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Functional and structural MRI signature of the induced migraine attack

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"Medicina è ripareggiamento de' disequalati elementi;
Malattia è discordanza d'elementi fusi nel vitale corpo."

Leonardo Da Vinci

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Preface

Magnetic resonance imaging (MRI) studies have consistently shown a distributed pattern of static and dynamic functional brain alterations in migraine patients. Up to now, it is elusive whether these alterations may represent a migraine-specific or phase-specific modification occurring along the migraine cyclical experience.

This study aims to assess the functional and microstructural alteration of the grey matter which characterizes the brain of people living with migraine compared with a matched group of healthy subjects, particularly during the activation derived from an attack induced by nitroglycerin (NTG) administration.

Ten subjects suffering from episodic migraine without aura (EM, 5 female, 28,9 years old, 4.4 migraine days per month) underwent 3T MRI examinations consisting of four task-free functional MRI (fMRI) and T13D scan repetitions during subsequent phases of a nitroglycerin-induced migraine attack (baseline, prodrome, full-blown attack, recovery). Ten healthy subjects (HS, 4 female, 26.9 yo) were enrolled for reference as control and underwent the same pharmacological protocol. A non-parametric permutation test was run to detect significant functional connectivity (FC) changes between EM and HS subjects in the different attack phases. Secondly, a seed-based correlation analysis

(SCA) and a wavelet component analysis (WCA) were performed to focus the attention on the static and dynamic altered relationship between the thalamus and the rest of the brain, during the different phases of the migraine attack. Moreover, 3D-T1 images were processed to obtain subject-specific GM density (GMd) maps. A non-parametric permutation test was run to detect significant GMd differences in EM compared to HS during the attack phases. Finally, fMRI data were tested for correlations with the clinical variables collected.

At baseline, EM subjects showed a significantly altered FC within the posterior cerebellum, frontal/prefrontal cortex, and cingulate cortex. The thalamus, instead, expressed its pivotal role from the prodromal phase, exhibiting an altered coupling with the brainstem, the cingulate cortex, and the cerebellum's posterior part over the migraine cycle. Moreover, WCA proved a loss of synchronisation between the thalami and the salience network, observed mainly during the prodrome and full-blown phases. These findings further support the idea that a temporal change in thalamic function occurs over the experimentally induced phases of NTG-induced headache in migraine patients.

From a structural point of view, EM subjects showed significantly reduced GMd in the insula, inferior parietal lobe, and superior and middle temporal gyrus, compared to healthy subjects. An increased density was instead observed in the cingulate cortex, middle frontal gyrus and the limbic system, but it did not significantly change during the different phases of the attack.

Migraine-like pain induction caused a profound alteration of the FC, which persisted over recovery. The results point to the involvement of the cerebellum - a multiple effector system integrator and a ruler of pain perception modulation – and the frontal/prefrontal cortex. The changes observed in these areas may also explain the cognitive impairment associated with the migraine ictal phase. Finally, the microstructural modification here discussed suggests that migraine is associated with significant GM volume loss in key areas for pain processing, possibly reflecting alterations in the local dendritic complexity caused by the disease.

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1 Introduction

Migraine

Several types of headaches are recognised and identified with precise diagnostic criteria by the International Classification of Headache Disorders (ICHD) (Vincent et al., 2018), with migraine (EM) being the most burdensome one (M. Ashina et al., 2021). Migraine is a disorder of sensory processing (Goadsby et al., 2017b) which represents the second leading cause of disability in the population under 50 years old (Stovner et al., 2022) affecting mostly young women.

The majority of people living with migraine experience a range of different phases during an attack, as summarized in Figure 1 (Dodick, 2018). In the beginning, mood swings, yawning and food cravings could characterize the prodromal phase, which may be followed by an aura phase. Aura is a set of transient focal visual or sensory-motor symptoms, which usually precede or sometimes accompany the headache. Following this, the full-blown attack phase occurs with pulsating, severe head pain associated with nausea, photo/phonophobia, and worsened by movement. The full-blown headache pain phase is followed by the postdromal/recovery phase. Despite having been described in literature for decades, the features, pathophysiological relevance and clinical implications of prodromal and postdromal phases (recovery) have been largely neglected (Bose et al., 2018; Karsan et al., 2018).
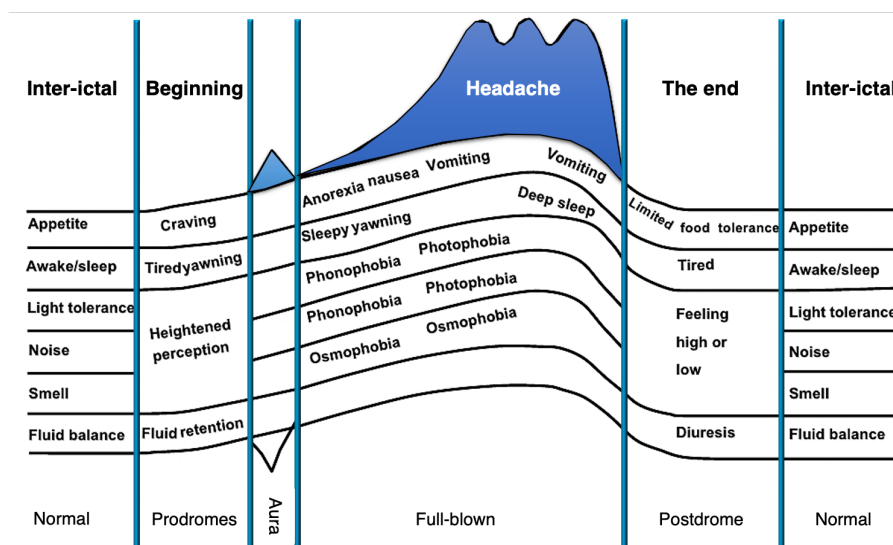

Figure 1 - The migraine attack: signs and symptoms occurring during the different phases, (mod. from Blau 1992)

Yet, migraine pathophysiological aetiology is poorly known, hindering the development of effective therapies. Indeed, migraine can be considered as a cyclical dynamic functional disorder (Peng et al., 2022) that affects several neural systems and neuroanatomic structures, involving multiple pathways, and ultimately leading to the excessive activation of trigeminovascular afferents in the meninges with the local release of the vasoactive peptide calcitonin gene-related peptide (CGRP) (Goadsby et al., 2017b).

Over the last two decades, migraine research studies have witnessed remarkable scientific efforts and advances, which unfortunately have not led to the identification of reliable biomarkers of disease or response to treatments (Ferroni et al., 2018). In this framework, Magnetic Resonance Imaging (MRI) offers in vivo non-invasive imaging biomarkers of the brain structure and functioning that can be exploited to study the pathophysiology of migraines. Neuroimaging studies have changed the way we understand migraine, highlighting a key pathogenetic role for brain connectivity alterations (Messina et al., 2022). Functional imaging techniques, from positron emission tomography (PET) to magnetic resonance imaging (MRI) including structural 3DT1 acquisition for a morphological evaluation or task-related functional magnetic resonance imaging (fMRI), resting-state fMRI (rs-fMRI) and arterial spin labelling (ASL) allow assessing the volumetric, metabolic, and hemodynamic changes that are coupled with regional neural activity. Functional connectivity (FC) rs-fMRI data provide information about the interplay between different brain areas, spatially separated but actively modulating their activities together. Taken together, structural, and functional abnormalities in cortical and subcortical areas involved in pain processing, have proven to be peculiar to the migraine experience. In this framework, advanced neuroimaging techniques, applied to the study of headache patients, have shed light on the possible mechanisms responsible for the initiation and propagation of the attacks, paving the way to a comprehensive analysis of the cortical and subcortical brain activity during the different phases of the attack. Firstly, moving beyond the initial idea of the involvement of one single brain area, such as the ventral dorsal pons as a migraine generator. More recently, introducing the current idea of network disruption. Networks, more than a single structure, are cyclically altered and therefore contribute to migraine pathology (Peng et al., 2022).

Assessing the functioning of the central nervous system of patients living with migraine is challenging because the cyclical occurrence of attacks, which evolve over time with subsequent phases, introduce a high degree of variability in the analysis and heterogeneity in the results. As well-reviewed by Messina et al, (Messina et al., 2022) there are three major designs of MRI studies in migraine:

A. Case-control studies analysing the intercritical phase: people living with migraine are scanned outside of attacks and compared to healthy subjects.

B. Human migraine provocation models of the ictal phase: patients are scanned on a headache-free day and are subsequently challenged with a migraine-like inducing drug. This paradigm allows a high level of control over the different phases of the attack and permits a direct comparison of it with the pain-free state at baseline.

C. Case-control studies of the spontaneous ictal phase: patients are evaluated during a spontaneously occurring attack and on a headache-free day.

Inducing the attack

A migraine attack is generally considered spontaneous when it occurs without any tangible trigger or precipitating factors. However, many patients will frequently report known factors they believe trigger their migraine attacks, or at least increase their likelihood of occurring (Martinelli et al., 2022). Over the last 30 years, this knowledge has led to the investigation of experimental settings to induce a migraine attack: environmental or experimental – endogenous and exogenous – chemical triggers of migraine have proven to be the most reliable approach to studying this primary headache disorder. Several substances and different targets have been studied for this purpose, as reported in table 1. These are largely vasoactive and many are present at the nerve endings of perivascular nerve fibers innervating the cranial vasculature from the sensory, parasympathetic, and sympathetic nervous systems and are released as a consequence of activation, although not all of them appear to be released during migraine (Goadsby et al., 2017a). Acting on vascular smooth cells, these molecules result in an outflow of potassium via the opening of adenosine triphosphate-sensitive potassium (K_{ATP}) channels and large conductance calcium-activated potassium (BK_{Ca}) channels (H. Ashina et al., 2022).

Cranio-vascular Nerve Fiber Localization	Vasoactive Neuropeptide/Neurotransmitter
Trigeminal sensory nerve fibers	Calcitonin gene-related peptide (CGRP)
	Substance P (SP)
	Neurokinin A (NKA)
	Pituitary adenylate cyclase-activating peptide (PACAP)
	Nitric oxide
Parasympathetic nerve fibers	Vasoactive intestinal polypeptide (VIP)
	PACAP
	Neuropeptide Y (NPY)
	Acetylcholine
	Nitric oxide
Sympathetic nerve fibers	Norepinephrine
	NPY
	ATP

Table 1 Summary of cranio-vascular nerve fibers with a putative role in migraine and the vasoactive neuropeptides/neurotransmitters involved, from (Goadsby et al., 2017a)

One of the most studied migraine triggers is nitroglycerin (NTG), a potent vasodilator that exerts its systemic effect by being converted to nitric oxide in the body by mitochondrial aldehyde dehydrogenase. NTG is used clinically mainly to treat heart conditions, such as angina pectoris and chronic heart failure. It is available in sublingual tablets, sprays, ointments, solutions for intravenous use and patches. Sicuteri et al. first described segregation in the headache response to NTG in healthy subjects and patients (Sicuteri et al., 1987). Healthy subjects tested as controls develop an immediate, low intensity non-specific holocranic and self-limiting headache that resolves within a few minutes; at variance, subjects with migraine develop a headache attack with migraine-like features after several minutes or hours, which may have a long duration. In 2004 the specificity of the headache response to sublingual NTG administration was tested on a large clinical population (Sances et al., 2004) proving a sensibility of 82.1% and a specificity of 96.2% in inducing migraine-like attacks in migraine sufferers. It was possible to describe the presence of a non-specific headache in 49.4% of the population studied within the first 60 minutes of the observation, while it took an average of 136.4 minutes to develop a specific headache response, characterised by phonophobia (84%), photophobia (78%) and moderate-to-severe vomiting (34%).

A striking similarity exists between spontaneous migraine attacks and NTG-induced headache attacks in people living with migraine: NTG-induced headache fulfils the IHS diagnostic criteria for migraine in a very high percentage of patients, responds to triptans and is prevented with migraine preventive drugs. Table 2 below compares the features of spontaneous and NTG-induced migraine attacks in relation to the diagnostic criteria of the International Headache Society and other relevant features.

Diagnostic criteria	Spontaneous attack	NTG induced attack
A. At least five attacks fulfilling criteria B-D	Yes	Not relevant
B. Headache attacks lasting 4-72 hours (when untreated or unsuccessfully treated)	Yes	Yes
C. Headache has at least two of the following four characteristics: 　　1. unilateral location 　　2. pulsating quality 　　3. moderate or severe pain intensity 　　4. aggravation by or causing avoidance of routine physical activity (*e.g.*, walking or climbing stairs)	Yes	Yes
D. During headache at least one of the following: 　　1. nausea and/or vomiting 　　2. photophobia and phonophobia	Yes	Yes
E. Not better accounted for by another ICHD-3 diagnosis.	Yes	Not relevant
Additional features non-included in the diagnostic criteria of HIS but clinically relevant: 　　- Premonitory symptoms 　　- Allodynia 　　-Subjective spontaneous-like characteristics	Yes Yes Yes	Yes Yes Yes

Table 2 Comparison of the clinical features of the spontaneous and induced NTG migraine-like attack. From Restani et al. 2012 (Restani et al., 2012).

Overall NTG provocative test is an easy, low-cost, and reliable method for supporting the diagnosis of migraine without aura, as well as for studying this primary headache in a clinical experimental setting.

Several studies have shown that the mechanism linking a vasodilating drug like NTG to migraine is much more complex than the simple effect on the endothelial cells, as it involves the following three pathways: direct relaxation of blood vessels by no-cGMP, neuromodulatory effects of nitric oxide (NO) formed by NTG and de novo synthesized NO via the NO synthase pathway. If the immediate headache occurring after NTG administration is likely associated with the direct NO-cGMP mediated vasodilatation of

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dural vessels (Akerman et al., 2002), more interesting are the mechanisms underlying the second, delayed headache. NTG acts both at central and peripheral levels, leading to the activation of the trigeminovascular system and central sensitisation (Laura et al., 2009), a condition considered as the leading mechanism in migraine pathophysiology, due to the release of multiple excitatory neurotransmitters including CGRP, SP, and neurokinin A from dural afferent terminals, resulting in neurogenic vasodilatation and aseptic inflammation. Furthermore, NTG activates specific structures in the central nervous system (CNS) that may explain the associated symptoms – like nausea, photophobia and phonophobia – which frequently occur during the NTG-induced delayed headache. This is the case of the observed neuronal activation in the area postrema and the hypothalamus in rat (Tassorelli et al., 1999) or human hypothalamus (Maniyar, Sprenger, Monteith, et al., 2014). Multiple pathways have been involved in this model, moving from the mentioned CGRP release to the liberation of the PACAP in sensory neurons of the trigeminal ganglia to the monoamines secretion in the brainstem nuclei (such as norepinephrine, dopamine and serotonin), to the dysfunction of the endocannabinoid system, which is strongly involved in the antinociceptive regulation (Restani et al., 2012). Conversely, the disadvantage of the activation of multiple pathways is that the administered drug (NTG as well as CGRP, PCAP38 and so on) *per se* can cause changes in the brain function as well as other adverse events, which may bias the analysis.

Overall, multiple lines of evidence from the animal model and the human experience suggest that NTG generates peripheral and central sensitisation via a cascade of different events as summarised in Figure 2.
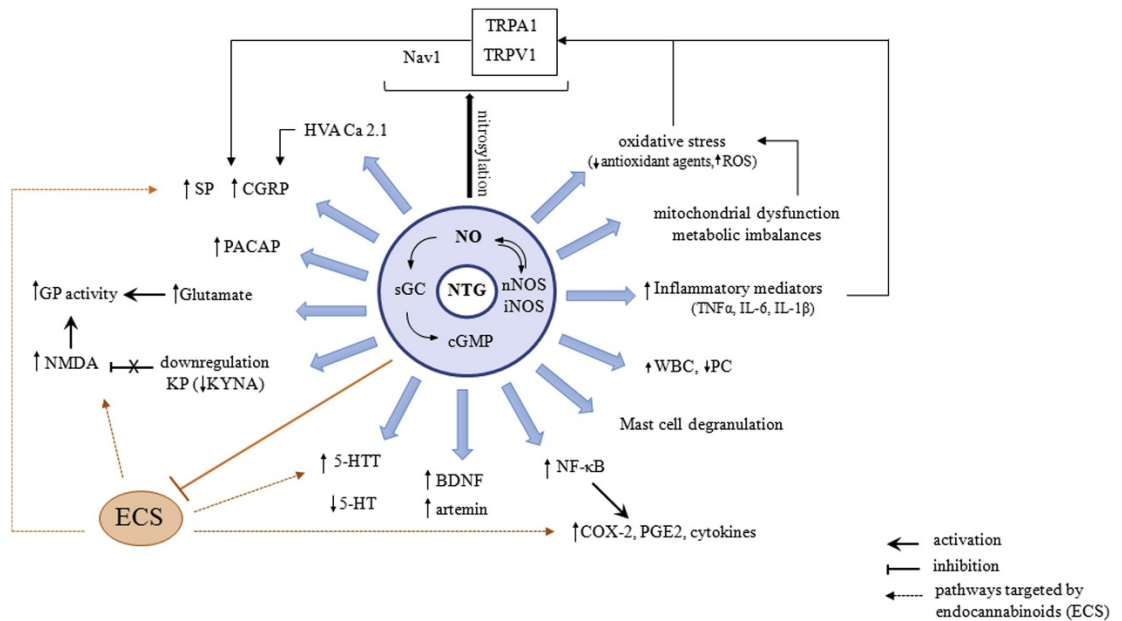
Figure 2 Schematic representation of the possible mechanisms that can be involved in NTG effects and that may be relevant for migraine pathophysiology. NTG- derived NO leads to the activation of NOS and cGMP pathways, which in turn induce modifications on multiple targets: ion channels, neuropeptides and neuro-transmitters release, inflammation, immune system activation, mitochondrial function, and oxidative stress. Altogether these pathways contribute to the generation of central sensitisation and related phenomena. Key: 5-HTT=serotonin transporter; ECS=endocannabinoid system; GP=glutamate pathway; HVA=high voltage-activated Ca+channels; KP=kynurenine pathway; NaV=sodium channels; PC=platelet count; WBC=white blood cell count. From Demartini et al. 2019

NTG: a translational model

Few clinical diseases have a true translational model and the NTG provocative paradigm can be easily realized both via oral, sublingual, transdermal or intravenous administration of the drug. This is considered a reliable surrogate of the spontaneous attack because:

- the majority of people living with migraine develop an attack that fulfils the IHS diagnostic criteria for migraine as described earlier;

- there is a clear dichotomized response in patients and healthy subjects (Sances et al., 2004; Sicuteri et al., 1987);

- the attacks can be prevented or aborted by common anti-migraine drugs like Valproate and Sumatriptan (Fullerton et al., 1999; Thomaides et al., 2008);

- the typical premonitory symptoms and migraine-associated symptoms occur in association with cranial pain (*e.g.* nausea and photophobia) (Maniyar, Sprenger, Schankin, et al., 2014).

The NTG-induced migraine-like attack is a robust human and animal model that has proved useful also in the neuroimaging field: first of all using PET(Afridi, Matharu, et al.,

2005). The brainstem involvement during NTG-induced headaches has been documented as compared to those obtained during a spontaneous migraine attack (Weiller et al., 1995). Significant brainstem activation was seen in the dorsal pons and rostral medulla. During the migraine attack, other areas of activation included: the anterior cingulate cortex (ACC), bilateral insula, bilateral cerebellar hemispheres, prefrontal cortex, and putamen. Similar responses were observed during spontaneous migraine attacks (Afridi, Giffin, et al., 2005; Weiller et al., 1995) which suggests a central action of NTG, proven and detailed by Karsan et.al.(Karsan et al., 2020).

This model has been extensively studied also with other neurophysiological approaches. What has been observed is that the NTG infusion causes changes in the trigeminal nociceptive reflex and in evoked cortical response (recorded as visual evoked potentials), which are both comparable to those obtained during a spontaneous migraine attack (Laura et al., 2009). Moreover, the NTG-induced cortical and spinal changes cause the facilitation of pain transmission in migraine subjects (Perrotta et al., 2011).

Taken together, these observations show a remarkable overlap of clinical, pharmacological and neuroimaging findings obtained during spontaneous migraines and NTG-induced headaches in people living with migraine without aura, which strengthens the adoption of NTG-induced headaches as the tool for exploring the pathophysiology of primary headaches in experimental settings.

Magnetic resonance imaging

Neuroimaging studies have changed the way we understand migraine, suggesting the possible role of different areas of the brain. From the pivotal SPECT and PET studies, brain imaging technology has evolved toward MRI, which is widely available and safe. Using high-resolution acquisition MRI techniques, widespread not only structural but also functional abnormalities have been revealed in cortical and subcortical areas involved in multisensory processing.

Functional changes

Functional MRI (fMRI) allows measuring brain activity by detecting changes associated with variations in the cerebral blood flow (CBF) (and volume) when the brain is engaged in performing a specific task (*e.g.,* moving a hand). This technique relies on the fact that CBF and neuronal activation are coupled (Hillman, 2014). The most

frequently used MR imaging method to produce information related to brain functioning is called BOLD (Blood Oxygenation Level Dependent) contrast imaging. When an area of the brain is in use, the blood flow to that region increases, leading to an increase in the local BOLD levels as well. For this reason, the BOLD signal is used as a non-invasive and indirect measure of changes in neuronal activity. Through different post-processing approaches, it is possible to study functional connectivity (FC), which is defined as the temporal dependency (or correlation) between spatially remote neurophysiological events (Friston et al., 1993).

Functional imaging techniques can be divided into two distinct categories: task-based or task-free analysis. The first approach (e.g., task-based fMRI) measures the brain responses to a specific action or stimulus, while the second one (e.g., task-free fMRI) analyses the FC when the brain is "at rest", which means that subjects are asked to lie in the scanner awake, but without thinking or doing anything in particular. The task-free fMRI approach is better known as "resting state fMRI" as mentioned above. Indeed, by measuring FC between spatially distinct brain regions, rs-fMRI allows the identification of several networks, called resting state networks (RSNs), in which separate brain areas show MR signal temporal correlations at very low frequency (< Hz 0,02), in the absence of any specific external stimulations (Biswal et al., 1995; Damoiseaux et al., 2006). When the subject is not actively completing a task, but for example, is experiencing pain all over the acquisition, the condition is better addressed as task-free fMRI. This technique allows the identification of intrinsic brain activity (basal activity) and connectivity by scanning a patient's brain at rest with three major approaches. The first approach is based on the so-called *independent component analysis* (ICA) (Beckmann et al., 2005; Smith et al., 2009) which allows determining group differences without a previous anatomical hypothesis to identify the RSNs (data-driven approach). The second approach is a region-of-interest (ROI) analysis, which is usually referred to as seed correlation analysis (SCA) The connectivity of particular predetermined areas, called seed region, is examined based on specific anatomical knowledge, in a sort of hypothesis-driven approach (Yeo et al., 2011). Finally, another data-driven method (regional homogeneity, ReHO (Jiang & Zuo, 2016)) gives the possibility of evaluating low-frequency BOLD fluctuations to characterise the regional homogeneity and the relative connectivity in the neighbouring voxels. All these approaches can be used to

assess neuronal activity changes, therefore giving insights into the functional organisation of pain-processing networks (Jenkinson & Chappell, 2017).

The role of neuroimaging in migraine:

Over the last three decades, functional imaging studies have provided several meaningful insights into the pathophysiological mechanism of migraine, moving from the concept of the rostral dorsal pons migraine generator to a more complex view where the functional networks abnormalities may lead to changes in different brain areas (Russo et al., 2017).

Dissecting the inter-ictal phase

The pain-free asymptomatic phase is the easiest to test. Therefore, most of the publications available are focused on this inter-critical and highly reproducible phase. Their focus is on the functional reorganization of the cortical and subcortical brain areas involved in abnormal pain processing in people living with migraine. The thalamus, periaqueductal grey (PAG), insula, prefrontal, anterior cingulate and primary-somatosensory cortex, as well as the amygdala and the cerebellum, are the structures where most commonly an altered FC is described after a noxious stimulus is presented to patients in a pain-free state (Skorobogatykh et al., 2019). Task-related studies have also shown the involvement of the superior-anterior middle temporal gyri, (Antal et al., 2011) and perigenual anterior cingulate cortex, (Russo, Tessitore, Esposito, et al., 2012) in terms of an increased connection between nociceptive parts and the PAG. Considering the clinical point of view, the extent of these abnormalities positively correlates with headache frequency and disease duration (Mainero et al., 2011). Not only, but these structures are also involved in sensory discrimination, emotional and cognitive pain processing, proving an impaired activation of the ascending and descending nociceptive networks. Moreover, in task-free resting-state functional connectivity studies, several other networks appeared to be altered: the default mode network (DMN) (Tessitore et al., 2013), the limbic, the salience (SN) (M. J. Lee et al., 2019), the frontoparietal networks, the executive-control network (ECN) (Russo, Tessitore, Giordano, et al., 2012) and the sensorimotor networks (SMN) (S. Ashina et al., 2021; Colombo et al., 2019). Interestingly, also the visual network appears to be

altered even during the intercritical phase (Vincent et al., 2003) as are widespread regions involved in the visual processing, like the middle frontal gyrus, the insula, the anterior cingulate cortex, the superior parietal lobule, and the cerebellum (S. Ashina et al., 2021). In this framework, however, the clinical correlations are heterogeneous.

Overall, the results of these fMRI studies in the asymptomatic phase provide insights into the neural constitutive mechanisms sustaining migraine pathophysiology, proving how the function interplay of different brain areas in people living with migraine is altered. Whether functional brain alterations in the asymptomatic phase represent brain traits that predispose to the development of migraine or a brain state secondary to the recurrence of migraine attacks is still a matter of debate.

Dissecting the prodromal phase

The prodromal phase and the early stage of the migraine painful attack are associated with changes in the hypothalamic activity and several intrinsic functional connectivity networks involved in pain processing (Messina et al., 2022). Indeed a seminal 30-daily fMRI study (Schulte & May, 2016a) showed how the hypothalamic activity, as a response to trigeminal nociceptive stimulation, is altered during the 24h before pain onset, e.g., it increases towards the next migraine attack. More importantly, the hypothalamus showed altered functional coupling with the spinal trigeminal nuclei and the region of the migraine generator, *e.g.*, the dorsal rostral pons during the preictal day and the pain phase of spontaneous migraine attacks.

An altered hypothalamic activity was recorded also after migraine-like attacks induced by different substances, such as PACAP38, NTG and oral glucose administration. Moreover, PACAP38 disrupts the salience, sensorimotor and DMN during the early phase of the relative migraine-like attack, (Amin & Hougaard, 2015) specifically disturbing the coupling between the somatosensory and the visual cortices. Finally, Karsan et al. (Karsan et al., 2020) presented a study with NTG-triggered migraine-like attack addressing the prodromal symptoms, proving increased functional connectivity between the bilateral thalami, the right precuneus and cuneus. Moreover, there was a decreased functional connectivity between the pons and the limbic lobe. Contrary to the previous descriptions, this study also included the hypothalamus as a

region of interest (ROI) in the seed-based correlation analysis but could not find any FC alterations.

Dissecting the aura phase

The aura phase represents the most challenging and volatile phase to be recorded. In this rapidly evolving stage associated with the cortical spreading depression phenomenon (Leao, 1944), for example, the visual cortex is hyper-excitable in association with the description of positive aura symptoms aura (e.g. scintillations), whereas there is reduced neuronal activity during reported negative visual aura symptoms (e.g. scotomas). In the human brain, this phenomenon was firstly described in 1981 with a SPECT study (Olesen J, Larsen B, 1981) that showed focal cortical hyperaemia, followed by spreading oligemia, in a patient evaluated during an aura. In 2001 there was the first article using fMRI (Hadjikhani et al., 2001) which tried to investigate a migraine aura, induced by physical exercise. At the beginning of the visual aura phase, a focal increase in the BOLD signal was described in the V3A area, possibly reflecting vasodilation, followed by a slow progression of the BOLD signal change over the occipital cortex, congruently with the retinotopy of the visual percept. Following the same retinotopic progression, the BOLD signal then weakened, probably mirroring vasoconstriction after the initial vasodilation.

Dissecting the full-blown headache phase

Only a few studies have used fMRI to investigate brain activity changes in the ictal phase of a migraine attack. They helped describe how the central trigeminovascular or the ascending pain pathways (composed of the spinal trigeminal nucleus in the pons, thalamus, sensorimotor and visual cortices) present disruptions in the normal functional network architecture. The Italian contribution in this area is significant thanks to the experiences of Coppola and his colleagues (Coppola et al., 2016) who studied FC changes in 13 migraineurs without aura during migraine attacks compared to 19 healthy subjects (HS) using the ICA approach. During a spontaneous migraine attack, people living with migraine showed decreased FC between the executive-control network and the dorso-ventral attention network (dVAN). Furthermore, weaker ECN connectivity was related to higher monthly headache frequency in patients suffering from migraine. The

reduced FC between cognitive and attentional networks during the full-blown phase is quite interesting since it might relate to the difficulties with memory and attention that are often experienced by patients during attacks. Furthermore, the authors described a negative correlation between the mean FC calculated within the ECN and headache frequency, indicating that reduced network FC might relate to a higher disease burden. Whereas in HS there was a relationship between stronger FC in areas of the dVAN and lower bilateral thalamic fractional anisotropy (FA) measures, this relationship was absent in migraine patients, potentially indicating a functional decoupling of thalamocortical control networks during the attack. This abnormal decoupling could subtend the known ictal impairment of cognitive performance and suggests that the latter might worsen with increasing attack frequency. In the following sub-analysis regarding the same subjects (Coppola et al., 2018), a stronger FC was found between the medial prefrontal cortex and the posterior cingulate cortex, the medial prefrontal cortex and the insula and between the insula and the inferior parietal lobule. In people living with migraine, during the attack, the FC strength between the medial prefrontal cortex and the insula regions was negatively related to the perceived pain intensity.

FC within the salience, sensorimotor network, default mode network, executive and attention cerebral networks were modified during migraine attacks, which may reflect the cognitive, painful, and emotional symptoms described by the patients. Abnormal connectivity between these large-scale networks and brain regions important for the emotional processing of pain, including the insula, could be considered an adaptive response in the setting of acute pain. It could be inferred that these abnormal couplings allow for a greater focus on acute pain, even at the expense of impaired higher-order functions (Coppola et al., 2020). However, in the setting of a chronic pain condition like migraine, these responses might be maladaptive if they lead to an excessive focus on the pain experience. Since the authors compared ictal migraine patients to HS only, and not to interictal patients, caution is mandatory since it was not possible to determine if these changes were specific to the attack phase of migraine or if they were general features of migraine pathophysiology.

In 2017, an investigation of 16 patients was carried out during spontaneous attacks of migraine with visual aura vs the relative pain-free condition (Hougaard et al., 2017). During the attacks, FC increased between the dorsolateral pons and the ipsilateral

primary somatosensory cortex corresponding to the head and face somatotopic areas as well as between the visual area V5 and the ipsilateral middle frontal gyrus of the "symptomatic" hemispheres (*e.g.,* contralateral to the perceived visual aura symptoms). Finally, another SCA study was carried out in 2018 (Amin et al., 2018) highlighting an altered right thalamocortical network during spontaneous attacks of migraine without aura relative to the inter-ictal state.

Dissecting the recovery phase

Only recently the research community has focused attention on the postdrome phase describing how the visual cortex, but not the hypothalamus, remains hyperexcitable even 24h after the end of the painful phase. In detail, Schulte et. al., (Schulte et al., 2020; Schulte & May, 2016a) reported that during the recovery phase, the visual cortex responded to noxious stimulation with higher activation than during the headache or asymptomatic phases, with no deactivation compared to the pain-free inter-ictal phase. Overall these results may explain why some patients are still light-sensitive even after the conclusion of the headache ictal phase (Messina et al., 2022).

Overall, the results of these studies support the notion that migraine attacks are a disorder brain's sensory processing. In this framework, several brain regions cyclically modify their functional coupling producing the complex phenomenology experienced by the patient (Peng et al., 2022). Under this condition, the thalamus emerges as a pivotal relay station in the migraine cycle both from a structural and functional perspective. Anatomically it is detrimental to the processing of the ascending nociceptive information, via the trigemino-thalamocortical pathway, from lower brain areas to various cortical regions. Furthermore, the thalamus plays a key role in central sensitisation in migraine pathophysiology and its nuclei are involved in the complexity of migraine manifestation, such as widespread allodynia or photophobia.(Burstein et al., 2010)

Taken together, the several neuroimaging studies previously mentioned could be summarised in the following Figure 3 by Messina et. al. (Messina, Filippi, et al., 2018), in which the structures involved in the different stages of the migraine attack are presented in a template. It is worth noting that all the studies evaluated the static functional changes addressing the specific phase of interest reported.

- *Prodrome*: hypothalamus, pons, spinal trigeminal nucleus and visual cortex.(Schulte & May, 2016a)
- *Aura*: visual cortex (Arngrim et al., 2017)
- *Full-blown headache*: ACC, cerebellum and PAG (Mehnert & May, 2019), hypothalamus (Schulte & May, 2016a), pons (Amin et al., 2018), spinal trigeminal nucleus and visual cortex (Schulte & May, 2016a), middle frontal, somatosensory and temporo-occipital cortex (Hougaard et al., 2017)and thalamus (Amin et al., 2018);
- *Recovery*: visual cortex (Schulte & May, 2016a);
- *Interictal phase*: ACC, amygdala (Jia & Yu, 2017), cerebellum (Mehnert & May, 2017), hippocampus (Chanraud et al., 2014), hypothalamus (Schulte & May, 2016a), insula frontal, temporal and somatosensory cortex, thalamus (J. Zhang et al., 2017), PAG (Jia et al., 2017), temporo-occipital and visual cortex (X. Zhang et al., 2011).
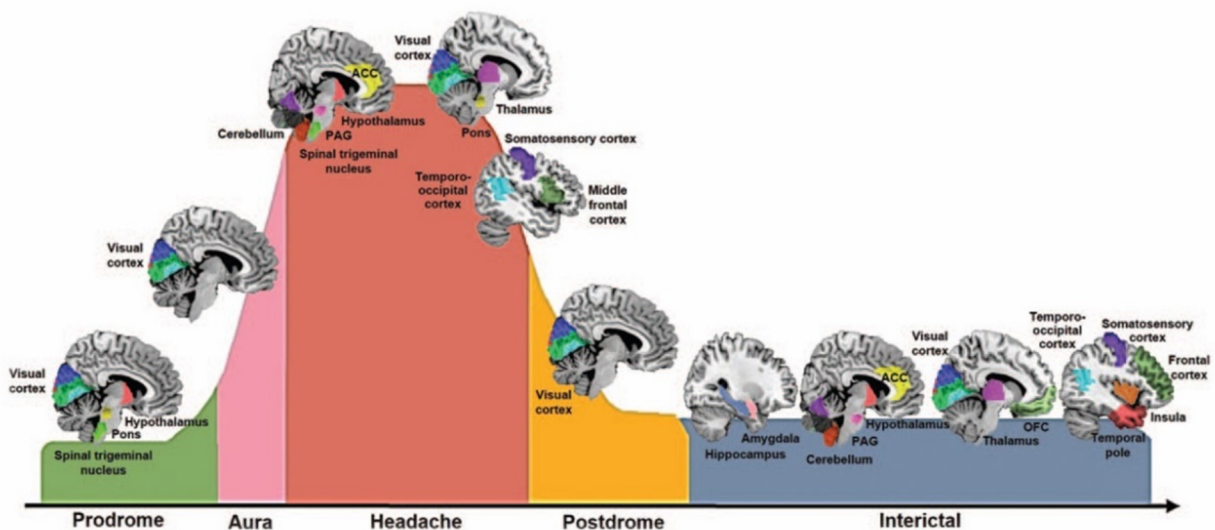

Figure 3 A schematic illustration of the main brain regions described by recent studies as key areas involved in the various migraine phases, represented on a high-resolution T1-weighted template. (Messina, Filippi, et al., 2018)

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<u>Alteration of migraine dynamic activities</u>

Most studies regarding resting-state functional connectivity rely on methods that assume temporal stationarity of the signal across the duration of the scan. However, evidence from both task-based fMRI studies and animal electrophysiology experiences suggests that FC may exhibit dynamic changes within time scales of seconds to minutes. In this framework, other advanced methods used to analyse the time-frequency fluctuations have been developed to characterize temporal varying FC between different regions/networks and applied to psychiatric and neurological pathologies such as multiple sclerosis (Valsasina et al., 2019). New approaches involving wavelet decomposition, for example, the wavelet-coherence analysis (WCA) (Chang & Glover, 2010), represent a powerful method to assess transient or non-stationary processes to gain insights into the brain's temporal dynamics. In particular, WCA has been applied to evaluate large resting state networks' altered dynamic in patients affected by an autism spectrum disorder (Bernas et al., 2018), but only rarely to study the dynamic activity of pathological brains. Wang et al. (Wang et al., 2017) for example, used it to compare people living with chronic migraine in the interictal phase and matched HS as controls, highlighting robust and significant differences (in terms of a reduction) in temporal dynamics FC between left medial-orbitofrontal vs left posterior-cingulate and left medial-orbitofrontal vs inferior-temporal gyri. These areas are core parts of the DMN, ECN, SN, and SMN.

Another recent application of these principles was published by Wei et al. (Wei et al., 2022). Resting-state fMRI studies have already revealed altered static functional connectivity of the visual cortex in migraine, as proven by Tedeschi et al. (Tedeschi et al., 2016), remarkably proving the differences in neural activities in the visual cortex between patients with migraine and HS. In this context, Wei et al. showed how the dynamic functional interaction was disrupted within the visual cortex and in the relationship with other brain networks, such as DMN, SN, ECN, subcortical network, and SMN. Moreover, decreased dynamic functional alterations within the visual cortex were significantly found to be correlated with anxiety performance, which may indicate that the dysfunction of the visual cortex contributes to the emotional impairments in patients living with episodic migraine with aura during the attack-free period.

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<u>Structural changes</u>

To date, many efforts have been made to explain structural changes in the brain with studies addressing exclusively the interictal phase. Neuroimaging studies often revealed white matter hyperintensities or white matter abnormalities, silent infarct-like lesions, ischemic lesions (stroke-like) as well as volumetric changes in the grey and white matter in the brain of those living with migraine.

Voxel-based morphometry (VBM) is one of the key neuroimaging techniques to assess macroscopic grey matter (GM) asymmetries with high regional specificity. VBM is an analysis workflow for structural MRI scans that allows for voxel-wise comparison of local grey matter concentrations between two groups of participants. In a 2017 study (Palm-Meinders et al., 2017) 83 patients with and without aura were investigated, finding decreased grey matter volume in the visual areas V3 and V5 of the extrastriate cortical areas of the right occipital gyrus (Brodmann area 19) in patients compared to healthy subjects. An analysis of migraine subgroups (*e.g.,* migraine with or without aura, active or inactive disease, low or high attack frequency) displayed roughly the same pattern of differences in these areas compared to controls, therefore indicating that these changes might have been present throughout life and migraine with and without aura might share common pathophysiologic mechanisms.

In exploratory whole-brain analyses, several authors have also identified structural differences in other cortical and subcortical areas that are particularly involved in sensory processing, such as a volume loss in the bilateral insula, frontal/prefrontal, temporal, parietal and occipital cortices, the anterior cingulate cortex, basal ganglia, and the cerebellum (J. H. Kim et al., 2008; Qin et al., 2019; Rocca et al., 2015). Migraine attack frequency and migraine disease duration were correlated with GM reduction in individuals with migraine in the frontal, temporal and parietal lobes, the limbic system and the ACC, the brainstem and the cerebellum (S. Ashina et al., 2021; Schmitz, Admiraal-Behloul, et al., 2008). Compared to people living with episodic migraine, chronic patients had also a more prominent volume loss in the ACC, possibly highlighting an association between attack frequency and the degree of grey matter reduction (Valfrè et al., 2007). Finally, in patients suffering from migraine with aura, an increased grey matter density (GMd) was observed specifically in PAG and dorsolateral pons (Rocca et

al., 2015). Overall, these structural differences might contribute to the dysfunction of the transmission and modulation of noxious information, trigeminal nociception, and conduction and integration of multimodal information in the brain of those living with migraine. Whether structural abnormalities might either predispose to migraine or be a consequence of the disease is still unclear (Jia & Yu, 2017).

To better understand the dynamics of grey matter abnormalities, a longitudinal study (Messina, Rocca, et al., 2018) was carried out to map modifications of GM volume in a cohort of 25 patients affected by migraine over 4 years of observation showing that, compared to controls, patients with migraine had higher GM volume of the regions of the frontotemporal lobes and lower volume in the cerebellum (quadrangular lobule) at baseline. At follow-up, the patients developed an increased volume of fronto-temporo-parietal regions, which was more evident in patients with higher attack frequency and longer disease duration at baseline. The patients with an increased attack frequency at follow-up, experienced both increased and decreased volume in nociceptive regions. Another interesting finding was that the level of pain intensity, baseline disease duration, and the number of migraine attacks could influence GM volume changes in cortical visual areas. These data support the notion that the brain of patients affected by migraine can be remodelled over time, suggesting that cellular and molecular mechanisms can occur in response to patients' disease severity.

The large body of evidence collected over the years through neuroimaging and neurophysiological studies has changed the way we understand migraine, highlighting a key role of brain connectivity alterations in its pathophysiology. Their application in studying headache patients has shed light on the possible mechanisms responsible for the initiation and propagation of the attacks, focusing on the hypothalamus and the brainstem. In order to better understand the cyclical alteration of this disorder, it is possible to hypothesize an orchestrating role for the thalamus in altering cortical and subcortical brain activity during the different phases of the attack.

2   Aims

The overall aim of the present study is to investigate the static and dynamic cortical changes of the ictal and inter-ictal phases of migraine using advanced MRI techniques combined with the NTG provocation model to further improve the knowledge of migraine pathophysiology.

To this end, we assessed:

- the migraine-specific and phase-specific central nervous system alterations in representative clinical populations compared with a matched group of healthy subjects;
- the patterns of altered activation during an attack induced by NTG administration;
- the microstructural alterations of the grey matter which characterize the brain of people living with migraine, particularly during the activation derived from an attack induced by NTG administration.

Primary endpoints:

I.   Depict the static changes in brain activity during an NTG-induced migraine-like attack in a representative clinical population by comparing the effect with a group of matched healthy subjects.

II.   Assess the patterns that present a microstructural alteration of grey matter density at cortical levels across the group and different phases of the migraine-like attack.

Secondary endpoints:

I.   Better address the role of the thalamus over the different phases of the migraine cycle.

II.   Correlate the functional changes occurring during the migraine-like experience with the relative clinical and anamnestic features.

III. Corroborate the use of the NTG-induced attacks paradigm as a reliable instrument combined with advanced MRI approaches.

Exploratory endpoints

I.   Depict the dynamic functional changes in the thalamocortical activity during a migraine-like attack.

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3 Methods

Subjects

Subjects living with episodic migraine (EM) were consecutively recruited over three years at the Headache Science Centre (a tertiary referral centre) of the IRCCS Mondino Foundation in Pavia, Italy. The inclusion criteria were:

- age between 18–60 years;
- diagnosis of EM without aura developed before the age of 50 with a moderate-to-high intensity of attacks;
- no migraine attack in the previous 24 hours from the beginning of the acquisition;
- no current prophylactic treatment for migraine prevention;
- no use of benzodiazepines or non-steroidal anti-inflammatory drugs or triptans in the 24 hours before the beginning of the acquisition;
- no caffeine or stimulant administration during the day of acquisition;
- historical antimigraine efficacy of non-steroidal anti-inflammatory drugs.

The exclusion criteria were:

- overuse of acute medication for headache;
- diagnosis of cluster headache;
- a diagnosis of tension-type headache with a frequency of more than 2 days per month;
- any chronic pain condition or disorders other than migraine;
- major psychiatric disorders such as depression, bipolar affective disorder and schizophrenia;
- cardiovascular diseases contraindicating the use of NTG;
- blood pressure hypotension;
- closed angle glaucoma;
- anaemia;
- pregnant women or breastfeeding women;
- frequent use of benzodiazepines;
- any neuroradiological pathological findings, different from those related to the disease, at a previous MRI scan of the head.

Healthy Subjects (HS) were consecutively recruited over the years among workers and students of the IRCCS Mondino Foundation in Pavia, Italy and among partners of headache patients seeking healthcare at its Headache Science Centre (a tertiary referral centre). The inclusion criteria were:

- age between 18–60 years;
- no diagnosis of migraine nor family history of migraine disorder within two orders of relationship
- no use of benzodiazepines or non-steroidal anti-inflammatory drugs or triptans in the 24 hours before the beginning of the acquisition;
- no caffeine or stimulant administration during the day of acquisition.

All exclusion criteria listed above were applied for HS as well.

The study was approved by the local Ethics Committee, it was registered online in the ClinicalTrial.gov database (NCT04503083) and all subjects provided written informed consent before enrolment in the study. The acquisitions were carried out from the first semester of 2019 till 2022 and were delayed due to the COVID-19 pandemic outbreak.

Clinical assessment

An expert neurologist assessed the subjects' health status with a clinical evaluation. Migraine diagnosis was made according to the International Classification of Headache Disorders, 3rd edition version ICHD-III (Olesen, 2018). Headache qualities were evaluated and noted for each subject during their progression through the protocol.

NTG-induction paradigm

Before the NTG challenge visit, subjects with EM were informed about the possibility that the procedure might induce a spontaneous-like attack and were instructed to recognise the different features of the prodromal and full-blown phases of the attack. On the day of the challenge, the enrolled EM subjects underwent both clinical and imaging assessments within the NTG-induction procedure, which included a detailed recording of symptoms at serial (15 min.) intervals and 4 MRI sessions, all performed on the same day at specific time points during the evolution of the NTG-induced attack. The evaluation of the pain intensity was performed with a Numeric Rating Scale (NRS) scoring from 0 to 10 (mild: 1–3; moderate: 4–6; severe: 7–10).

MRI acquisition

All subjects underwent four MRI examinations in a single day using a 3T Siemens Skyra scanner (Siemens, Erlangen, Germany) with a 32-channel head coil. The total acquisition time (AT) of each MRI scan was about 18 min. The MRI acquisition protocol was the same during every scan and included: 1) fMRI: multi-band T2*-weighted Gradient Echo echo-planar (GRE-EPI) sequence (TR/TE = 3010/20 ms; MB = 2, voxel size = 2.5 mm^3 isotropic, FOV = 224 mm^2, 60 slices, 200 volumes; 2) a high-resolution 3D sagittal T1-weighted (3DT1) scan for anatomical reference (MPRAGE sequence: TR/TE/TI = 2300/2.96/900 ms, flip angle = 9°, FOV = 256 mm^2, 156 slices, voxel size = 1 mm^3 isotropic). During each fMRI acquisition, participants were asked to keep their eyes closed and neither to think about anything in particular nor to sleep, to acquire a resting state condition, which can only be considered "represented" in the baseline pain-free scan. The lights inside the scanner room were switched off to reduce the photophobia experience. All patients had been fasting for at least 3 hours before each scan.

The MRI acquisition protocols are shown in Figures 4 and 5. In detail, the study protocol consisted of six consecutive steps as follows: (1) Scan 1—Baseline: acquired in a pain-free condition during interictal period at least 72 h resolution of any prior migraine attack; (2) NTG administration: supervised administration of 3 sublingual tablets of 0.3 mg and monitoring of vital signs every 15 min during the first hour; (3) Scan 2— Prodrome: this was acquired at the occurrence of at least two of the typical symptoms of the prodromal phase out of a list of possible features; (4) Scan 3—Full Blown: acquired during the migraine-like full-blown headache attack (e.g., NRS ≥ 5); (5) Recovery: after the full-blown scan, the patient was treated with an effective non-steroidal anti-inflammatory drug (NSAID); (6) Scan 4—Recovery: acquired during the recovery phase (e.g., headache resolution, NRS ≤ 1).

Healthy subjects (HS) were challenged with the oral administration of NTG after the patients group. As we did not expect the occurrence of a migraine-like attack in the HS we devised a timely matched scheme of scans, where the median scan time since NTG administration for EM was calculated and applied to the HS.
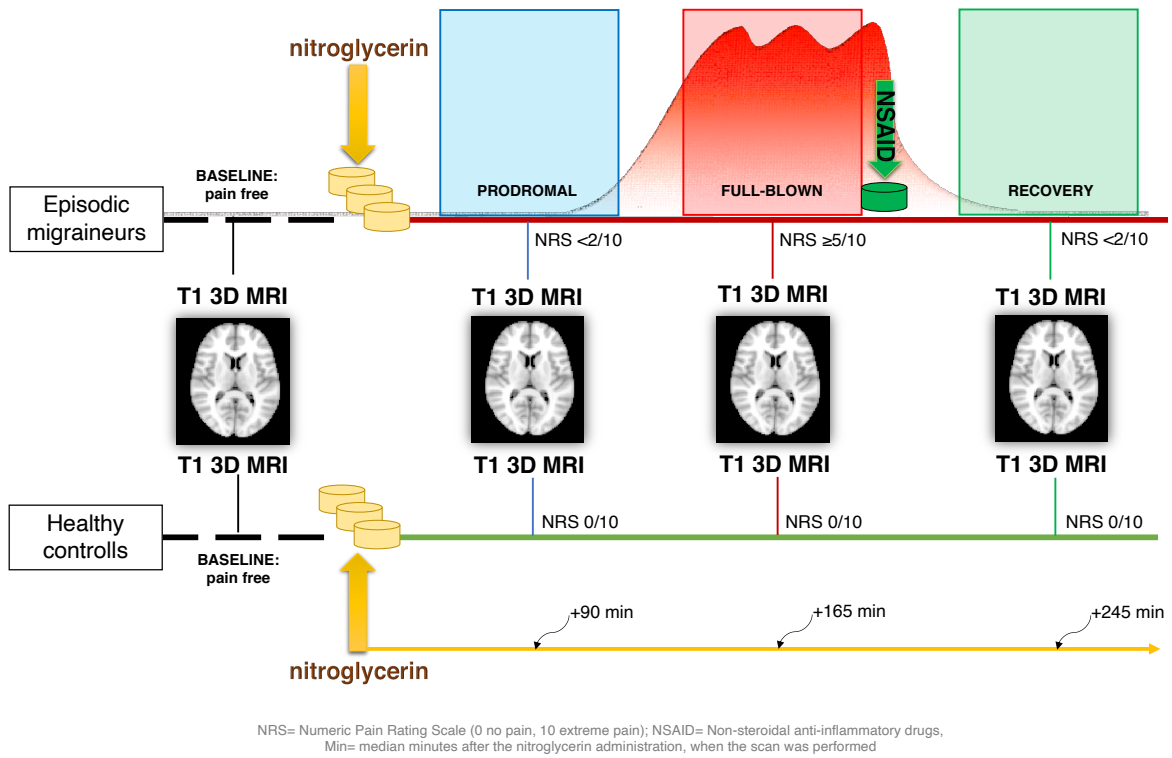
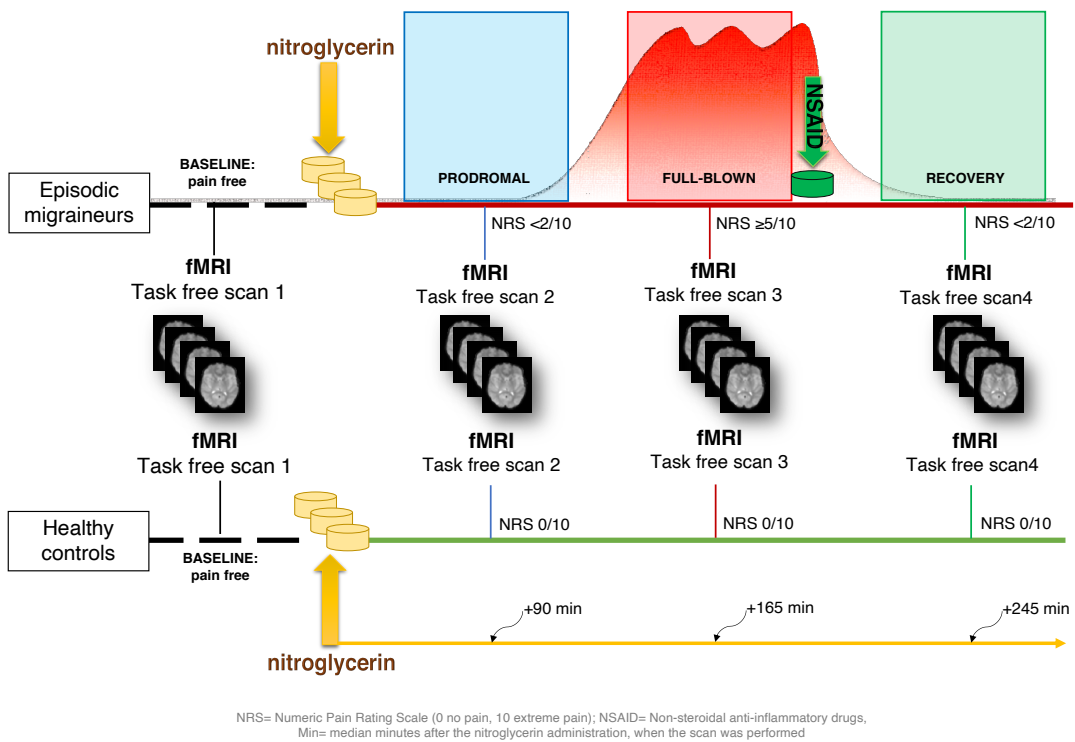Figure 4 Protocol design for the structural MRI acquisition.

Figure 5 Protocol design for the functional MRI acquisition.
     All subjects underwent four scans with a 3DT1 (Figure 4) and task-free fMRI acquisition (Figure 5), temporally matched with a nitroglycerin-induced migraine attack: pain-free baseline, prodromal, full-blown attack, and recovery. None of the HS developed a migraine attack following the nitroglycerin challenge. NRS: numeric pain rating scale. NSAID: non-steroidal anti-inflammatory drug

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fMRI analysis

Data pre-processing

All fMRI data were pre-processed using FSL (FMRIB Software Library, version 5.0.9, http://www.fmrib.ox.ac.uk/fsl/) and MATLAB (v. R2022a, The Mathworks, Inc., Natick, MA) as described in Castellazzi et al. (Castellazzi et al., 2020). Individual pre-processing steps consisted of motion correction, brain extraction, spatial smoothing using a Gaussian kernel of a full-width-at-half-maximum (FWHM) of 5 mm, and high-pass temporal filtering equivalent to 120 s (0.008 Hz). For each subject, individual fMRI volumes were linearly registered to the corresponding structural 3DT1 scan and subsequently to standard space (MNI152) using the NiftyReg toolbox (http://niftyre.g.,sf.net). To reduce the nuisance effects of non-neuronal BOLD fluctuations, the white matter (WM) and the cerebrospinal fluid (CSF) signals were regressed out of fMRI data

Resting-state networks (RSNs) identification

Pre-processed functional data, containing 200-time volumes for each subject, were temporally concatenated across subjects to create a single 4-dimensional data set to run the group-independently component analysis (group-ICA) analysis via MELODIC, with an automatic estimation of the number of independent components (ICs). At this level, some of the ICs were identified as noise while others as RSNs, based on previous literature (Beckmann et al., 2005; Cole, 2010; Damoiseaux et al., 2006; Smith et al., 2009). Group-ICA decomposes data into spatial maps that are the ICs relative to the total processed dataset, or the multi-subject ICA components. At a group level, the IC maps are the same for each subject and are used as inputs for the subsequent dual regression analysis to calculate the statistical inference among groups or baseline and the other scans within each group.

Between-group RSNs comparison and global alterations assessment

A non-parametric permutation test referred to as "dual regression" (Binnewijzend et al., 2012; Filippini et al., 2009; SMITH & NICHOLS, 2009) was then applied to compare group-specific FC maps at each scan point (baseline, prodromal, full-blown and recovery) for each IC map. The RNSs comparison between-group allows us to describe the migraine-specific pattern of global alterations during the task-free acquisitions

occurring at very low frequencies (< 0,02 Hz). For this reason, the definition of the resting state networks at baseline was pivotal for the subsequent analysis, to evaluate the migraine-specific alteration of the basal activity within the network which characterises these patients. Considering that, first, the analysis tested the statistical differences between HS and EM using two comparisons or contrasts (HS > EM and HS < EM). In order to define phase-specific global alterations during the entire migraine-like cycle, we then investigated the presence of significant differences in RSN FC within the groups (EM, HS) between baseline and each of the other scans (prodromal, full-blown and recovery) with six further contrasts: baseline > prodromal, baseline <prodromal, baseline > full-blown, baseline< full-blown, baseline > recovery and baseline < recovery.

In this study, each dual regression analysis was carried out on the total ICs using age, gender and disease duration as additional covariates included in the general linear model (GLM). The statistical inference at the group level was performed using 5000 permutations. The resulting statistical maps were family-wise error (FWE) corrected for multiple comparisons, implementing threshold-free cluster enhancement (TFCE) (SMITH & NICHOLS, 2009) using a significance threshold of at least $p \leq 0.05$. After that, the final statistical maps were saved as *tstatFC* maps.

To study the overall FC changes within the brain, for each considered contrast we calculated a global map of alteration, which unifies in a single map of alteration all the FC changes observed in the identified RSNs. Finally, the resulting maps were visualised using the Xjview toolbox (http://www.alivelearn.net/xjview) and for noise reduction purposes, a cluster size threshold of 50 voxels was applied.

RSNs correlations with clinical scores

a correlation analysis between fMRI finding and clinical scores was performed in order to evaluate the impact of the clinical sign and symptoms on the global brain activity. Specifically, the possible correlation was evaluated between the FC alteration and the clinical feature characterising each phase (such as the prodromal symptoms) and the neurological history of each patient.

For each contrast, the global map of alteration was then used to calculate the correlation between FC changes and clinical scores, as listed in the supplementary material eTable 1. For this analysis, we started using the voxels surviving the

FEW/TFCE-corrected thresholds ($p \leq 0.05$) in the resulting *global* maps of alteration. We then used these maps to mask the subject-specific 4D pre-processed task-free fMRI data to calculate the grand mean FC effect for each subject.

Pearson's correlation analysis was carried out using the Orange Data Mining tool (Demsar et al., 2013) to obtain a numerical value of the correlation strength between the grand mean FC effect values previously calculated and clinical scores.

Seed-based correlation analysis (SCA)

In order to better address the role of the thalamus over the rest of the brain during the different phases of the migraine cycle, a seed-based correlation analysis was carried out. Considering each subject, the pre-processed parcellated fMRI was treated with a SCA approach, implemented as voxel-wise multiple regression analysis. Specifically, for each seed ROI (e.g., left thalamus, right thalamus), the average fMRI time course was extracted and used as a reference for the cross-correlation analysis (e.g., the SCA). For each seed ROI, the result of this operation was a set of four SCA maps, labelled SCA-baseline, SCA-prodrome, SCA-full-blown and SCA-recovery map, where the intensity value of each voxel reflects the Pearson correlation coefficient between the voxel time-course and the reference time-course. SCA maps were then used for statistical testing to assess the group mean effect (ME) using a non-parametric permutation test, referred to as the "dual regression" technique using 5000 permutations and age, gender and disease duration as additional covariates in the analysis (Castellazzi et al., 2018). The resulting statistical maps (tstatME) were corrected for threshold-free cluster enhancement (TFCE) and multiple comparisons using the family-wise error (FWE) procedure. A statistical threshold of $p < 0.05$ was considered significant. Finally, the resulting ME maps were visualised using the Xjview toolbox (http://www.alivelearn.net/xjview) and for a noise reduction purpose, a cluster size threshold of 50 voxels was applied.

Secondly, to better evaluate the possible consequences of structural alteration over the brain activity of those people living with migraine, the same process was applied considering, as a seed, the map of grey matter density reduction at baseline in EM, as calculated through the method explained in the next paragraph.

Voxel-based morphometry (VBM)

In order to assess microstructural alteration of grey matter density at cortical levels across the group and different phases of the migraine-like attack, a voxel-based morphometry approach was applied.

The structural data were analysed with FSL-VBM (Douaud et al., 2007) (http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FSLVBM), an optimised VBM protocol (Good et al., 2001) carried out with FSL tools (Smith et al., 2004). First, structural images were brain-extracted and grey matter-segmented before being registered to the MNI 152 standard space using non-linear registration (Andersson et al., 2007). The resulting images were averaged and flipped along the x-axis to create a left-right symmetric, study-specific grey matter template. Second, all native grey matter images w ere non-linearly registered to this study-specific template and "modulated" to correct for local expansion (or contraction) due to the non-linear component of the spatial transformation. The modulated grey matter images were then smoothed with an isotropic Gaussian kernel with a sigma of 4 mm. Finally, voxelwise GLM was applied for group/scan comparisons using permutation-based non-parametric testing, correcting for multiple comparisons across space. Specifically, the statistical inference at the group/scan level was performed using 5000 permutations. The resulting statistical maps were family-wise error (FWE) corrected for multiple comparisons, implementing threshold-free cluster enhancement (TFCE) (SMITH & NICHOLS, 2009) using a significance threshold of at least $p \leq 0.05$.

Wavelet coherence analysis (WCA)

Coming to the possible dynamic functional changes in the thalamocortical activity during a migraine-like attack, a wavelet coherence analysis was applied to the acquired scan. The WCA is based on wavelet transform coherence (WTC), which allows analysing of the coherence and phase lag between two time series as a function of both time and frequency (Torrence & Compo, 1998). More specifically, WTC decomposes a time series in the time-frequency domain by successively convolving the time series with the scaled and translated versions of a mother wavelet function (Mallat, 2008). In Torrence et al (Torrence & Compo, 1998), $R2(s,\tau)$ denotes the local correlation coefficients in time (τ) and wavelet scale (s), between two signals X and Y. The correlation coefficients are

found by applying a wavelet cross-spectrum between the two signals and measuring the common power between the signals at various scales (s) and time (τ). The phase difference between X and Y is calculated with arg(R2(s,τ)). The results of these operations are pooled in a scalogram, which is a map of wavelet coherence between the two signals (see Figure 6).
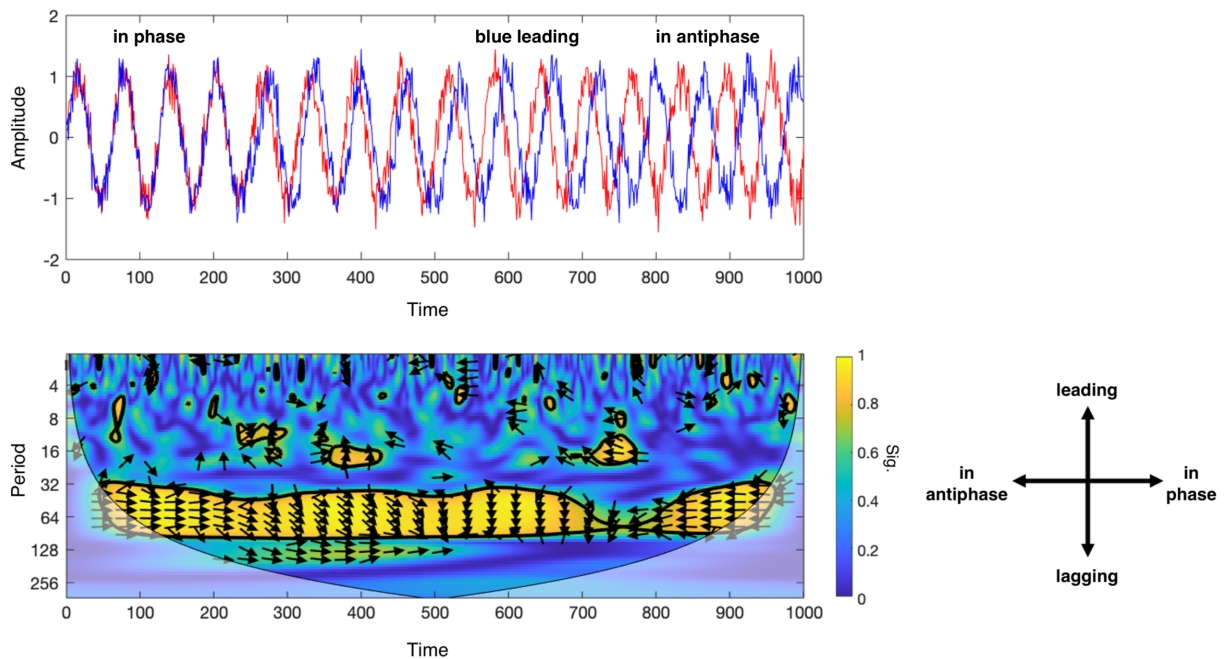

Figure 6 Description of the Wavelet Coherence Analysis (WCA). Top left: example of phase coherence between two sinusoidal signals (in blue and red), from in-phase (on the left) to antiphase (on the right) condition during a defined set of acquisition time; bottom left: scalogram that outputs from WCA for the two sinusoidal signals. The X-axis indicates the progression of time (which may be expressed in seconds, minutes or number of volumes if referred to MRI scans), and the y-axis is the scale (in Fourier periods). The map threshold is set at 95% confidence by a thick black curve based on Monte Carlo tests. Regions outside the cone of influence appear in faded colour. The Colour scale reflects the statistical significance of the wavelet coherence between the two signals. The phase angle between the ROI time series at a particular location in the time-frequency plane is indicated by an arrow. Bottom right: legend for the interpretation of the direction of the arrows (phase arrows). Phase arrows pointing: right – signals are in phase; left – signals are in antiphase; down – blue signal leading red signal by 90°; up – blues signal lagging red signal by 90°.

In this study, WCA was performed between the spontaneous oscillations of SN and bilateral thalami, each taken individually. To this end, a subset of episodic patients was selected as a subpopulation for the exploratory analysis (EM1 to EM5). The map of the structures involved in their SN, obtained from ICA, was then regressed into each subject's space to give the SN time-course for each fMRI scan, for a total of four SN time-courses per subject. After that, the subject-specific SN time-courses were temporally concatenated to form a single time-course fMRI signal of 800 points. Similarly, for each subject, the average fMRI time-courses respectively from the left and right thalamic areas were extracted for each scan as described in the Seed-based correlation

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analysis section and then temporally concatenated into single time-courses. The concatenated SN and thalamic signals were then averaged across subjects and the resulting time-courses were finally used as input for the WCA approach. WCA scalogram maps were produced using the Wavelet Coherence toolbox in MATLAB (http://www.glaciology.net/wavelet-coherence) (Grinsted et al., 2004). Specifically, WCA was performed using the complex Morlet wavelet as the mother wavelet, enabling us to obtain phase information and therefore allowing visualization of directionality in the dynamics between signals (in-phase, leading, lagging, or anti-phase) (Torrence & Compo, 1998). The significance of the resulting coherence coefficients was tested against wavelet coherence of random red noise signals using Monte Carlo methods with 1000 surrogate data set pairs as described in Bernas et al.(Bernas et al., 2018).

Non-Imaging statistics

Statistical analyses were carried out using SPSS (version 21.0; SPSS, Chicago, IL, USA). Demographic and behavioural continuous data are expressed as median, ± standard deviation. They were first tested for normality using the Shapiro-Wilk test and differences between EM and HS were assessed, with different tests depending on the typology of the variables (binary, normally or non-normally distributed). χ2-test was performed to compare frequency distributions of gender in the two groups, as well as the diagnosis of tension-type headache or ongoing anxiety. Non-parametric Mann-Whitney U-test was performed to test differences between paired groups in age and tension-type headache frequency. Correlations between imaging variables such as the mean FC effect and the clinical variables were estimated with the Orange Data Mining tool (Demsar et al., 2013).

4 Results

Clinical results

Fourteen patients affected by episodic migraine without aura, according to the ICHD 3 criteria, and fifteen HS considered as controls, were included in the study (demographic data are shown in table 3). All subjects completed the NTG study: 11 EM patients developed a headache attack fulfilling the criteria for migraine (Vincent et al., 2018), while one of the HS reported a migraine attack over 12 hours after NTG administration and was therefore excluded from the analysis. A baseline scan was performed starting at noon. Overall, 13 subjects between HS and EM developed an early non-specific headache after NTG administration, respectively 6 HS and 7 EM, which lasted an average of 25 minutes for EM and 75 minutes for HS before spontaneous resolution. Due to an imaging technical problem, 1 subject in the EM group and 2 HS were not considered for the analysis and 1 HS was excluded as well, because of evidence of WM alteration in the basal scan. For these reasons, their clinical data were discarded as well. The final analysis population included 10 EM patients vs 10 HSs.

Patients reported a migraine frequency of 4.0 ± 2.6 attacks per month. Six of them presented tension-type headache as comorbidity occurring on 1.0 ± 1.3 days per month compared with 7 HS who presented a diagnosis of infrequent tension-type headache (median frequency 0.5 ± 0.7). None of the patients was using any preventive migraine drugs and all subjects used an NSAID or triptan drug as abortive treatment with no ongoing or history of medication overuse. Three EM patients reported comorbid generalised anxiety disorder, which did not require daily pharmacological treatment, as well as 2 HS. No other relevant medical history record was reported in the subjects analysed. All 10 patients who developed the NTG-induced headache attack not only fulfilled the ICHD III criteria for a migraine episode, but the clinical features were also directly comparable with the anamnestic characteristics described by each patient. No statistically significant differences were found when comparing each condition feature using the χ^2 test ($p > 0.05$).

As illustrated in the demographical table 3 and the following table 4 regarding the clinical features of the patients during the acquisition, the median time between NTG administration and the scans at the prodromal phase was 90 minutes (range 39 to 143 minutes) with an average delay from the onset of the symptoms and the scan of 12 minutes. The median time between NTG administration and the scans at the full-blown phase was 165 minutes (range 100 to 218 minutes) with a median delay of 21 minutes. Migraine features were recorded by a trained clinician immediately after the scan. Aggravation by the movement was assessed by walking within the waiting room. The last scan, at recovery, was performed in patients after a median of 85 minutes (range 55 to 110 minutes) from the administration of an NSAID, when the second scan was completed. Overall, the last scan for the recovery phase was performed at a median time of 245 minutes after the NTG administration. In one case, 10mg intramuscular metoclopramide was administered due to severe nausea.

All HS were scanned after 90, 165 and 245 minutes since NTG administration.

	EM (10)	HS (10)
Woman, n	5	4
Age, y	28.9 ± 5.96	26.9 ± 2.88
migraine frequency, n. of days per month	4.4 ± 2.7	
disease onset, y	13.2 ± 4.5	
disease duration, y	20.2 ± 7.9	
comorbid tension type headache, n (%)	6 (60%)	7 (70%)
tension type frequency, n. of days per month	1.0 ± 1.3	0.5 ± 0.7
comorbid anxiety, n (%)	3 (30%)	2 (20%)
comorbid depression, n (%)	0	0

Table 3 Summary of the demographical and anamnestic characteristics of the population studied. Median, ± standard deviation

participant ID	age	sex⚘	aspecific headache∫	prodromal scan, minutes post NTG	prodromal features at em1 scan †	NRS* prodromal	Full-blown scan, minutes post infusion	NRS*/side full blown	migraine features during scan^	abortive drug	postdrome scan, minutes post abortive	NRS* recovery
EM1	29.4	1	1	90	0/0/0/1/0/0/0/0/1/0	2	218	7/R	0/0/1/1/1/1	NSAID	94	1
EM2	26.9	1	1	39	1/0/0/1/1/0/0/0/0/0	2	165	5/R	0/0/1/0/1/1	NSAID	65	0
EM3	32.2	0	1	125	0/0/0/0/1/0/0/1/1/0/0	2	170	6/L	1/0/1/0/0/1	NSAID	88	1
EM4	33.2	1	0	44	1/1/0/0/0/0/1/0/0/0	2	100	8/B	1/0/1/0/1/1	NSAID	110	0
EM5	45.4	0	0	86	0/0/0/0/1/0/0/1/1/0/0	1	121	6/B	1/0/1/1/1/1	NSAID	106	1
EM6	27.6	1	1	143	0/0/0/0/1/0/0/0/0/0/0	2	178	5/R	1/0/1/0/0/1	NSAID	85	0
EM7	24.2	0	0	113	1/0/0/1/0/0/0/1/0/0/0	0	205	5/L	1/0/1/0/0/1	NSAID	71	0
EM8	26.0	0	1	75	1/0/0/0/1/0/0/0/0/0/0	2	150	7/L	0/0/1/1/0/1	NSAID	55	0
EM9	32.0	1	1	100	0/0/0/0/1/0/0/1/1/0/0	2	125	7/R	1/0/1/1/0/0	NSAID	78	0
EM10	28.3	0	1	90	1/0/0/1/1/0/0/1/0/0/0	2	218	5/r	1/0/0/0/0/1	NSAID	94	0
median	28.9	5F5M		90		2*	145	6*			85	1*

Table 4 Clinical features of the migraine-like experience

⚘ Sex: 1 = male

† 1 = presence of yawning/irritability/mood swing/sleepiness/tiredness/loss of appetite/food desire/nausea/stiffness/thirst/urinary retention

^ 1 = presence of nausea/vomiting/photophobia/phonophobia/aggravation by movement/throbbing pain

*NRS: numeric pain rating score 0–10 (mild: 1–3; moderate: 4–6; severe: 7–10) collected at the beginning of the scan.

∫ aspecific headache: 1 = presence of a transient feeling of holocranial headache after NTG administration, not resembling a migraine attack

Functional MRI results (fMRI)

Identification of the resting state networks

ICA processing on rs-fMRI images resulted in 25 independent components (ICs), 19 of which were classified as RSNs based on their frequency spectra and spatial patterns (Castellazzi et al., 2014; Cole, 2010; Damoiseaux et al., 2006). The remaining 6 components probably reflected artefacts, such as movement, physiological noise, or cerebrospinal fluid (CSF) partial volume effects.

The identified 19 RSNs were: medial visual network (*MVN*), lateral visual network (*LVN*), precuneus network (*PN),* superior precuneus network (*PNsup*), sensory-motor networks area M1 (*SMNm1),* and area S2 (*SMNs2*), auditory network (*AN*), executive control network (*ECN*), default mode network (*DMN*), anterior default mode network (*DMNa*), frontal cortex network (*FCN*), language networks (*LN* anterior (*a*) and posterior (*p*), right (*R*) and left (*L*) ventral attention networks (*VAN*), salience network (*SN*), task-positive network (TPN), basal ganglia network (BGN) and cerebellar network (*CBLN*).

Comparison between groups

Results of the comparison between the global mean FC in EM patients and HS (p≤0.05, TFCE corrected) are shown for each induced migraine administration phase in Figure 7.

At baseline, people living with episodic migraine scanned in a pain-free condition, presented reduced global FC (Figure 8), when compared to HS, among the following structures: left (L) posterior cerebellum, especially the CRUS II, left (L) middle frontal gyrus (MFG) and the left precuneus. Areas of reduced FC also involved the left cingulate gyrus (particularly the posterior part) and the superior temporal gyrus. On the contrary (Figure 11), clusters of increased global FC in EM at baseline highlighted the relationship between the posterior
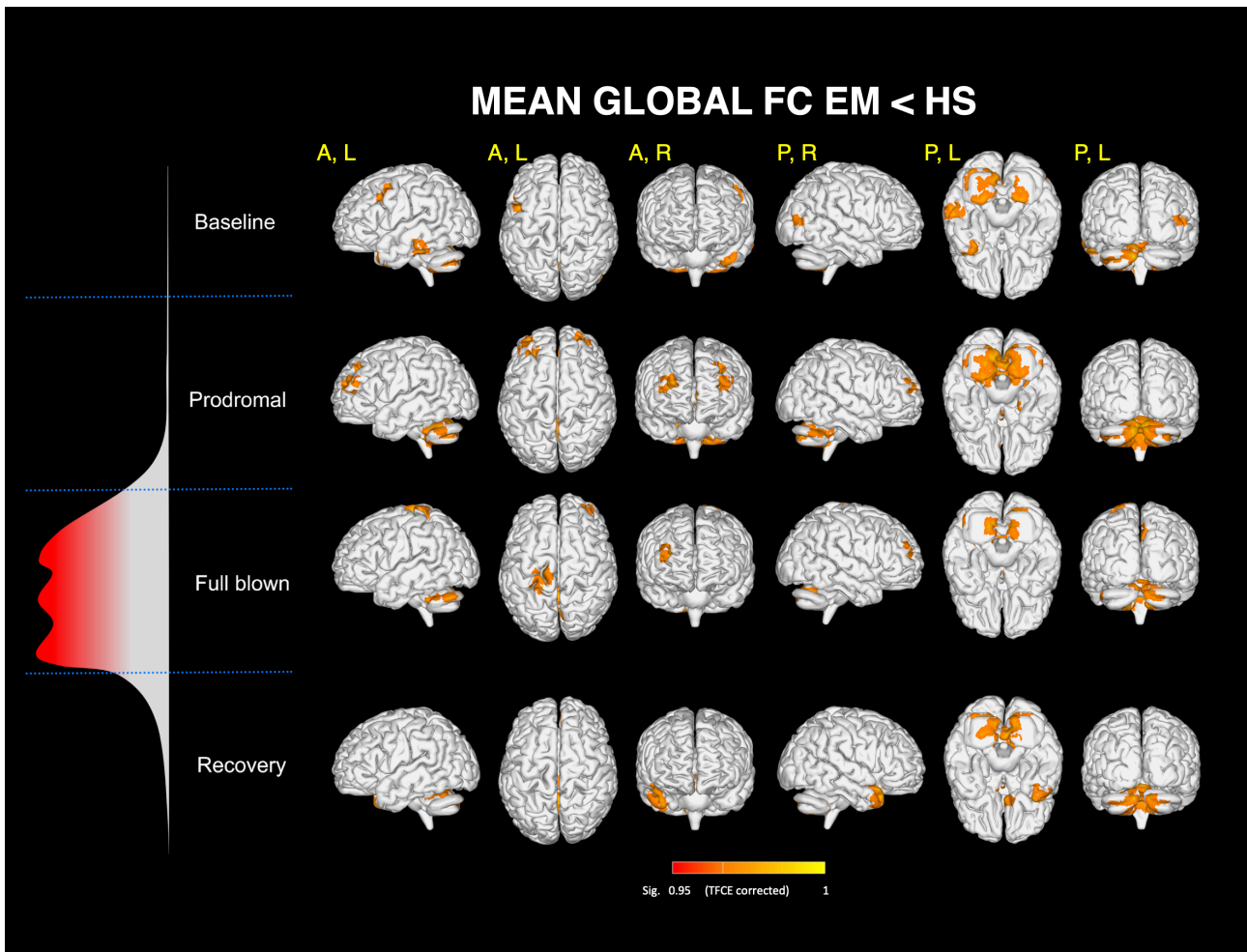
41

Figure 7 Global functional connectivity (FC) describing the area where mean value is statistically reduced among the patients with episodic migraine (EM) compared to the healthy subjects (HS).
Legend: Anterior (A), Posterior (P), Left (L) Right (R), p≤0.05, TFCE corrected.

cerebellum, the middle and inferior temporal gyrus (prevalently but not exclusively on the right side) the angular and supramarginal left gyri in the parietal lobe. Finally, it involved also the bilateral superior and middle frontal gyrus.

After baseline, during the prodromal phase of the migraine-like attack-induced administration, the mean global FC was persistently reduced among the posterior cerebellum (crus I, II) anterior bilateral cerebellum lobe and the superior/middle frontal gyri (particularly on the right side). Notably, the cluster of reduced FC persisted including the cingulate cortex (particularly in the anterior part) and highlighted the involvement of the thalamus (mainly on the left side). On the other hand, the cluster of increased FC in EM vs HS was restricted during the prodromal phase and limited to the posterior cerebellum, the left inferior temporal gyrus, and the middle frontal gyrus.

Focusing on the full-blown phase, the involvement of the posterior cerebellum persisted in its relationship with the superior and middle frontal gyri, as well as the

thalamus and the cingulate gyrus, but the cluster was less extended. On the other hand, the areas of increased FC among EM involved the left posterior cerebellum, the parietal lobe (specifically the inferior and superior parietal lobe, strongly on the left side), the temporal lobe within the superior and inferior temporal gyri (prevalently on the left side), the supramarginal gyrus and finally the frontal lobe (inferior, superior, and middle frontal gyri).

Finally, during the postdrome at the recovery phase, areas of reduced global FC were still strong among the posterior cerebellum (crus I, II), the cingulate cortex (in particular the left posterior portion), and the left thalamus. Additionally, it was possible to describe a cluster attack between the inferior and superior right temporal gyri. Moreover, the area of increased FC among the EM compared with the HS was redistributed but still had its peak in the posterior lobe of the cerebellum, in its relationship with the left precuneus, supramarginal gyrus, inferior temporal gyrus and the frontal (middle and superior gyrus) and parietal lobe (inferior and superior) bilaterally but with a stronger FC value on the left side.

Overall, the comparison between EM patients and HS highlighted the constitutive relationship between the posterior cerebellum (namely crus I e II), the frontal lobe (middle and superior frontal gyri) and the cingulate cortex. The extent of this alteration varied during the migraine-like experience also introducing the participation of the left thalamus, which persisted over the prodromal phase till the recovery phase. The parietal lobe instead, namely the angular e supra-marginal gyri, expressed an increased FC within the other structures listed above, in particular during the full-blown, but lasting over this phase.
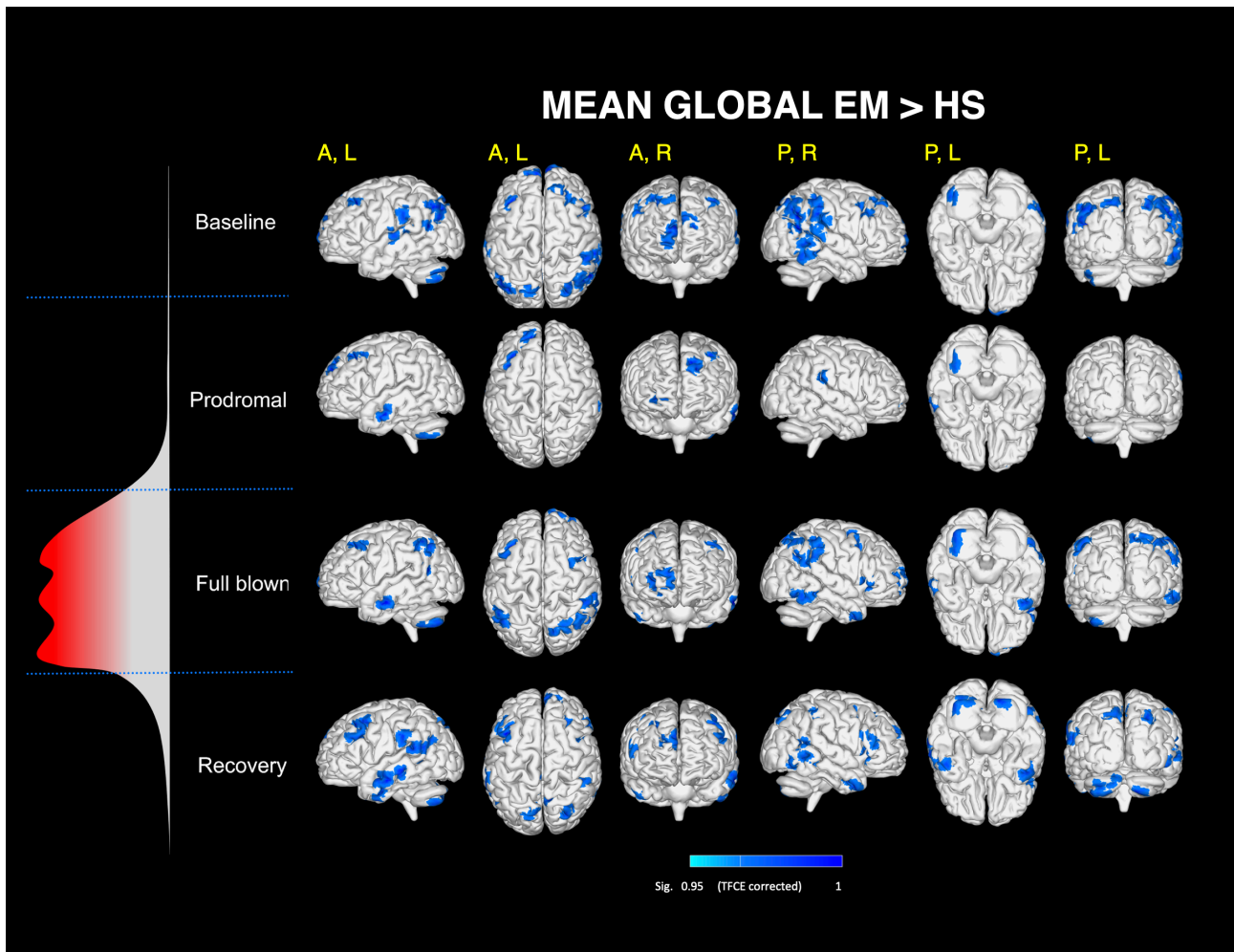
Figure 8 Global functional connectivity (FC) describing the area where mean value is statistically increased among the episodic migraine (EM) population compared to the healthy subjects (HS) Legend: Anterior (A), Posterior (P), Left (L) Right (R), p<0.05, TFCE corrected

Correlations with the clinical variables

Considering the resulting meaningful map of the structures involved in weaker or stronger connectivity among groups, the relative global FC was calculated and correlated to the clinical variables collected. As shown in table 5, people living with migraine demonstrated a statistically significant correlation between FC alteration, migraine frequency, and tension-type headache frequency over the entire migraine cycle. When comparing EM with HS, structures with a stronger FC showed a negative correlation with migraine and a positive one with tension-type headache frequency. Interestingly, when analysing the mean FC among the structures involved in a weaker functional relationship, an opposite correlation was found. In addition to this, the prodromal phase also presented a correlation with the intensity of the discomfort during the Scan 2 acquisition (NRS Scan 2) and the duration of the aspecific headache induced by the NTG

contrast	feature	Pearson correlations	p value
		Baseline (Scan 1)	
em<hs	migraine frequency	+0.839	p≤0.05
em<hs	tension-type headache frequency	- 0.073	p≤0.05
em>hs	migraine frequency	- 0.595	p≤0.05
em>hs	tension-type headache frequency	+0.298	p≤0.05
		Prodromal (Scan 2)	
em<hs	tension type headache frequency	-0.476	p≤0.05
em<hs	NRS scan 2 (prodromal)	-0.027	p≤0.05
em<hs	migraine frequency	+0.171	p≤0.05
em<hs	duration aspecific headache	+0.029	p≤0.05
em>hs	duration aspecific headache	+0.405	p≤0.05
em>hs	prodromal symptoms onset after NTG induction (min)	+0.207	p≤0.05
em>hs	NRS scan2	+0.147	p≤0.05
em>hs	migraine frequency	+0.147	p≤0.05
		Full-blown (Scan 3)	
em<hs	NRS scan3 (full-blown)	-0.632	p≤0.05
em<hs	migraine frequency	+0.455	p≤0.05
em<hs	prodromal symptoms onset after NTG induction (min)	+0.263	p≤0.05
em<hs	tension type headache frequency	-0.18	p≤0.05
em>hs	tension type headache frequency	+0.446	p≤0.05
em>hs	prodromal symptoms onset after NTG induction (min)	+0.332	p≤0.05
em>hs	migraine frequency	-0.186	p≤0.05
em>hs	duration aspecific headache	-0.186	p≤0.05
em>hs	NRS scan2 (prodromal)(prodromal)	+0.14	p≤0.05
		Recovery (Scan 4)	
em<hs	tension type headache frequency	-0.562	p≤0.05
em<hs	prodromal symptoms onset after NTG induction (min)	+0.427	p≤0.05
em<hs	NRS Scan 3 (full-blown)	-0.403	p≤0.05
em<hs	migraine frequency	+0.375	p≤0.05
em<hs	full-blown symptoms onset after NTG induction (min)	+0.139	p≤0.05
em>hs	migraine frequency	-0.422	p≤0.05
em>hs	duration of aspecific headache	+0.354	p≤0.05
em>hs	tension type headache frequency	+0.19	p≤0.05
em>hs	NRS Scan 3 (full-blown)	+0.035	p≤0.05

Table 5 . Statistically significant correlations in the EM group between clinical variables and mean global FC resulting from each comparison.

administration. The latter correlated also with the map of stronger FC among EM during the full-blown and

recovery phases. The FC in the full-blown phase instead, specifically correlated with pain intensity during the Scan 3 acquisition and the time between the NTG administration and the onset of the prodromal symptoms. These features correlated also with the FC during the recovery phase. Finally, the global FC map of weaker connectivity in EM at Scan 4 positively correlated with the time between the NTG administration and the advent of the full blown phase.

Table 6 summarises the statistically meaningful correlations observed between the FC of the HS and the duration of the aspecific headache induced by the NTG administration, as well as the frequency of the comorbid tension-type headache.

contrast	feature	Pearson correlations	p value
Baseline (Scan 1)			
em<hs	tension type headache frequency	+0.65	p≤0.05
em>hs	tension-type headache frequency	+0.124	p≤0.05
Prodromal (Scan 2)			
em<hs	duration aspecific headache	+0.371	p≤0.05
em<hs	tension type headache	+0.274	p≤0.05
em>hs	duration aspecific headache	-0.15	p≤0.05
em>hs	tension type headache frequency	-0.094	p≤0.05
Full-blown (Scan 3)			
em<hs	tension type headache frequency	+0.822	p≤0.05
em<hs	duration aspecific headache	+0.653	p≤0.05
em>hs	tension type headache frequency	+0.193	p≤0.05
Recovery (Scan 4)			
em<hs	tension type headache frequency	+0.803	p≤0.05
em<hs	duration aspecific headache	+0.767	p≤0.05

Table 6 Statistically significant correlations in HS group between clinical variables with the mean global FC resulting from each comparison.
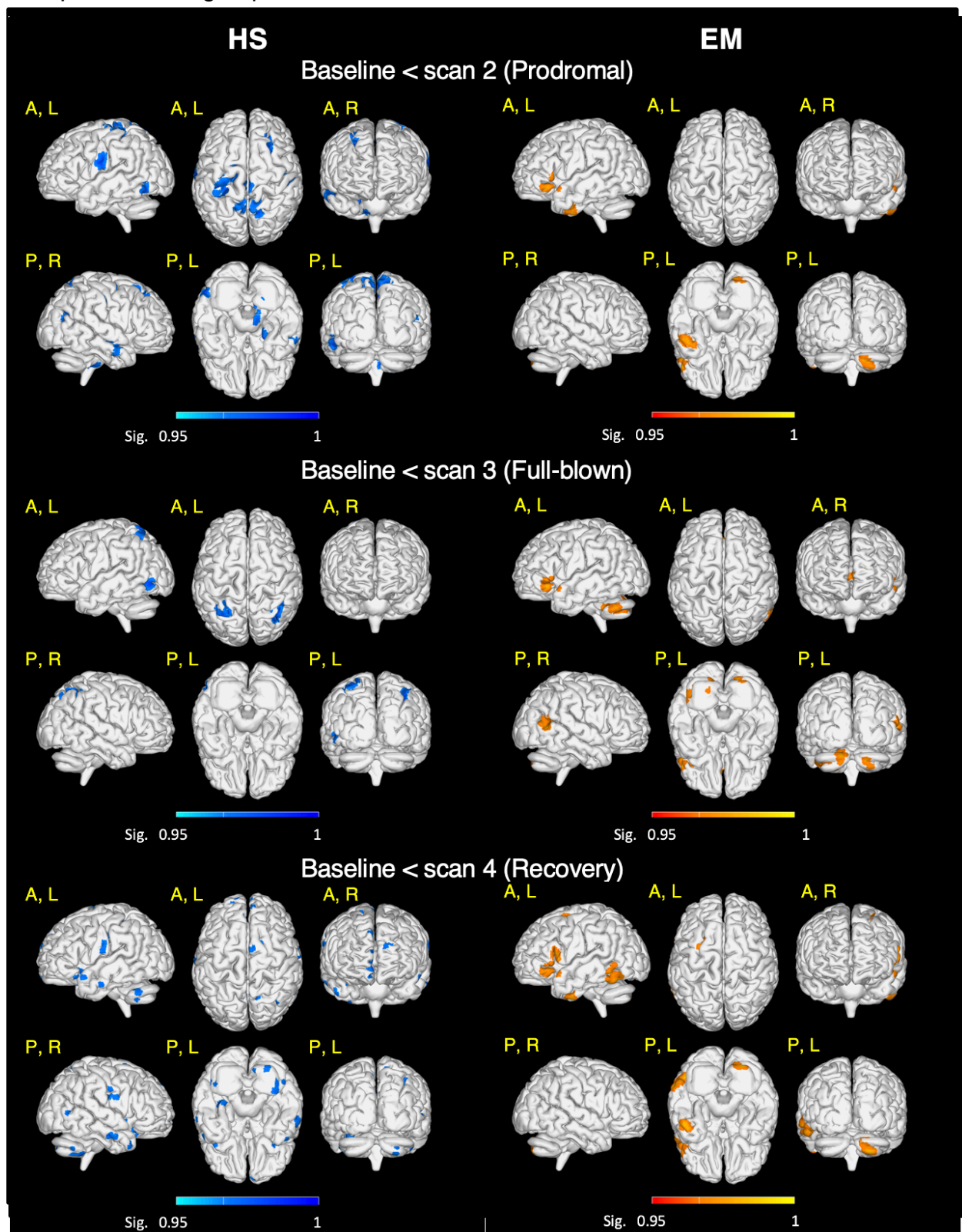
Comparison intra group

Figure 9 Comparison of the mean functional connectivity (FC) of each scan with the baseline acquisition. The results express the area where FC is stronger during each scan, compared to the pain-free baseline condition. Each comparison is calculated within the same group. Only statistically significant results are presented (p≤0,05 TFCE corrected). Anterior (A), Posterior (P), Left (L) Right (R)
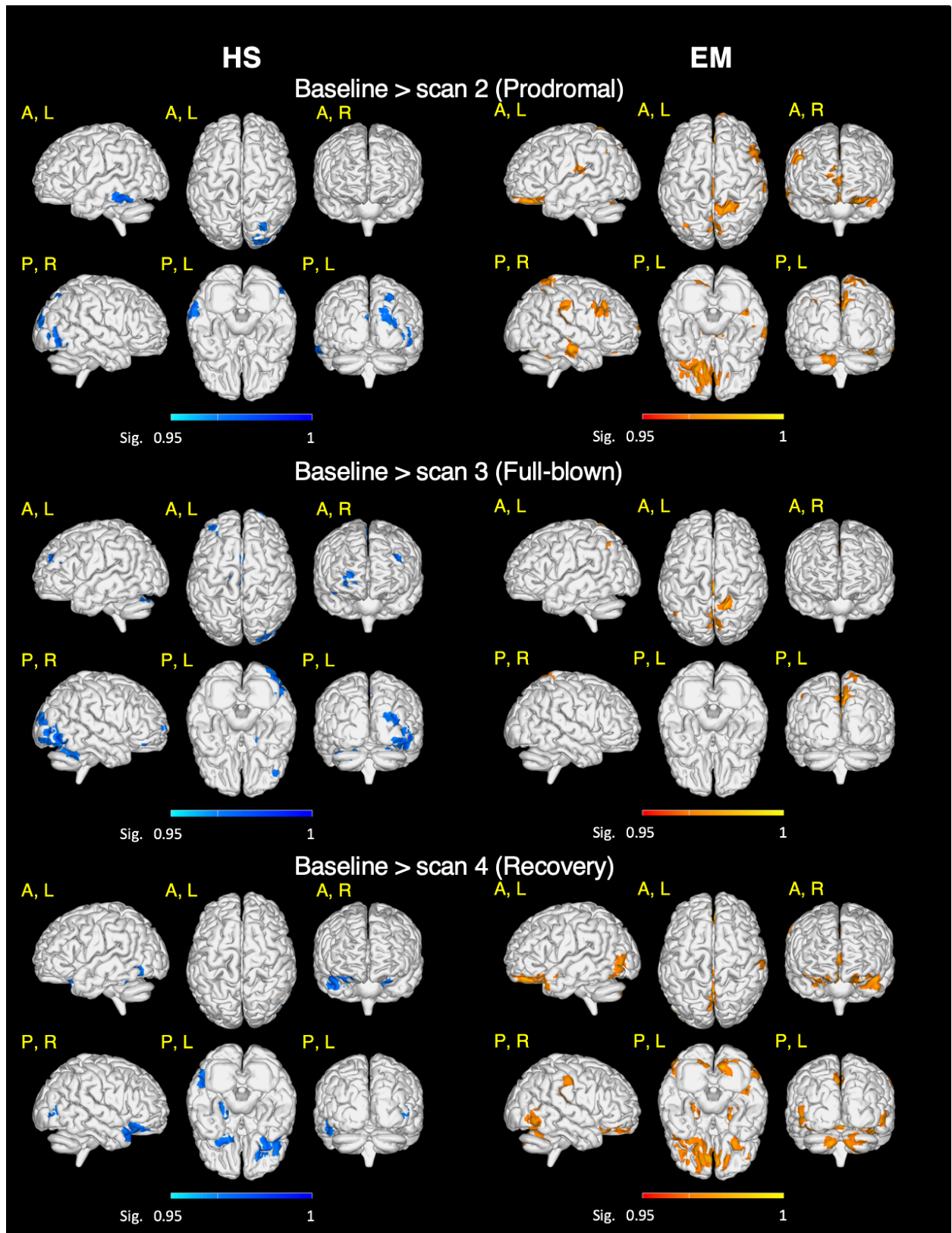
Figure 10 Comparison of the mean functional connectivity (FC) of each scan with the baseline acquisition. The results here presented, express the area where the FC is weaker during each scan, compared to the pain-free baseline condition. Each comparison Is calculated within the same group. Only statistically significant results are presented (p≤0,05 TFCE corrected). Anterior (A), Posterior (P), Left (L) Right (R)

When comparing every scan (prodromal, full-blown and recovery) with the relative pain-free state at baseline, it was possible to describe an FC alteration peculiar to the migraine experience. Figure 9 shows the structures where the FC is stronger during each scan compared to the baseline scan, while Figure 10 is the opposite.

During the prodromal phase, a reduction of the FC was observed in the posterior cerebellum (namely the Crus I and II), the left precuneus, the cingulate cortex (in its middle portion) and the left rectal gyrus. On the contrary, areas of increased FC were found in the right crus II, frontal inferior orbital gyrus, and inferior temporal gyrus. Over the full-blown phase, EM showed increased FC when compared to the baseline in the posterior cerebellum (crus I and II, right side) and the cingulate cortex (anterior portion). Only a modest FC reduction was observed within the left precuneus, cingulate cortex and left parietal lobe. Coming to the recovery phase, the FC alteration persisted over the posterior part of the cerebellum, and FC reduction was evident also within the bilateral inferior rectal gyrus, as well as the left precuneus and left cingulate cortex. On the other hand, areas of increased FC were observed within the left inferior frontal orbital gyrus and the inferior temporal gyrus.

Overall, the functional activity of the crus I and II in the posterior cerebellum was significantly altered within the entire migraine-like cycle, with a different directionality of the FC changes during each phase. The prodromal and recovery phases resembled one another for the same reduction of FC within the posterior cerebellum, the rectal gyrus. Overall, the activity of the cingulate cortex and precuneus appeared to be strongly associated with the entire migraine cycle and its altered FC persisted also during the recovery. Conversely, the increased FC in the anterior cingulate cortex on the left side appeared to be peculiar to the full-blown phase.

Considering the HS group, the comparison between scans produced sparse and inconstant results with an increased FC during Scan 2 compared to the first scan before the NTG administration in the inferior frontal gyrus and the precuneus. A reduced FC was observed instead considering the inferior temporal gyrus and the occipital cortex. Comparing the third scan with the baseline acquisition, the reduction of FC was evident in the supplementary motor area, while the increase in FC was highlighted in the ITG

and bilateral angular gyrus. Finally, the comparison of the fourth scan with the baseline scan described only a reduction of the FC in the superior and inferior temporal gyrus. Overall, the differences were sparse and inconstant, comprising the motor and visual cortex and areas not involved in pain processing.

Correlations with the clinical variables

Table 7, on the following page, describes the statistically significant correlations between the global FC, calculated for each comparison within the group of people living with migraine, and the collected clinical variables. As previously highlighted, also in this setting the FC maps correlated with the migraine and tension-type frequency as well as the duration of the aspecific headache induced by the NTG administration. During each scan over the migraine cycle, the structures presenting an altered FC showed a significant correlation with the time between the NTG administration and the onset of the prodromal symptoms. The FC resulting from the comparison of the prodromal phase with the pain-free condition also presented a positive correlation with the intensity of the discomfort during Scan 2 acquisition during the prodromal phase (NRS Scan 2). During the full-blown and recovery phases instead, the global FC correlated with the intensity of the pain during the acquisition and the time between the NTG administration and the advent of the full-blown phase.

contrast	feature	Pearson correlations	p value
prodromal > baseline	duration aspecific headache	-0.367	p≤0.05
prodromal > baseline	prodromal symptoms onset after NTG induction (min)	-0.334	p≤0.05
prodromal > baseline	migraine frequency	-0.194	p≤0.05

contrast	feature	Pearson correlations	p value
full-blown> baseline	full-blown symptoms onset after NTG induction (min)	+0.359	p≤0.05
full-blown> baseline	tension type headache frequency	+0.229	p≤0.05
full-blown> baseline	duration aspecific headache	+0.184	p≤0.05
full-blown> baseline	migraine frequency	-0.173	p≤0.05
full-blown> baseline	NRS Scan 3(full-blown)	+0.055	p≤0.05

contrast	feature	Pearson correlations	p value
recovery>baseline	tension type headache frequency	+0.436	p≤0.05
recovery>baseline	time from NTG administration	+0.377	p≤0.05
recovery>baseline	full-blown symptoms onset after NTG induction (min)	+0.327	p≤0.05
recovery>baseline	migraine frequency	-0.278	p≤0.05

contrast	feature	Pearson correlations	p value
prodromal < baseline	NRS Scan 2 (prodromal)	-0.61	p≤0.05
prodromal < baseline	prodromal symptoms onset after NTG induction (min)	+0.586	p≤0.05
prodromal < baseline	tension type headache frequency	+0.303	p≤0.05
prodromal < baseline	duration aspecific headache	-0.1	p≤0.05
prodromal < baseline	migraine frequency	+0.046	p≤0.05

contrast	feature	Pearson correlations	p value
full-blown < baseline	NRS Scan 2 (prodromal)	-0.347	p≤0.05
full-blown < baseline	tension type headache frequency	+0.343	p≤0.05
full-blown < baseline	prodromal symptoms onset after NTG induction (min)	+0.28	p≤0.05
full-blown < baseline	NRS Scan 3 (full-blown)	+0.164	p≤0.05
full-blown < baseline	migraine frequency	+0.152	p≤0.05

contrast	feature	Pearson correlations	p value
recovery<baseline	prodromal symptoms onset after NTG induction (min)	+0.375	p≤0.05
recovery<baseline	migraine frequency	-0.204	p≤0.05
recovery<baseline	NRS Scan 3 (full-blown)	+0.201	p≤0.05
recovery<baseline	duration aspecific headache	+0.189	p≤0.05

Table 7 Correlation of the clinical variables collected for the subjects living with migraine with the mean global FC resulting from each comparison with the pain-free condition at baseline. Only statistically significant correlations (p≤0.05) are presented

Structural MRI (VBM) results

Comparison between patients and healthy subjects

Grey matter density (GMd) alterations were observed during the entire migraine-like experience when comparing HS with patients. Area with increased GMd in patients vs HS (Figure 11) were located in the limbic cortex, the middle frontal gyrus, the postcentral gyrus, and the cingulate cortex. Notably, also the parahippocampal gyrus consistently resulted structurally alternated. Moreover, it was possible to describe a reduction of the GMd among patients (Figure 12) in the inferior parietal lobe, the fusiform gyrus (during Scans 3 and 4) the middle and superior right temporal gyrus and the homolateral insular cortex.
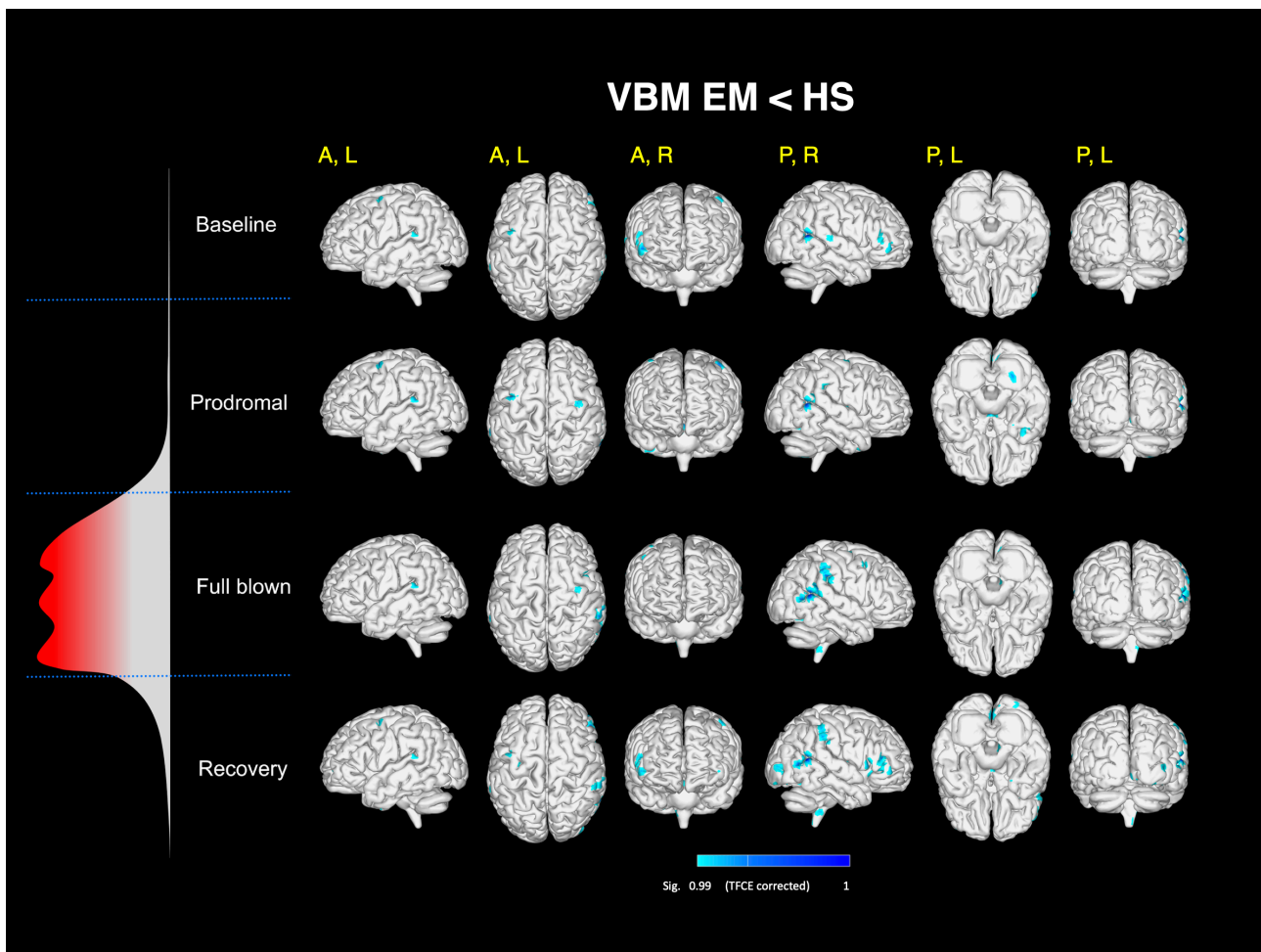

Figure 11 Grey matter density (GMd) alteration calculated through voxel-based morphometry (VBM) approach, describing the area where mean value is statistically reduced among the episodic migraine (EM) population compared to the healthy subjects (HS) Legend: Anterior (A), Posterior (P), Left (L) Right (R). Sig p≤0.01, TFCE corrected
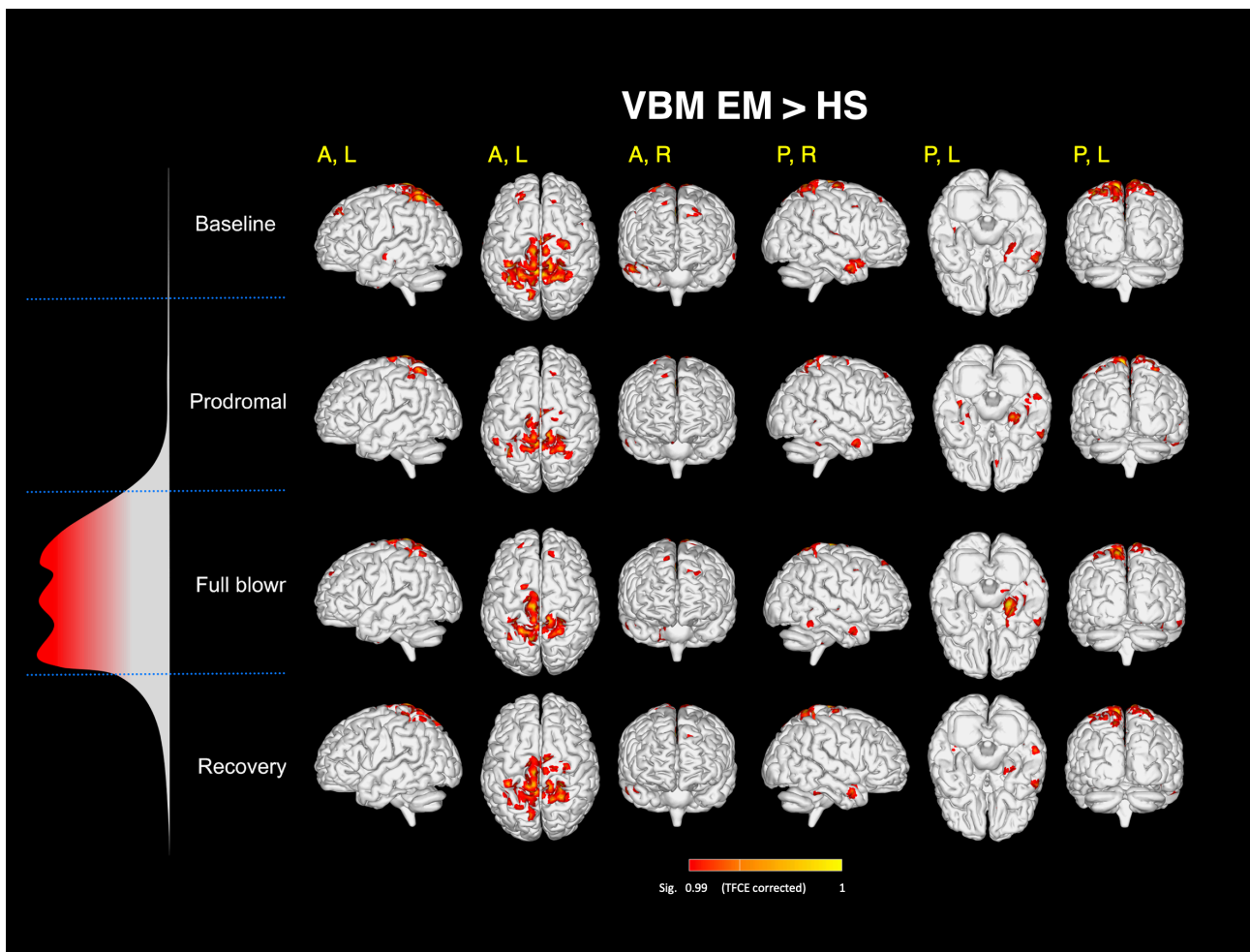
Figure 12 Grey matter density (GMd) alteration calculated through voxel-based morphometry (VBM) approach, describing the area where mean value is statistically increased among the episodic migraine (EM) population compared to the healthy subjects (HS) Legend: Anterior (A), Posterior (P), Left (L) Right (R). Sig p≤0.01, TFCE corrected.

Intra group comparison: phase by phase

When comparing every single scan during the migraine experience (i.e., prodromal, full-blown and recovery) vs the pain-free condition, neither in patients nor in HS it was possible to detect a meaningful GMd alteration, when thresholding at p≤0.05 threshold. Only some spotting results could be obtained using a threshold of p<0.1, which were not statistically significant. Therefore, we can conclude that grey matter density does not change through the induced migraine-like attack or due to the NTG administration.

Seed-based correlation analysis (SCA)

<u>Seed: baseline grey matter density reduction</u>

Considering the map of GMd reduction in EM at baseline as seed, SCA was performed and the maps of relative functional connectivity changes at baseline and at the full-blown phase were calculated. The results are highlighted in Figure 13.
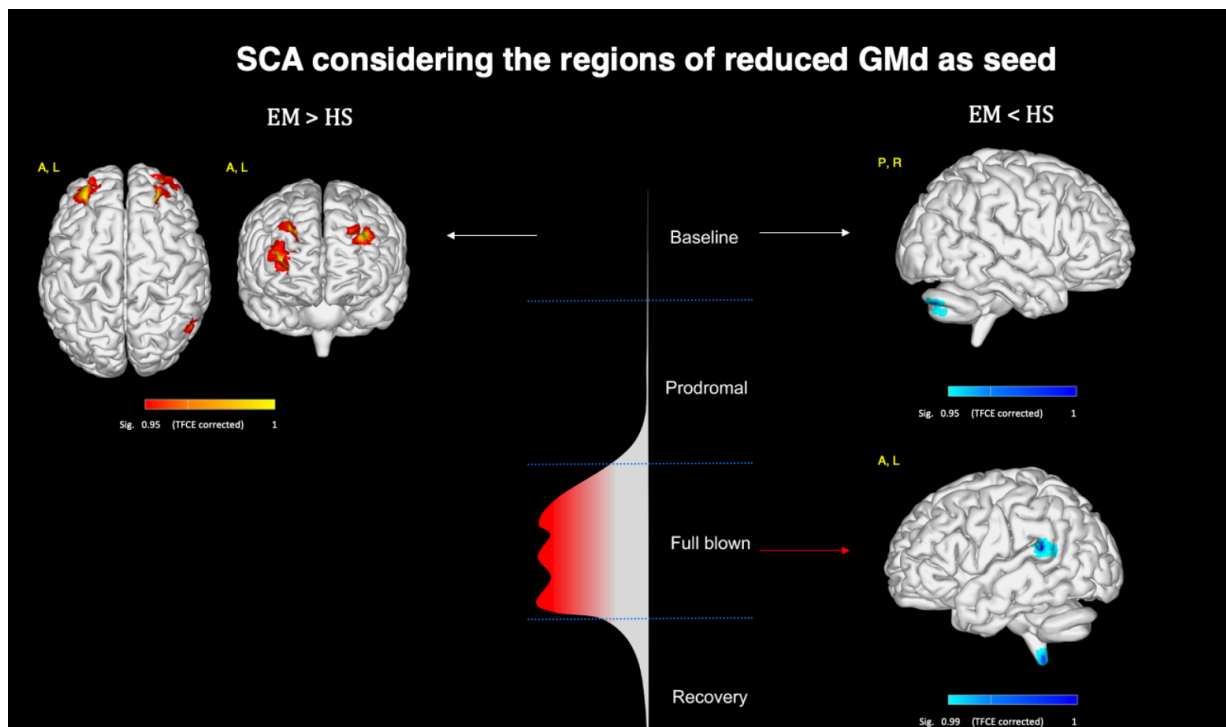

Figure 13 Seed-based correlation analysis (SCA) considering as seed the map of grey matter density (GMd) reduction at baseline in EM compared to HS, calculated through voxel-based morphometry (VBM) approach. The seed includes the left precentral gyrus, the middle and superior bilateral temporal gyrus, and the left inferior parietal lobe. Only statistically significant changes are presented in the comparison between healthy subjects and patients. Legend: Anterior (A), Posterior (P), Left (L) Right (R). Sig p≤0.05 or lower, TFCE corrected.

At baseline, EM presented a meaningful (p≤0,05) FC decrease between the areas of GMd reduction and the posterior cerebellum (crus I, II R) and an increased FC with the bilateral middle and superior frontal gyrus, as well as the right frontal inferior opercular gyrus and the angular gyrus.

Over the full-blown phase instead, a strongly statistically significant (p≤0.01) FC reduction was observed considering the relationship between the area of structural GMd in EM and the left superior temporal gyrus as well as the posterior portion of the rostral medulla.

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<u>Seed: Thalamus</u>

<u>Comparison between groups</u>

In EM patients, at baseline, both thalami presented stronger FC correlation with the supramarginal gyrus and the homolateral superior frontal gyri. Moreover, the left thalamus showed weaker FC correlation with the superior temporal gyrus. Coming to the prodromal phase, the functional relationship between the thalami and the crus I in the posterior cerebellum became stronger than the one observed in HS, together with a stronger interplay between the left thalamus and the homolateral cerebellar tonsil. Interestingly, the left thalamus presented a peculiar functional alteration characterised by a weaker FC correlation with the bilateral dorsal portion of the pons and the precuneus. The alteration in the functional relationship between the left thalamus and the dorsal pons was evident over the entire ictal phase of the migraine-like attack, but with a change in directionality of the coupling over the full-blown phase (increased) in contrast with the observed reduction during both the prodromal and recovery phase. The change in FC directionality was evident also considering the relationship between the posterior part of the cerebellum (Crus II) and the right thalamus: stronger FC correlation compared to HS was observed not only at baseline but also during the prodromal phase and it was restored at recovery. During the full-blown instead, a reduction of the relative FC strength was observed among the mentioned structures. Moreover, the full-blown phase presented a stronger functional interaction between the right thalamus and the precuneus, precentral gyrus and superior frontal gyrus in EM compared to HS. Strikingly, the functional interaction of the thalami and the middle temporal gyrus as well as the cingulate cortex during the prodromal phase was like the one observed during the recovery phase.

<u>Comparison intra groups</u>

Considering the group of people living with migraine, thalamic activity was evaluated along the different phases, in comparison to the pain-free state at baseline, as shown in Figure 14 for the right thalamus and Figure 15 for the left thalamus. During the prodromal phase, both thalami expressed a weaker FC correlation with the superior and middle frontal gyri, as well as the cingulate cortex. Interestingly, the right thalamus presented a peculiar relationship, characterised by a reduction of their FC strength, with the posterior

part of the cerebellum (homolateral crus I, II) and the right spinal trigeminal nucleus, caudal portion. The right thalamus persisted in a weaker FC correlation coupling with the cerebellum and MFG as well as SFG. Both thalami coupled with the precentral gyri during the full-blown phase and, notably, the left thalamus coupled also with the orbitofrontal gyrus with a stronger FC correlation compared to the pain-free condition. The recovery phase expressed instead the persistence of a weaker FC correlation between both thalami and the homolateral spinal trigeminal nucleus, caudal portion, as well as dorsal portion of the pons (right only) and posterior cerebellum (crus I and II, right). Finally, it was worth noticing the persistent functional alteration of the relationship between the thalami and the frontal cortex which lasted from the prodromal over the recovery phase.
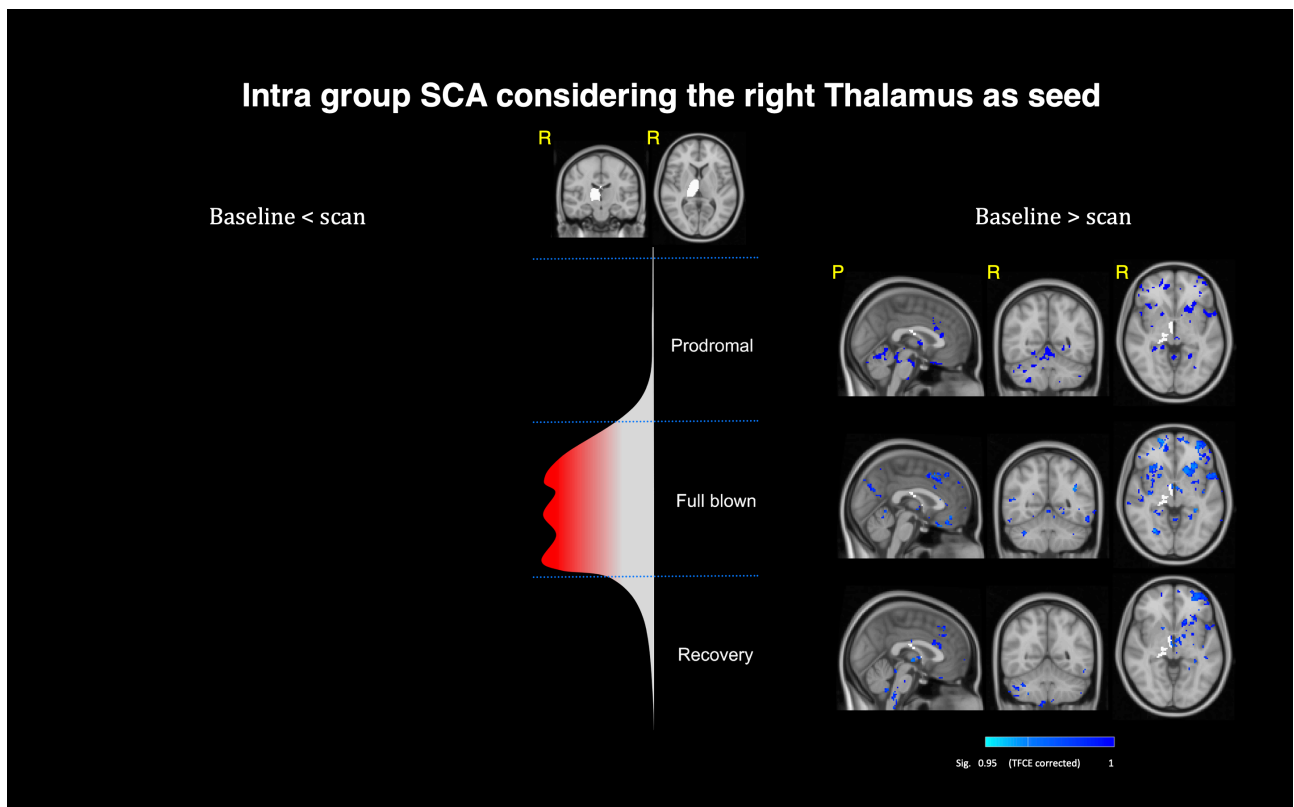

Figure 14 Seed-based correlation analysis (SCA) considering as seed the right Thalamus (in white). The results here presented, express the map of structures with a weaker FC correlation (in blue) with the right thalamus during each scan, compared to the pain-free baseline condition. Each comparison Is calculated within the same group Only statistically significant changes are presented. Legend: Posterior (P), Right (R). Sig p≤0.05, TFCE corrected.
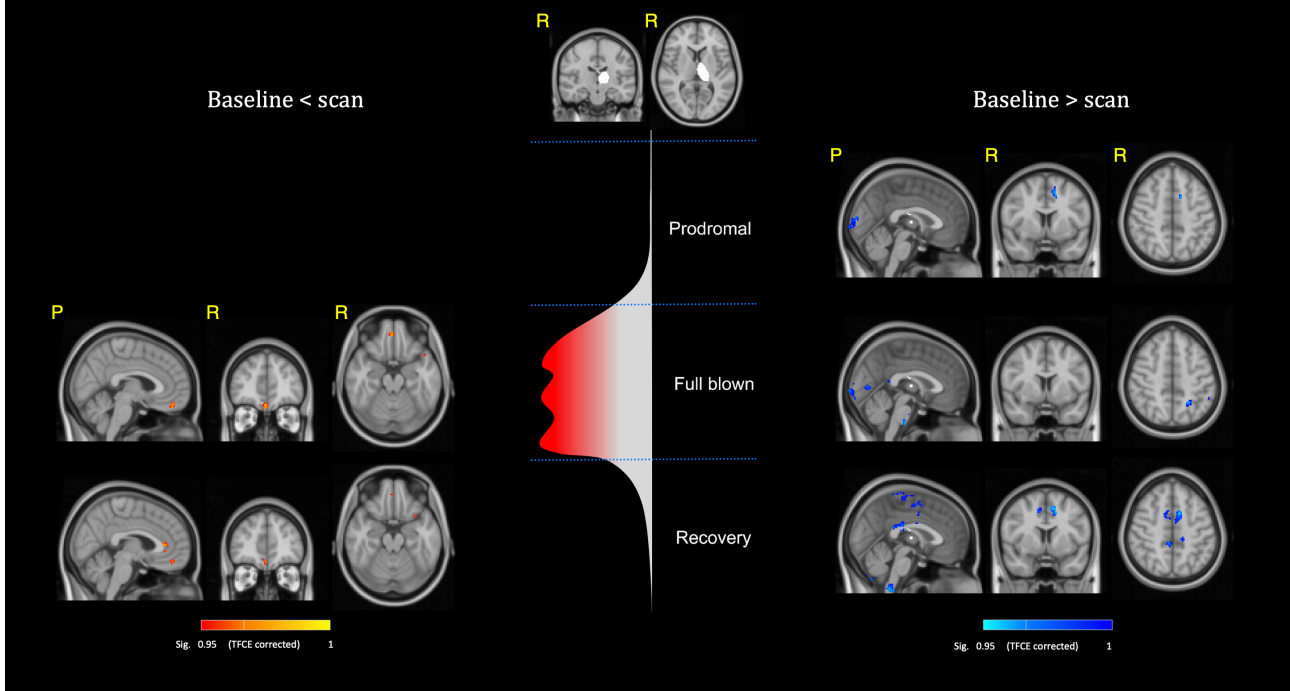
Figure 15 Seed-based correlation analysis (SCA) considering as seed the left Thalamus (in white). The results here presented, express the map of structures with a weaker (in blue) and stronger (in yellow) FC correlation with the left thalamus during each scan, compared to the pain-free baseline condition. Each comparison Is calculated within the same group Only statistically significant changes are presented. Legend: Posterior (P), Right (R). Sig p≤0.05, TFCE corrected.

Wavelet coherence analysis (WCA)

Considering the first 5 patients acquired as an exploratory population, WCA was applied to both thalami and SN, showing significant dynamical changes in their functional interaction during the experience of the NTG-induced headache attack (see Figure 16). At baseline, bilateral thalamic fMRI signals resulted in antiphase with SN, gradually losing phase synchronisation during the prodromal and full-blown phases, with the thalamic signals prevalently leading the SN. This loss of phase-coherence reached its peak during the full-blown phase. During the full-blown phase, both thalami showed only signs of phase lag with SN. During the recovery phase, the relationship between the thalami and the SN was in phase and gradually restored qualitatively resembling the baseline profile in the scalogram.
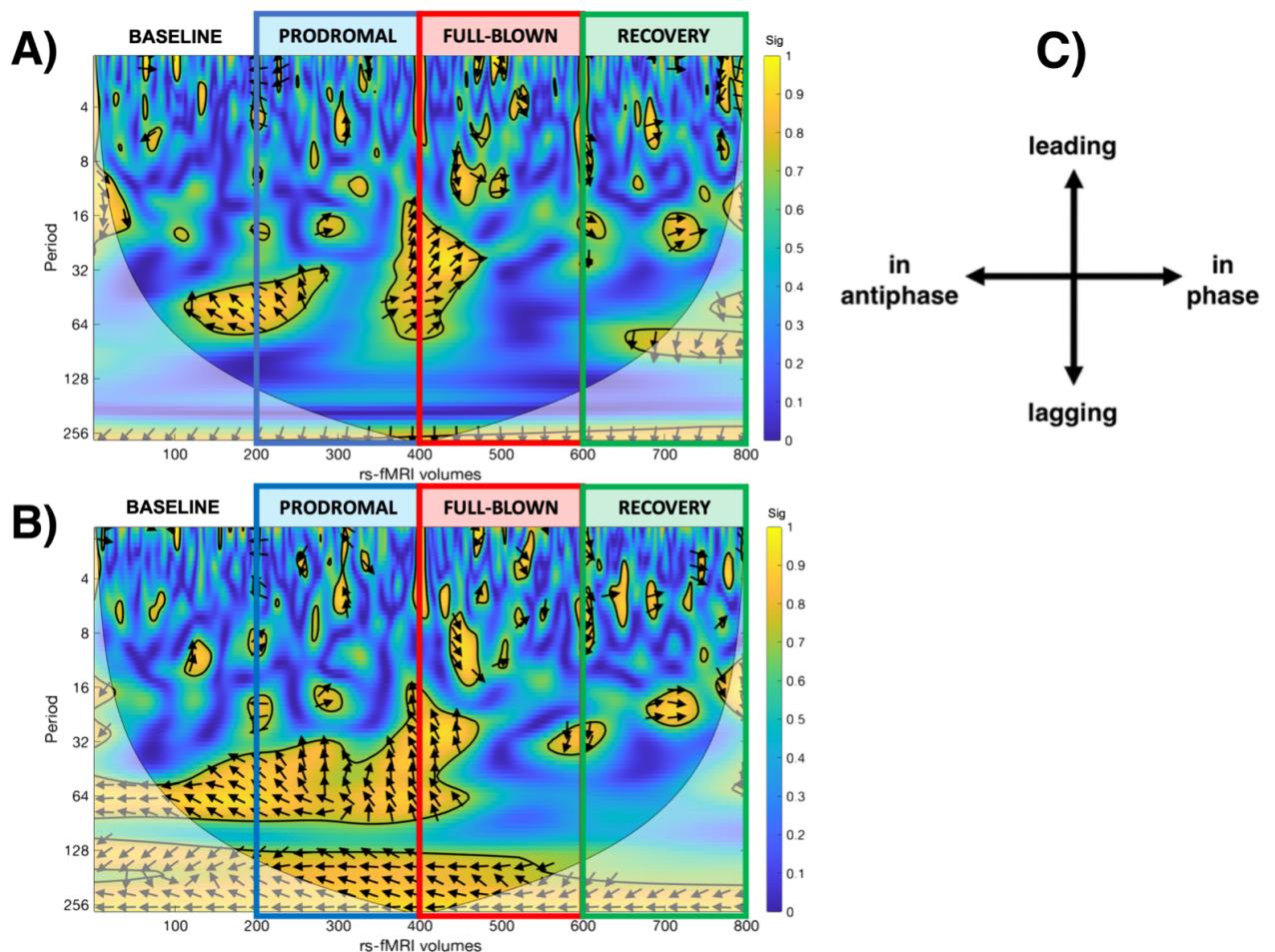

Figure 16 Scalogram representing the statistically significant WCA analysis results showing the dynamic correlation with the salience network during each phase of the study (baseline, prodromal, full-blown, recovery), considering the (A) left thalamus and the (B) right thalamus as a seed. (C) legend for the interpretation of the direction of the arrows (phase arrows).

5 Discussion

The strength of this study is the combination of the experimental NTG human model of migraine with advanced magnetic resonance imaging approaches and novel mathematical algorithms, providing further support to the hypothesis that migraine is a dynamic functional disorder involving multiple brain structures. The major finding of this study was the characterisation of structural and functional cortical connectivity alterations during an NTG-induced migraine-like headache attack under the key control of the thalamus. Specifically, in the group of episodic migraine patients our findings can be summarised as follows:

- the relationships between the posterior part of the cerebellum (crus I and II), the middle and superior frontal gyrus and the cingulate cortex were peculiarly altered over the entire course of the events;

- the [left] thalamus showed reduced functional activity starting from the prodromal phase, especially with the posterior cerebellum, the frontal gyri, and the cingulate cortex. The SCA analysis showed that the thalami, the brainstem, the cingulate cortex, and the posterior part of the cerebellum exhibited an altered functional coupling with one another. Furthermore, WCA analysis further highlighted changes and disruptions in the dynamic functional interactions between the thalamus and the different brain areas that are involved in salience pain processing;

- compared to the pain-free condition, the reduced FC areas within the precuneus, cingulate cortex and crus I/II represented another peculiar observation of the prodromal and recovery phases of the NTG migraine-like attack. Contrastingly, during the full-blown phase, the activity of the posterior cerebellum showed increased FC within its relationship with the frontal inferior orbital gyrus and the anterior portion of the cingulate cortex;

- functional alterations were correlated with the migraine frequency and the intensity of pain perceived over the different phases;

- from a structural point of view, the brain of people living with migraine was characterised by a reduced grey matter density in the insula, inferior parietal lobe, and superior and middle temporal gyrus, compared to HS. An increased density

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was instead observed in the cingulate cortex, middle frontal gyrus and the limbic system, but did not significantly vary along the migraine attack experience;

- finally, these areas of cortical volume loss in people living with migraine were associated with altered functional connectivity with the frontal gyri and the posterior part of the cerebellum even in a pain-free condition.

Functional connectivity fMRI data provide information about the interplay between different brain areas. Their application in studying headache patients has shed light on the structures responsible for the initiation and propagation of migraine attacks, paving the way for a comprehensive analysis of the cortical and subcortical brain activity during the different phases of the attack (De Tommaso et al., 2021). Over the last decade, several studies have investigated this disease from a neuroimaging point of view, but the considerations on its different phases have too often been a generalisation due to the difficulties in collecting information from spontaneous attacks and extremely varying scan times (table 8).

Study	Study design	Scan time	Notes
Amin et al. 2017	Spontaneous- MwoA	Range: 4.0 – 15.5 h	
Hougaard et al. 2017	Spontaneous- MwA	Mean: 8.2h	
Coppola et al. 2016	Spontaneous- MwoA	Initial 6 h	
Amin et al. 2015	MwoA induced with PACAP-38	Fixed timepoint: 130 minutes	Median migraine onset attack: 270 minutes. Only 5 patients present features fulfilling the ICHD3 criteria for a migraine attack during the fMRI
Karsan et al. 2020	MwoA induced with nitroglycerin infusion	According to clinical evaluation	The premonitory scan was conducted in the presence of 3 or more premonitory symptoms on the symptom questionnaire on the NTG-triggered visit

Table 8 Summary of the scan time in different fMRI studies. MwoA = migraine without aura; MwA = migraine with aura

The chance to induce spontaneous-like attacks with NTG in migraine patients has prompted the possibility to assess the central nervous system involvement in the different

phases of the attack. The strength of this study lies in its design, which allows an evaluation of all the different phases of the attack not only in contrast with a matched population of HS but also within the same protocol and on the same subject during the inter-critical pain-free state, thanks to the reproducibility of the NTG paradigm. In line with the pioneering experience published by Karsan et. al., the present study proves the feasibility of this approach and the potential of task-free fMRI to investigate the changes occurring during the different stages of the migraine attack.

In this study, several regions were involved following NTG administration over time. The peculiar interaction between the posterior cerebellum, the frontal and the cingulate cortex is aberrant throughout the migraine experience. The intervention of the thalamus occurs specifically during the ictal phase. It orchestrates the activity of several structures being a fundamental point of relay for sensory processing in migraine (Valenzuela-Fuenzalida et al., 2021). The mentioned structures are discussed below in the context of thalamic involvement/connectivity over the migraine experience, as well as the peculiar coupling between themselves.

The importance of the thalamus in migraine has been largely investigated for its role in central sensitisation (Burstein et al., 2011). Amin et. al. with an fMRI seed-based correlation study in a spontaneous attack (Amin et al., 2018) reported a significant thalamic involvement in the cortex activity modulation during the ictal phase of the induced migraine attack. The described structures were the superior parietal lobule, insular cortex, primary motor cortex, supplementary motor cortex, orbitofrontal cortex, and primary somatosensory cortex (S1). Coppola et al. (Coppola et al., 2016) compared the thalamic structural alteration and relative FC changes to the resting state networks (RSNs) at a cortical level, highlighting the decoupling of thalamocortical control over the network in the ictal migraine phase. In line with these findings, we found a significant alteration in the interactions between the thalami and the whole brain during the ictal phase of the migraine attack. This finding furtherly supports the idea that thalamic ascending and descending modulation of trigeminal nociceptive input may fluctuate, contributing to pain perception during the attack.

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Precuneus. As previously mentioned, in the context of the induced migraine-like attack protocols, the pivotal study by Karsan et. al. in 2020 (Karsan et al., 2020) explored the use of fMRI applied to NTG administration. It proved the feasibility of this approach in assessing the functional alteration occurring in the prodromal phase focusing on different regions of interest as seeds. The reported findings during the premonitory phase of the NTG-induced headache attack are in line with the functional involvement of the precuneus (part of the DMN, known to play a role in sensory integration) (Utevsky et al., 2014) and cuneus (an area of the visual cortex also thought to be involved in multisensory integration and cognitive processing) proposed by Karsan et al. From an anatomical point of view, the 'limbic' portion of the precuneus relates to the anterior nuclei and lateral dorsal nucleus of the thalamus as well as the cingulate cortex. The widespread connectivity of the precuneus, a structure known to be involved in higher association regions, suggests an important role in integrating both internally and externally driven information (Cavanna & Trimble, 2006). Based on this, an altered precuneus activity has been proposed to be associated with the depressive tendency in people living with migraine (Ma et al., 2018). Focusing on the relationship between the precuneus and the thalamus, thalamocortical pathways are likely involved in sensory sensitivities as well as allodynia and photophobia. In this study, no clinical feature and no prodromal symptom correlated with the global FC alteration. Cognitive impairment instead was not tested. On the other hand, some evidence supports the notion that thalamocortical pathways are involved in visuospatial and cognitive dysfunction during the prodromal phase (Goadsby et al., 2017b; Karsan & Goadsby, 2018). In the present study, the reduced FC within the precuneus, cingulate cortex and crus I/II represents the hallmark of the prodromal and recovery phases of the NTG-induced migraine-like attack, proving a positive correlation with migraine frequency. These observations were consistent with previous findings since the abnormal dynamic FC between the pulvinar nucleus, the visual cortex and the precuneus, were significantly correlated with migraine frequency (Tu et al., 2019).

The above-mentioned reduction in global FC during the prodromal phase negatively correlates with the presence and intensity of discomfort during the prodromal phase (NRS ≤ 2). This might represent a confounding factor in the analysis since it might be the expression of an early phase of the painful full-blown stage, which is rapidly approaching.

To minimise this possibility, all patients were carefully instructed on how to recognise the prodromal symptoms and a list was provided to help familiarise themselves with the possible manifestations. The requirement of at least two typical prodromal symptoms, as well as the delay between the onset of the symptoms and the actual beginning of the scan, could have led to an early phase of the painful migraine phase.

Cerebellum. Growing evidence from structural and functional neuroimaging studies suggests a cerebellar involvement in migraine, in particular of Crus I, II and Vermis VI (Kros et al., 2018). The cerebellum is highly connected with migraine-related brain regions such as the frontal cortex, thalamus, midbrain, brainstem, and spinal cord (Mehnert et al., 2017). Indeed, the cerebellum is described as an integrator of multiple effector systems. Therefore it is not to be considered only for its motor control and coordination role, but also affective processing, pain modulation, as well as sensorimotor processing (Noseda, 2022). It is even suggested that it has a modulating role in pain perception, (Moulton et al., 2010) but the extent of its implication in headache, and specifically in migraine, is not fully understood.

The data here presented further highlight the strong relationship between the thalamus and different structures of the posterior cerebellum. Structures such as the Crus I and II are well known for their role in non-motor processes such as attentional/executive integration (Guell & Schmahmann, 2020). The same posterior structures in this study were proven to be functionally coupled with the frontal cortex over the ictal migraine experience. From an anatomical point of view, neurons in cerebellar nuclei send divergent, excitatory axonal projections to various thalamic nuclei, including extensive innervation of migraine-related thalamic areas (Bostan et al., 2013). Through these connections, the cerebellum has been shown to dramatically affect thalamocortical network activity (Kros et al., 2015) even in the frontal lobe. MRI tractography findings showed that cerebro-cerebellar connections to the frontal and temporal cortex are contralateral to each other and include the cerebellum-thalamo-cortical and cortico-ponto-cerebellar pathways (Palesi et al., 2017). This structural evidence, therefore, supports the hypothesis of a cerebellar involvement in higher cognitive functions. Specifically, the cerebellar contribution to migraine pathophysiology has also been suggested through the co-activation of the periaqueductal grey and the posterior

cerebellum (Crus I and Crus II areas) during trigeminal pain stimulation in patients experiencing a migraine attack (Mehnert & May, 2019). Cerebellar Crus I and Crus II areas are strongly connected to the association cortices, especially the prefrontal and posterior parietal cortical areas. They were proven to be engaged in cognitive and emotional representations, showing overlapping activity between aversive behaviour and heat pain exposure (Moulton et al., 2010).

Cingulate cortex. The cingulate cortex is a forebrain structure in mammals and its anterior portion (anterior cingulate cortex (ACC)) is a crucial structure involved in various higher functions, such as nociception, chronic pain, cognition, and emotions (Tsuda et al., 2017). Its role is evident in our study, where it appears to be not only functionally coupled with the thalamus during the prodromal and full-blown phases but also involved in changing the direction of the interaction during the recovery phase. Moreover, the interaction between the thalamus and the cingulate cortex is a piece of robust evidence, being present also after the HS comparison, particularly during the prodromal and recovery phase. ACC involvement is migraine- and phase-specific. From an anatomical point of view, ACC also receives sensory inputs from the thalamus and subcortical areas1. It projects sensory outputs to the motor cortex, amygdala, midbrain areas, PAG, rostral ventromedial medulla, and spinal dorsal horn (Dai et al., 2021). In the animal model of recurrent headache, the anterior cingulate cortex showed increased functional connectivity with the cerebellum (Jia et al., 2019), in line with the evidence here presented of an altered FC among these structures. Overall, in the headache framework, the functional alteration of the ACC's activity is associated with chronic migraine, acute noxious stimuli, and pain-like aversive behaviours (Schwedt et al., 2015). In addition, Karsan et al. in their NTG study focused their attention on the cingulate cortex, which was negatively coupled with the pons and the limbic lobe, as well as with frontal cortical regions during the premonitory phase and during the full-blown phase, compared to baseline. It is possible therefore to speculate that ACC actively participates in pain modulation as well as determining the behavioural correlates during prodromal and postdrome phases.

Frontal/prefrontal cortex. The frontal lobe acts as a higher-order pain processing hub in the descending pain modulatory system, within its connection with the medial temporal cortex, hypothalamus, brainstem, and amygdala (Rempel-Clower, 2007). Several neuroimaging studies have shed light on the interventions of the superior, middle frontal gyri and the prefrontal cortex in people living with migraine, not only during the inter-critical phase (Schwedt et al., 2014; Skorobogatykh et al., 2019) but also as a response to trigeminal heat stimulation in migraine subjects with cutaneous allodynia (Russo, Tessitore, Esposito, et al., 2012). Moreover, several neuroimaging approaches and neuropsychological investigations have indicated that people living with migraine display frontal lobe-related cognitive impairment, particularly those suffering from chronic migraine, not only during the migraine attack but also during the inter-critical phase (S.-H. Lee et al., 2021). In this context, it is possible to interpret the observed altered FC involving the frontal/prefrontal cortex and the posterior portion of the cerebellum in EM compared to HS, as the expression of the related cognitive impairments typical of the ictal phase, which include working memory and executive function deficits (Schmitz, Arkink, et al., 2008).

Brainstem. Pivotal PET studies support the role of the pons in migraine genesis, in particular, the dorsal rostral pons area (Afridi, Matharu, et al., 2005). It was suggested that the pathophysiology and genesis of migraine attacks are probably not just the results of one single "brainstem generator" (Borsook & Burstein, 2012). In line with this hypothesis, but differently from Amin et al. (Amin et al., 2018), the present data support the notion of the fundamental coupling between the pons structures and the thalamus, suggesting how the alteration of the activity among them leads not only to the premonitory phase but also regulates recovery after the NTG-induced headache attack.

The spinal trigeminal nucleus, considering its caudal portion, is a structure in the medulla that receives information about deep/crude touch, pain, and temperature from the ipsilateral face. This study proved it to be coupled with the right thalamus during the prodromal and recovery scan. In this context, an Australian study (Marciszewski, Meylakh, Di Pietro, Mills, et al., 2018) assessing resting-state fMRI alterations in people living with migraine, showed an increased FC in the spinal trigeminal nucleus during noxious orofacial stimulation, and a reduced FC in its caudal portion with the rostral

ventromedial medulla (RVM). Additionally, during the interictal phase, patients displayed reduced FC in PAG matter and increased FC PAG connectivity with the RVM. These data are not directly comparable with those presented in this paper, but they further support the hypothesis that brainstem activity and sensitivity, within the pain-modulating circuitry, fluctuate across the migraine cycle. Moreover, the same group performed a structural MRI analysis (Marciszewski, Meylakh, Di Pietro, Macefield, et al., 2018) to compare the anatomy of the brainstem during the interictal phase in HS and patients. In patients, they found both reduced GM volumes and increased diffusivity in the spinal trigeminal nucleus, the dorsal portion of the pons and in the areas of the descending pain modulatory system, including PAG, and the medullary raphe. These structural findings have not been described before in other bigger cohorts (Palm-Meinders et al., 2017) or longitudinal studies (Messina, Rocca, et al., 2018), but provide further support to the hypothesis that anatomical changes in the brainstem may underlie, or be the effects, of the activity fluctuations of the circuitry due to the disease. Migraine attack generation, therefore, includes spontaneous fluctuations of complex network's interplay, involving the brainstem circuitry, the thalamus and the hypothalamus, even though the latter was not confirmed in this population (Schulte & May, 2016b).

Structural alterations:

Voxel-based morphometry studies of the human brain can detect structural changes in the grey matter, representing either a constitutive trait characterizing a population or a response to external stimuli or states of disease. In this framework, VBM was therefore used to reveal microstructural alterations of the grey matter density which might represent a hallmark of brain plasticity over time (Naegel et al., 2017). An increase in GMd may reflect structural brain plasticity because of exercise and learning. A decrease in GMd suggests central reorganization processes that, in chronic pain syndromes, may be represented by the degeneration of anti-nociceptive brain areas. Overall, these microstructural alterations may reflect: (1) an increase/decrease in cell size and neural or glial cell genesis/degeneration (Kempermann et al., 1997), (2) an alterations in the dendritic complexity or changes in the number of synapses (Trachtenberg et al., 2002), or, more simply, (3) changes in water content (Reiss, 2004). In this study where we evaluated single phases of a migraine-like attack, it was not possible to detect a

statistically significant change over the different phases, in comparison to the baseline condition. On the other hand, constitutive traits of alteration were detected when GMd was compared between EM and HS. In line with the available literature, volume loss was demonstrated in the insula, middle frontal gyrus, middle and frontal temporal gyrus, inferior parietal lobe and occipital cortices in patients with migraine compared to controls (S. Ashina et al., 2021). Usually correlated with attack frequency and disease durations, these GMd reductions could be the consequences of repeated episodes of ischemia caused by cerebral blood flow irregularities during both the ictal and interictal phases of migraine (Bashir et al., 2013). The critical pain experience could therefore worsen these GM alterations, possibly promoting degeneration of the anti-nociceptive pain system.

To investigate whether volumetric changes may indicate functional brain remodelling in migraine, the map of GMd reduction in EM at baseline was used as a seed for SCA over the migraine cycle. Once again, it was possible to depict an altered coupling within the posterior cerebellum, the left superior temporal gyrus, and the rostral portion of the medulla, as well as an increased FC with the prefrontal cortex. These findings involve structures well known for their role in the pain connectome and could not be merely downgraded as interstitial fluid modifications, considering their functional link within the ascending and descending pathways specifically depicted in EM and not in HS. On the other hand, the transient and deceitful GMd changes over the migraine cycle could hypothetically be the result of water-dependent changes in cell volume. These GMd changes might be hardly detected as short latency grey matter changes in VBM analysis but were not statistically meaningful in this analysis.

WCA Approach

To better elucidate the dynamic changes of the full-blown phase, where a comprehensive disruption of the FC coherence between the thalamus and the rest of the brain has been reported in spontaneous migraine attacks (Di Lorenzo et al., 2016), the WCA approach was applied to assess the dynamic changes during the NTG-induced headache.

WCA assesses the dynamic FC changes between the signals, addressing when, and how frequently, the activity of the two regions/networks co-vary and display phase-locked behaviour, suggesting a functional interaction between them (Chang & Glover, 2010).

Indeed, thalamocortical communication at rest was found to be not static, but rather dynamic with the bilateral insulae, prefrontal cortices, and dorsal anterior cingulate cortex. These structures are recognised to be part of the salience network, which has shown a temporal instability in its activity in people living with migraine (Veréb et al., 2020). The data here presented suggests that, during the prodrome and full-blown phase of the NTG-induced headache attack, the subjects espress profound dynamic changes in the interactions between thalami and the SN, nowadays labelled as the pain connectome (M. J. Lee et al., 2019; Legrain et al., 2011; Sotgiu, 2001). Remarkably, the WCA scalogram depicts a scenario in which the thalamus appears to be leading the salience network signal, therefore suggesting a possible role of the thalamus as a pacemaker for the pain matrix functional connectivity (Di Pietro et al., 2018; Sotgiu, 2001). Additionally, the scalogram clearly shows the absence of phase coherence between the thalamus and the SN during the NTG-induced headache full-blown phase, suggesting a severe disruption of the interaction within the pain circuitries.

Overall interpretation of the results

Over the last decades, it was suggested the thalamus does not simply act as a relay station during sensory processing. Instead, a robust network of cortico-thalamic feedback neurons exists and dynamically influences sensory processing (Briggs & Usrey, 2008). The alteration of the rhythmical coupling between the thalamus and the cortex (or the brainstem nuclei) brought to the theorization of the 'thalamocortical dysrhythmia' (TCD) model. In this model, an alteration of the underlying physiological oscillatory interplay of the thalamic activity is hypothesised to be the mechanism causing different manifestations present in specific neurological disorders (e.g., pain, tinnitus) (Vanneste et al., 2018) as well as neuropsychiatric disorders (e.g., depression or psychotic disorders present in Parkinson disease and Lewy bodies dementia) (Di Pietro et al., 2018; Llinás et al., 2005; Onofrj et al., 2019). The pain framework represented a peculiar chapter where TCD has been suggested to play a leading role in modulating central nociception (Walton & Llinás, 2010), chronic low back pain (Tu et al., 2020), and neuropathic diabetic pain (Cauda et al., 2009).

Several studies and different techniques, from magnetoencephalography (MEG) (Ren et al., 2019) to electroencephalography (EEG) (Coppola et al., 2007) and nowadays to fMRI, already suggested the possible role of thalamocortical dysrhythmia in leading to the dysfunction of multisensory integration consequently causing disturbances in sensory, cognitive, and motor neural processes in patients suffering from migraine (Coppola et al., 2016; Tu et al., 2019). An example where TCD was recognised is visual snow syndrome (VSS). VSS refers to the presence of achromatic dots with at least one associated visual symptom of palinopsia, photopsia, nyctalopia and entoptic phenomena as well as non-visual symptoms such as tinnitus, migraine, and tremor. A recent Australian study using MEG-MRI showed how the rhythmical brain activity in the primary visual cortex was both hyperexcitable and disorganized; this result was consistent with a condition of TCD (Hepschke et al., 2022).

Overall, the thalamus plays a pivotal role in orchestrating nociceptive and non-painful symptoms, along with the anterior cingulate cortex, the frontal/prefrontal cortex, and the cerebellum. Taken together, the data presented in this thesis, corroborate the hypothesis that an altered thalamocortical interaction could be the major contributor to abnormal multimodal sensory processing and cognitive impairment during the migraine attack. For

example, in the premonitory phase, common cognition-associated complaints include difficulty concentrating, finding words, or having a diminished capacity for tasks requiring working memory (Gil-Gouveia & Martins, 2019; Karsan & Goadsby, 2018). At least in part, these disabilities can be attributed to network dysfunctions that include the cerebellum and its reciprocal functional connectivity with prefrontal, limbic, and autonomic cortical areas (Noseda, 2022). Although not clinically specifically tested in this study, the migraine-specific altered coupling among cerebellar, limbic, and frontal/prefrontal structures was proven.

Taken together, the findings here presented support the idea of the pivotal interplay between the thalamus and those structures and networks well known for their role in migraine processing. In addition to this, it also highlights their dynamical modulation, disruption and finally restoration throughout the headache ictal experience. These are migraine-specific changes in the signal and appear to be peculiar to each phase of the migraine experience. Therefore, it is possible to speculate that the thalamocortical temporal correlation of the activity, so fundamentally involved in the manifestation of the symptoms, is progressively changed during the ictal phase of a migraine attack. It is possible therefore to sustain the hypothesis that, once migraine is generated, the thalamocortical dysrhythmia leads or influences the experience throughout all the subsequent phases, until its conclusion. This hypothesis needs to be further confirmed on a larger sample size population and possibly with also other neurophysiological approaches, such as dynamic causal modelling, to reach a comprehensive understanding of the causal architecture of migraine.

Finally, future studies on larger cohorts of patients will also be required to better address the importance of the relationship between the subthalamic nuclei and other potential key regions in migraine pathophysiology.

Limitations

Some considerations on the limitations of this study need to be mentioned.

[1] The relatively small number of subjects limited the choice of the analyses. However, since the results are in line with the literature, it is possible to assume that the proposed approach represents a powerful and reliable tool to investigate functional changes in the brain during a migraine attack.

[2] NTG is an active drug which may affect the BOLD signal dynamics, independently of headache occurrence. For this reason, it has to be noted that prodromal scans were acquired 90 min after NTG oral administration, and full-blown scans 165 min after NTG. Once orally administered, the onset of vasodilatory effects occurs within 1 to 3 min., with a max effect occurring within 5 min; moreover, NTG is primarily eliminated via metabolism in the liver and has a mean half-life of 2.6 min (K. H. Kim et al., 2022). Consequently, any direct vasoactive-related perfusion effects of NTG in the human brain should be excluded, even though it was described (Greco et al., 2011) as a persistent effect of NTG on the cortex up to 150 min after the administration in rats. Considering the presence of a control group of HS who underwent the same NTG paradigm, a direct comparison was performed between groups and the effect was erased.

[3] Reflecting about the difficulties of standardizing the scan(s) of all the evolving phases of a spontaneous migraine attack, the use of a migraine model represents both a limitation and the cornerstone of this study because it allowed the creation of a reproducible acquisition scheme. Experimental human and animal models of migraine have already yielded significant insights into brain structures that are essential to determine migraine symptoms and the recurrence of attacks. Among them, the reliability of the NTG model resides in its ability to reproduce headache attacks with features that are reminiscent of the spontaneous migraine attack (Greco et al., 2020). Given the statistical relevance of this comparison in the animal model, it is possible to assert that the brain changes observed were paralleled in the natural state even though a direct comparison would be required to fully prove this hypothesis. So far, no study was able to directly compare NTG induced headache with the spontaneous event on the other hand, the data here discussed are in line with the available literature.

[4] Aspecific headache induced by NTG. First, the incidence between groups did not differ. Secondly, through the direct comparison of the two groups, the effect of this discomfort should have been avoided in the imaging analysis, highlighting the migraine-specific alterations only. On the other hand, this was not possible in the intra-group analysis. Moreover, when the clinical features were tested for correlations, it was proved a persistent impact of the aspecific headache both on patients' and HS' FC. Even though the direct comparison between groups helps recognise the migraine-specific alterations, the data presented for the HS group supports the notion that NTG induces an experience that has a biological effect by itself, detectable still hours after the end of it. Even though recognisable, the effect in HS did not consistently involve any pain modulating structures and therefore it could be speculated that its effect is meaningless also for EM patients.

[5] Tension-type headache: although the incidence between groups did not differ, the biological effect of the comorbid tension-type headache, and specifically of its monthly frequency, was observed also in this study. Only a few studies have addressed this highly prevalent disease (S. Zhang et al., 2021) and it appears to be mandatory to accredit this painful condition among the confounding factors for the analysis.

[6] Brainstem: From a technical point of view, trying to depict functional or structural alteration targeting the brainstem raises many concerns since, from a neuroimaging point of view, this structure is more similar to the spinal cord than to the supratentorial structures. The detection of brainstem activations in functional imaging studies is challenging in several aspects: first, the brainstem is a very small area and single functional nuclei lie in a close spatial relationship to each other. To reliably attribute functional activations to each single brainstem nucleus, high spatial resolution rs-fMRI images are required, which would significantly increase the acquisition time. This would of course be in contrast with the ethical motivation to keep the length of the MRI scan as short as possible in consideration of the fact the patients are under a painful condition during the experiment. For this reason, a multiband echo planar imaging (MB-EPI) rs-fMRI acquisition has been implemented. This particular MRI sequence allows to acquire multiple slices (typically from 2 to 8) at the same time, therefore reducing the time required to record the entire fMRI exam (e.g., MB-EPI Rs-fMRI sequence allows the acquisition of 200 volumes in less than 5 min 30"). The brainstem is surrounded by large blood vessels and cerebrospinal fluid. This anatomical "layout" makes the brainstem

BOLD signal very susceptible to periodic signal fluctuations that are strongly correlated with the arterial pulse curve and breathing rate (e.g., physiological noise). When the heart rate and breathing changes correlate with the experimental paradigm, as often happens in pain experiments or when comparing the pain and pain-free state of people living with migraine, fMRI results may be biased. Therefore, in future studies, a proper recording of the physiological tracks during the MRI acquisition should be considered and used to perform a "nuisance regression" (physiological noise regression) on the Rs-fMRI images during the pre-processing step.

[7] Healthy subjects' phase-specific FC alterations. The comparison of each scan after NTG administration to the baseline condition allowed us to depict some inconsistent FC alterations in HS as well. Most of these results are considered irrelevant because localised in motor and visual areas and therefore possibly linked to misconducting behaviour by some subjects during the scan. On the other hand, it is not possible to exclude that some of these FC alterations are the consequence of the different pain processing in HS after the aspecific NTG-induced headache.

[8] This study did not include a placebo group as a control group, but an intra-group comparison was preferred as a first step to corroborate the use of the NTG paradigm.

[9] Overall, the comparison with healthy subjects could be considered a problem for the interpretation of NTG as a model for migraine. This consideration might be supported by the lack of a direct comparison with people having a spontaneous attack or a placebo group. On the other hand, the technical difficulties highlighted during the conduction of the spontaneous attack fMRI evaluation, namely the difficulty in standardising the scheme of acquisition, can not be easily overcome considering the naturally fluctuating nature of the disease and are beyond the scope of this project. Even the German study by May describing a spontaneous migraine attack approximates the prodromal and postdromal phases since there's a lack of coherent definition of them. Correctly addressing each alteration observed during the NTG induced migraine like attack helps define the peculiar migraine signature which will have to be proven during the spontaneous attack, once properly recorded. Finally, a standardised acquisition approach might also help to directly compare the results from different migraine models.

[10] Allodynia was not properly evaluated during the migraine like cycle therefore it was not possible to stratify the population based on this symptom.

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6 Conclusions

In conclusion, this thesis has shown migraine-specific and phase-specific alterations which could represent the functional and structural MRI signature of the induced migraine attack.

First of all, the thalamus plays a pivotal role in migraine from the prodromal phase. It exhibits an altered coupling with the brainstem, the cingulate cortex, and the cerebellum's posterior part over the migraine cycle. It is not only a static functional connectivity alteration, but it has been possible to depict a dynamical alteration through the application of the innovative WCA approach. WCA shows a loss of synchronisation between the thalami and the salience network, mainly appearing during the prodrome and full-blown phases. These results further support the idea that a temporal change in thalamic function occurs over the experimentally induced phases of NTG-induced headache in migraine patients.

Secondly, this thesis further highlights the involvement of the cerebellum - a multiple effector system integrator and a ruler of pain perception modulation – and the frontal/prefrontal cortex. The changes observed in these areas may explain the cognitive impairment associated with the migraine ictal phase.

Moreover, the microstructural modification here discussed suggests that migraine is associated with significant GM volume loss in key areas for pain processing (such as inferior parietal lobe and temporal lobe), possibly reflecting alterations in the local dendritic complexity caused by the disease.

Overall, understanding the functional organisation between subcortical and cortical areas in different phases of the migraine attack provides new insights into the abnormal sensory processing and integration over this cyclical event.

Future studies should address the peculiar role of each thalamic nuclei to better address their role in the migraine experience. Increasing the number of subjects evaluated and the possibility of having a placebo group appears to be the next fundamental step in the evaluation of migraine-specific alteration. Up to now, the data described in this thesis and the available literature, continue to contribute to a narrative

of migraine as a distinct, neuronal network disorder in which brain regions normally concerned with sensory processing alter in function so as to produce the complex, disabling symptoms that patients experience. Particular attention should be therefore paid also to the definition of the prodromal and postdrome as well as to the identification of clinical features able to better characterise their CNS correlates, since they are often important and invalidating symptoms which last over the end of the pain phase. For these reasons, deepening our knowledge regarding the central nervous system activation during the migraine attack might possibly suggest new pharmacological targets for its treatment.

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