reticulate; VTA: ventral tegmental area.

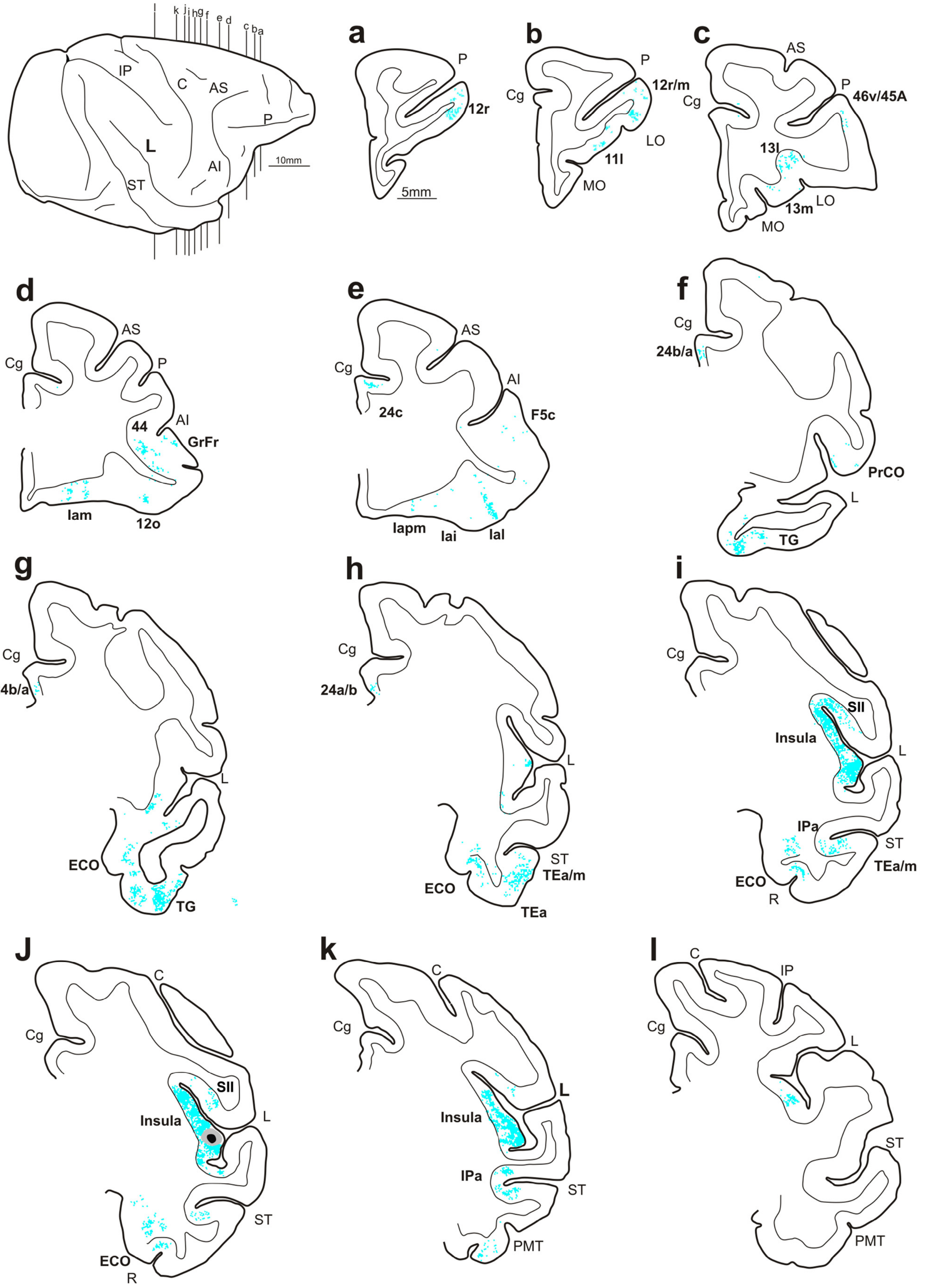


Figure C6: Distribution of the retrograde labeling observed following the FB injection in the ventral Idg, shown in drawings of coronal sections in a rostral to caudal order (a-i). the dorsolateral view of the injected hemisphere in the upper left part of the figure shows the levels at which the section were taken. Conventions and abbreviations as in figure C1 and C2.

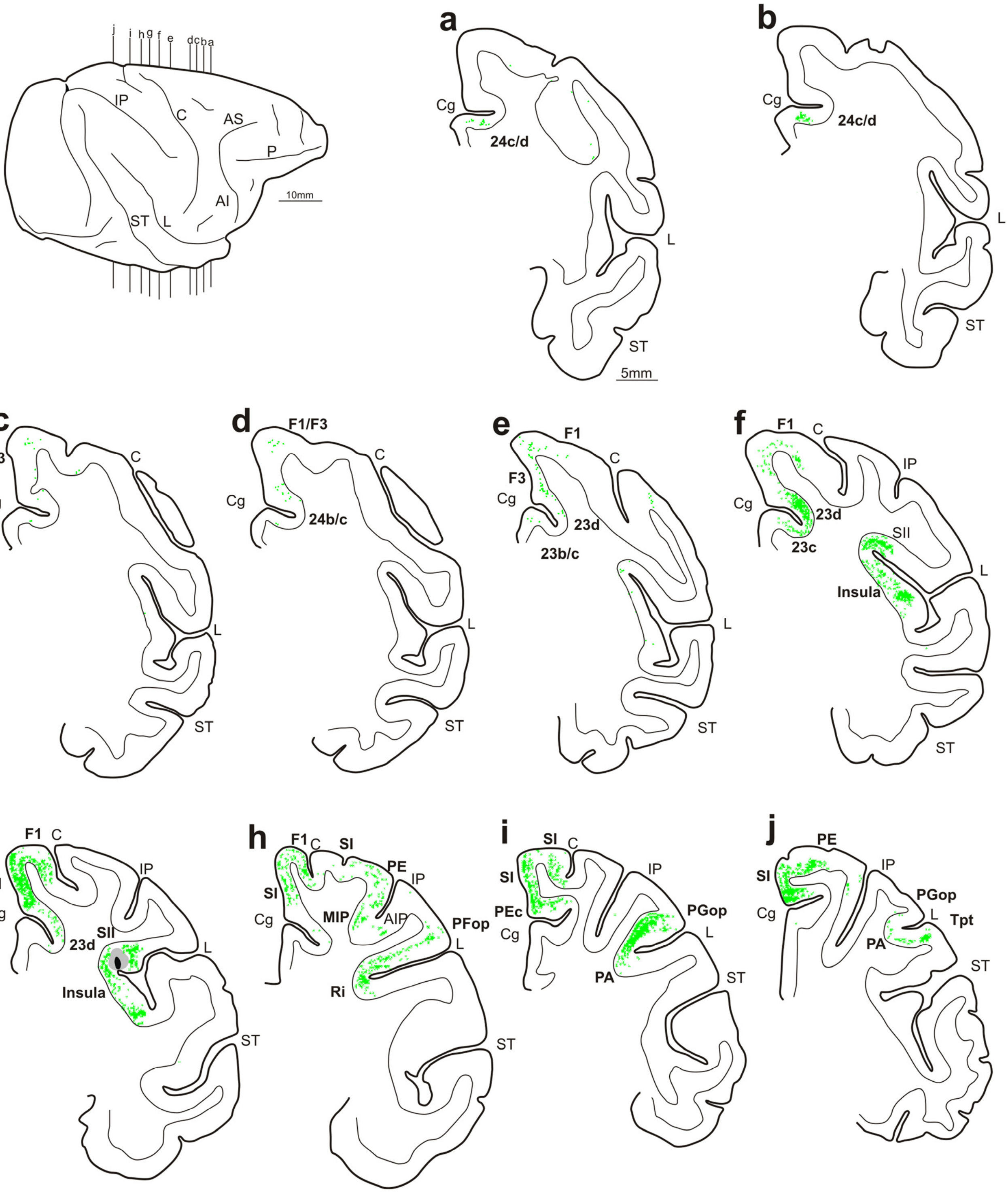


Figure C7: Distribution of the retrograde labeling observed following the DY injection, shown in drawings of coronal sections in a rostral to caudal order (a-j). The dorsolateral view of the injected hemisphere in the upper left part of the figure shows the levels at which the section were taken. Abbreviations as in figure C1 and C2.

V. DISCUSSION

The present study showed that the three investigated functional fields of the perisylvian regions display different connectivity patterns. These data hodologically complement the difference of the cytoarchitectonic organization of the studied regions and appear to be helpful in the understanding of the behavioral responses evoked by ICMS of the studied regions.

The agranular lateral insula as well as the areas of the caudo-lateral orbital cortex display reciprocal connections with the lateral orbital areas (14, 11, 13b, 13m), the prefrontal cortex (9, 46v, 12r, 12o, 12l, 12m) the anterior cingulate areas (24a/b/c, 32), the temporal gyrus, the supratemporal pole (STP), the inferior temporal gyrus (IPa, TEa/m, TEa) and the entorhinal cortex. The anterior complex has also connections with the amygdala (AB, L), with the hypothalamus, and the ventral tegmental area in the brain stem.

According to Gerbella and colleagues (2010), the labeling found in the prefrontal areas 8r, 8b, 8, (F6/F7) and the caudal part of the periprincipal area 46 are likely due to the tracer residues in the prefrontal cortex.

The connections of the middle dysgranular insula with the frontal lobe are limited to light to moderate labeling in the lateral orbito-frontal areas (12r, 12m, 11, 13l) and in the bank of the inferior arcuate sulcus (area 44 and GrFr), while with the cingulate cortex, the connections are limited to the ventral mid-posterior areas (24a/b/c). The connections with the temporal gyrus are less diffused than in the case of the anterior complex and are limited to the ventro-medial portion. Connections are present also with the inferior temporal gyrus (IPa, TEa/m and TEa). Also in this case there are connections with the amygdala (AB, L, and AB nuclei)

The posterior granular perisylvian complex (SII/Ig) has connections with primary somatosensory cortex, the primary motor cortex (F1), the mesial premotor cortex (F3), posterior parietal areas (PE, and PEc), the posterior cingulate areas (24c/d and 23b/c/d), the inferior parietal areas (MIP, AIP, PFop, and PGop), the retroinsular cortex, and the post-auditory cortex. Unlike to the anterior and posterior regions, the posterior complex doesn’t have connections with the temporal gyri or with the amygdala.

The results of the present study support the earlier description of the connections of the insula and inner perisylvian regions. In their study Mesulam and Mufson (1982) reported differences in the cytoarchitectonic distribution of the connections within the insula. In line with the findings of the present study they reported that connections with the orbito-frontal and frontal operculum are likely limited to the anterior insula while connections with somatosensory areas as well as with the parietal lobule areas are likely restricted to the posterior perisylvian complex. Unlike Mesulam and Mufson conclusions that cingulate connections are limited to the posterior insula, our results suggest a rostro-caudal gradient of the cingulate connections, where the anterior perisylvian complex is connected with the anterior cingulate areas, whereas posterior cingulate areas are connected with the posterior perisylvian complex.

The results on amygdaloid complex connections reported in the present study are partially in agreement with an earlier study (Mufson et al., 1981) where the authors reported that such connections appear to be limited to the anterior agranular-dysgranular insula, while in our study we show that amygdala is connected with both the most anterior portion of the perisylvian regions and the mid-posterior dysgranular portion.

The results of the anterior injection are in accordance with the results of the previous studies. On the one hand the present results confirm the findings of earlier study (Petrides & Pandya, 2001) where the injection site was localized in a position similar to that adopted in our study. On the other hand, the results are in agreement with the conclusion reported by Mesulam and Mufson that in addition to the shared cytoarchitectonic organization, the anterior insula and the lateral orbitofrontal regions share similar connectivity patterns. Actually our results due to the injection of the intermediate agranular insula and surrounding regions in the caudal orbital cortex had similar connectivity pattern showed in the study of Mesulam and Mufson following tracer injections in the anterior granular-dysgranular insula.

In comparison with Mesulam and Mufson study, in the present study, the injection in the dysgranular insula interested a more posterior portion. The results of this injection are partially in accordance with the previous study. In the present study the labeling was more restricted than in the earlier study. That could be due to the limited diffusion of the tracer. Like in the earlier study, connections were found with the frontal areas 12, 11, and 13, with the caudal orbito-frontal regions, regions ventral to the inferior arcuate sulcus (44, F5c), the ventral cingulate areas and superior and inferior temporal pole. Unlike to the earlier study, there are no connections with the inferior parietal lobule.

Despite the fact that the third tracer injection in the present study concerned a position that was not included in the previous studies, since the injection sites were mainly located in more lateral position (Cipolloni & Pandya, 1999; Disbrow et al., 2003) or concerned different functional fields (Friedman et al., 1986), our results are in agreement with these previous studies where connections were found with primary somatosensory cortex, primary motor cortex, mesial premotor and posterior cingulate cortex as well as with the inferior parietal lobule, the dysgranular and granular insula, and retro-insular cortex. The main difference between the present and previous data is the topographic distribution of the labeling, particularly in the somatosensory and motor areas, where the labeling distribution reflected the responses evoked by ICMS experiment.

VI. FUNCTIONAL CONSIDERATIONS

1. Anterior perisylvian complex

In the ICMS experiment of the present study we showed that the electrical stimulation of the anterior insula and adjoining perisylvian regions evoked a wide range of oro-alimentary responses including complex motor-like feeding behavior consisting in chewing, mouthing and/or swallowing movements, the alteration of the feeding behavior consisting in aversion or selective behaviors toward the food, or more explicit visceral behavior like in the case of the vomiting impulses. These behavioral responses were mostly accompanied with modulation of the autonomic functions identified by means of the bradycardia occurred during the ICMS. On the one hand these results support the hypothesis that the anterior insula and perisylvian regions are involved in the processing of various sensory and non-sensory aspects of feeding behaviors. On the other hand, the connectivity patterns of the anterior perisylvian regions, emerged from the present hodological study, seem to give explanation to the functional roles elucidated by the ICMS experiment.

The sensory-motor aspects of the evoked oro-alimentary behaviors could find support in the connections with areas 44 and F5c. A recent study (Petrides et al., 2005) showed that the area 44 is involved in oro-facial somato-motor responses where ICMS evoked jaw movements such as jaw closing, sequences of jaw opening and closing, and jaw movements with horizontal vector component. In the ventral premotor area F5c neurons tend predominantly to code mouth movements (Gentilucci et al., 1988). In particular, half of these neurons code mouth actions while the other half codes both mouth and hand actions (Ferrari et al., 2003). Furthermore, the anterior cingulate cortex hosts two facial representation areas that in collaboration with the facial representation areas within the motor and premotor cortices are involved in the control of facial movements of different functions such as chewing, swallowing, and social interaction (Morecraft et al., 2004). In particular, the facial representation areas in the anterior cingulate cortex are involved in the control of emotional expression.

The connections with the orbito-frontal, anterior cingulate areas as well as with the amygdala support the view of the involvement of the anterior perisylvian regions in neural circuits engaged in the processing of sensory, affective and motivational aspects of feeding behavior. Kadohisa and colleagues (2005) showed that neurons in the insula/frontal operculum (primary gustatory cortex), orbitofrontal cortex and amygdala responded to oral sensory stimuli (taste, texture, temperature). In insula/frontal operculum, stimuli are likely to activate different neurons while there are more convergence in the OFC and amygdala. According to Rolls (2005, 2007) neurons in the anterior insula and adjacent frontal operculum respond to the identity and intensity of taste independently from other aspects such as satiety. The OFC contains the secondary gustatory cortex where neurons not only code taste identity but other somatosensory taste aspects such as viscosity, texture or temperature. Some neurons respond to a combination of these aspects. Other neurons respond to the view of the food or to odors. Furthermore the OFC neurons represent the reward value of the food, given that the neural responses to the different sensory aspects decrease to zero if a given food is eaten to satiety. Furthermore, it was shown in humans that activation of the OFC and adjoining anterior cingulate cortex by odors (O'Doherty et al., 2000) and by liquid food (Krigelbach et al., 2003) is hunger-dependent, suggesting the presence of a tertiary gustatory cortex in the anterior cingulate cortex (Rolls, 2005; 2009).

Figure C8 is a schematic illustration of the taste pathway in primates, including humans, proposed by Rolls (2009) and modified according to our results. Unimodal processing provides cortical representation of the different sensory aspects of a given stimulus. The unimodal pathways converge in order to form multimodal representations, in disagreement with Rolls proposal, in the anterior insula, OFC and amygdala and then behavioral and autonomic responses to food presentation or intake are generated by means of later processing within the premotor areas, cingulate cortex, lateral hypothalamus and the basal ganglia. A very important element in the affective and motivational aspects of feeding behaviors is the ventral tegmental areas involved in the so-called reward circuit, further corroborating the notion of cognitive functions within the insula.

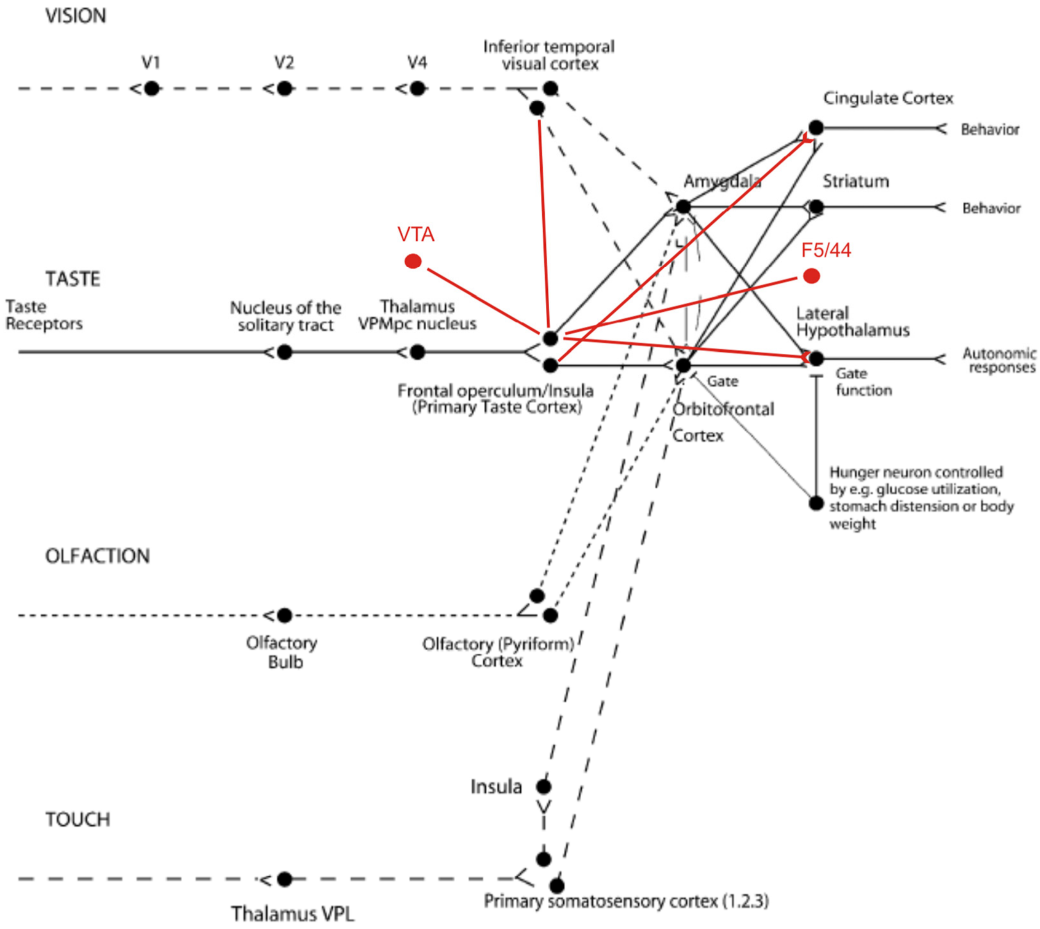


Figure C8: Schematic diagram of the taste pathway in primates including humans modified form Rolls, 2009. the red color indicate the added insular connections according to our results.

The connections of the anterior insula and adjoining perisylvian regions support the involvement of these regions in the control of the autonomic functions. The anterior cingulate cortex has an “affect division” including areas 25, 33 and rostral area 24. This division has robust connections with the amygdala and the peri-aqueductal grey, projects to autonomic brainstem nuclei and consequently plays a role in the control of the autonomic and endocrine function (Devinsky et al., 1995). The connections with the caudo- lateral hypothalamus confirm the involvement of the rostral agranular insula in the so-called “medial prefrontal” and “orbital prefrontal” networks (Charmichael & Price, 1996; Öngür & Price, 1998; Saleem et al, 2008) that are involved in the control of the regulation of autonomic and homeostatic responses (for more details see Introduction).

In 2003 Wicker and colleagues performed an fMRI study on humans in which participants inhaled odorants producing a strong feeling of disgust. The same participants observed video clips showing the emotional facial expression of disgust. Observing such faces and feeling disgust activated the same sites in the anterior insula and to a lesser extent in the anterior cingulate cortex. Thus, as observing a hand action activates the observer's motor representation of that action (Gallese et al. 1996), observing an emotion activates the neural representation of that emotion. The connections with the visual areas of the inferior temporal pole as well as with the anterior cingulate cortex and the ventral premotor cortex support the hypothesis that neural representation of behaviors such as disgust grimaces or vomiting impulses, evoked by ICMS in the anterior insula, could be activated by the observation of the same behaviors.

2. The middle ventral dysgranular insula

ICMS performed in positions corresponding to the injection site in the ventral dysgranular insula evoked communicative responses consisting in appeasing/submissive expressions that are lip smacking and teeth chattering. Such responses were evocable only when the electrical stimulation occurred during eye contact between the monkey and the experimenter. Even when the monkey was representing spontaneous facial expressions such as open-mouthed threat or avoidance, at the moment of the stimulation the monkey switched to the lip smacking interrupting the spontaneous expression. Neither of the two conditions (ICMS or eye contact) was able alone to evoke the lip smacking. Facing the monkey without stimulation evoked mostly an open-mouthed avoidance expression. Recently, the insula is thought to constitute the neural substrate of the representation of internal body states providing the bases for subjective feelings, consciousness of emotional experiences and self-awareness (Craig, 2002; 2009). The mental representation of oneself is considered as the first requirement in the awareness of a given object (in our case the object is the experimenter), the second requirement is the mental representation of the object, while the third requirement is the mental representation of the interrelationship between oneself and the object in the moment of interaction (Craig, 2009; Frith et al., 1999).

The present study showed that the middle ventral dysgranular insula has connections with the amygdala, the inferior temporal pole and the anterior cingulate cortex. All these brain areas are related in some way with the perception or the production of emotional facial expressions (Perrett et al., 1982, 1984; Rolls et al., 1982; Baylis et al., 1987; Gothard et al., 2007; Hadj Bouziane et al., 2008; LeDoux, 2000, 2003; Damasio et al., 2000)

Morecraft and colleagues (2007) stated, "the amygdala is a critical component of a neural network that interprets the emotional aspects of sensory stimuli, and contributes to the generation of appropriate responses to emotionally significant stimuli by binding perceptual representations of sensory stimuli with memory and emotional experiences".
As suggested by Morecraft, an "emotional stimulus" should be "significant" in order to generate a motor facial expression response. The electrical stimulation of the ventral dysgranular insula evokes a re-representation of the internal body states. This representation of oneself would allow the experimenter's face to be perceived (in the inferior temporal pole and amygdala) and interpreted (in amygdala) in relation to other emotional experiences and consequently a new appropriate response to the new signification of the stimulus can occurr. The motor output finds its foundations in the insular connections with the anterior cingulate cortex as well as in the amygdala connections with the face region in the anterior cingulate motor cortex, which, in turn, is connected with the facial nucleus (Morecraft et al., 2007-2001-1996) and with other facial representations of the motor cortex (Morecraft et al., 2004).

3. Posterior perisylvian complex

The DY injection site was located in the posterior perisylvian complex where ICMS evoked responses that involved mostly the lower extremity in both simple movements and miscellaneous responses categories. As we noted in the first part of the discussion, the hodological data of the present study confirm in general the connectivity patterns showed in previous studies. Furthermore, our data showed a robust selectivity within these connectivity patterns. This sector of the posterior perisylvian complex is connected to the caudal part of area F3 of mesial area 6, with the rostral dorsal and mesial portions of area F1, and with the posterior motor cingulate cortex. It was demonstrated that al these regions are hindlimb representation regions (Luppino et al., 1991) with hip, knee, ankle and digits movements being evoked by electrical stimulation of the caudal part of F3 as well as of the adjoining portion of F1 and cingulate cortex.

The posterior perisylvian complex has also connections with the primary somatosensory cortex (SI), mostly with its mesial portion which is a hindlimb representation region.

D- GENERAL DISCUSSION

I- General discussion and conclusions

The insula of Reil is a wide cortical region (~ 160mm² in rhesus monkey) buried in the depth of the sylvian fissure with an incomplete opercularization in non-human primates that reaches a complete opercularization only in the human brain. Researchers attributed to the insula and adjoining perisylvian regions in both monkeys and humans a very wide range of functions including autonomic and visceral functions, emotions, processing of various sensorial modalities (gustatory, olfactory, somatosensory, auditory).
Based on these observations, the present research was undertaken in order to assess the eventual role of the insula and inner perisylvian regions in each of these functions. Two complementary approaches were combined: intracortical microstimulation in awake free behaving monkeys and anatomical connection study. Two novelties were introduced in our ICMS study: the first one was the behavioral time scale stimulation strategy, consisting of the application of electrical stimulation up to 3sec in order to assess the role of the studied regions in the generation of complex goal-related behaviors. Furthermore, the electrical stimulations were applied in neutral conditions and in interfering conditions (when required) in order to assess the influence of the context (in particular the social interaction) on the generated behavioral responses. The second novelty was the introduction of the ultrasound imaging in all sessions of the experiment, allowing "live" monitoring of the localization of the microelectrodes at each stimulation. Consequently, it was possible to attribute an almost precise anatomical position of the tip of the electrode and then to match the evoked responses to the identified region.

In the anatomical study, three tracers were injected in three different sites where ICMS evoked significant responses for the understanding of the functions of the insula. This study, although preliminary, allowed to unveil the putative neural circuits in which are involved the studied regions and consequently to understand the mechanisms and the dynamics of the evoked responses. Moreover, the connection study allowed us to speculate on other presumed functions of the insula and inner perisylvian regions that we are unable to investigate with the ICMS study due to technical limitations.

Together the ICMS and connection studies allowed us to project the obtained results on the human insula in order to highlight its functions, which is the ultimate goal of any animal experimentation.

The present research showed as it was proposed that the insula and the inner perisylvian regions are involved in a wide range of functions. It was possible to distinguish diverse functional fields both along the rostro-caudal and dorso-ventral axis.

ICMS data showed that it was possible to evoke autonomic modulation (changes in the instantaneous cardiac frequency) almost from the whole structure, unlike what reported in earlier studies according to which autonomic responses could be evoked only in the anterior portion of the insula and adjoining regions (Kaada, 1949). Furthermore, our data showed that the most intense modulations were evoked in the ventral insula and lower bank, in contrast with Showers and Lauer (1961) who reported autonomic modulations only in the dorsal portion of the insula. The comparison between the cardiac responses evoked in the left and right hemispheres enabled us to show that the lateralization appears to deal more with the intensity of the modulation rather than with the sign of cardiac variation, as proposed by Oppenheimer (1992).

The oro-alimentary responses evoked in the anterior perisylvian regions confirm the results obtained from earlier monkey studies (Showers & Lauer, 1961) and from human ICMS studies as well as from clinical observations (Isnard et al., 2004; Catenoix et al., 2008). These data extend the traditional view of this region as a primary gustatory cortex where only sensorial aspects are processed (Rolls, 2005-2007-2009) to a more complex view according to which the anterior insula and adjoining regions are involved in different aspects of feeding behaviors including the sensory modalities, somato-motor aspects, affective and motivational aspects, and the autonomic modulations related to feeding behaviors. Our data suggest a topographic organization of these functions along the dorso-ventral axis, where sensory-motor aspects are represented dorsally and affective and motivational aspects ventrally. The generation of disgust-related responses and the connections with visual areas in the inferior temporal pole suggest that the anterior insula could include a neural substrate of mirror modulation in relation with the disgust, as it was demonstrated in humans (Wicker et al., 2003), although single neuron recording data are required to validate this hypothesis. The autonomic modulations evoked in this region as well as the anatomical connections confirm its involvement in the control of visceral and autonomic functions (Penfield & Faulk, 1955).

The ICMS data showed that with behavioral time scale stimulations, sensory-motor responses were evoked in the dorsal insula and the upper bank. A sensory-motor response could be a complex goal-related movement or a simple movement. The short train stimulations showed that the involved effectors followed a coarse somatotopic organization along the rostro-caudal axis. These data confirm previous monkey and human data (Showers & Lauer, 1961; Frontera, 1956; Isnard et al., 2004). The hodological study showed that the injected site in the dorsal portion of the posterior perisylvian sector has a selective connectivity pattern with motor and somatosensory areas. Together ICMS and connection data suggest a role of the studied regions into the mechanisms of somato-sensory perception and the generation of motor behavior.

One of most interesting outcomes of the present study was the communicative responses evoked by the ICMS in the mid-ventral dysgranular insula. These responses were evocable only under the condition of the direct interaction with the experimenter by means of eye contact. The fact that these responses were context-related and the connections of the corresponding region with areas involved in both the perception and generation of emotional facial expressions led to two considerations: the first is that the insula takes part in a neural network involved in the control of social interactions, possibly contributing to the establishment of hierarchies by means of communicative gestures. The second consideration is that the generation of such gestures is possible only if the monkey, at the moment of eye contact, has the appropriate internal body state to generate a given communicative gesture. As proposed by Craig (2002, 2009), the insula having a key role in interoception and self awareness is responsible of the re-representation of the "sense of the physiological condition of the body" which induces the monkey to react in determinate mode.

Among the responses classified under the miscellaneous responses category there were various stereotyped responses that can not be deciphered in term of goal-related or simple movements responses. Some of these responses was likely interpretable as painful, discomfort or agitation responses. It was possible to suppose that the visible part of some of these responses could be stereotyped reflex or reaction to uncomfortable sensations evoked by the electrical stimulation. Such sensations could be painful, discomforting or thermal. It was not possible to ascertain the validity of such hypothesis. Nevertheless, this hypothesis becomes very plausible if we take in consideration data from the human studies where it was demonstrated that the insula is involved in the coding of pain (Ostrowsky et al., 2002; mazzola et al., 2006-2009; Afif et al., 2008; Starr et al., 2009). In favor of this hypothesis are the biphasic and bradycardic responses we observed during the manifestation of such behavioral responses.

If the hypothesis of the painful and discomforting sensations evoked by the electrical stimulation is plausible, we can advanced the hypothesis of mirroring modality of the pain as suggested in humans (Singer et al., 2004-2009) and so suggesting a somatotopic organization of the representation of emotions in the ventral insula along the rostro-caudal axis.

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