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Thèse soutenue à Paris-Saclay, le 17 Septembre 2024, par

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THÈSE DE DOCTORAT

NNT : 2024UPASL045

Titre : Appropriation d'une prothèse de membre supérieur chez la souris

Mots clés : appropriation, illusion de la main en caoutchouc, optogénétique, stimulation corticale directe, modèle souris

Résumé : Les recherches sur l'appropriation corporelle sont essentielles pour le développement des prothèses. En effet, l'incapacité à s'approprier une prothèse entraîne inconfort et douleurs fantômes chez de nombreux patients. Pour améliorer l'acceptation et l'utilisation des prothèses, il est donc crucial de comprendre et de pouvoir manipuler ce sens d'appropriation.

Le modèle souris présente de nombreux avantages pour ces recherches grâce à ses comportements riches de membres supérieurs ainsi qu'aux technologies optogénétiques disponibles d'abord chez ce modèle. Ces techniques permettent une exploration précise du rôle du retour tactile dans l'appropriation des prothèses, et constituent une approche novatrice pour étudier ce phénomène.

Dans le cadre de cette thèse j'ai participé à la construction d'un prototype d'une prothèse motorisée à l'échelle de la souris qui peut être contrôlé par l'activité neuronale enregistrée à l'aide d'électrodes chroniques implantées dans le cortex moteur des animaux. L'étude de l'appropriation est particulièrement importante dans le cadre du développement de notre modèle de neuroprothèse, pour comprendre l'interaction de différents éléments sensoriels ou moteurs sur l'intégration d'un membre artificiel. Pour étudier cette question ma thèse s'est focalisée sur l'utilisation de méthodes comportementales exploitant des illusions perceptives pour manipuler l'appropriation de membres. Ainsi, dans l'illusion de la main en caoutchouc, grâce à des stimulations visuelles et tactiles synchrones, les participants s'approprient comme faisant partie de leur corps une fausse main placée devant eux, tandis que leur vraie main reste cachée.

Nous avons adapté cette illusion au modèle souris pour explorer le rôle du retour tactile dans l'appropriation des prothèses. Nous avons exposé des souris à ce paradigme, en les plaçant devant une prothèse ressemblant à leur patte, pendant que cette dernière est cachée. Après 2 minutes de stimulations, nous menaçons la patte et observons les réactions des animaux à cette menace avec une analyse automatisée de différents points d'intérêt de la face de l'animal. Les animaux montrent des signes d'appropriation envers la prothèse, démontrant que ce sens peut être étudié à ce niveau chez la souris.

Dans le contexte du développement de neuroprothèses, il est nécessaire de pouvoir fournir un retour tactile artificiel aux patients quand le membre en périphérie a été perdu. C'est dans cette optique que nous avons exploré la possibilité d'induire cette illusion via des stimulations corticales des régions sensorielles de la patte par optogénétique. Nous avons d'abord mené une étude d'observation des dynamiques corticales générées par des stimulations de la patte en périphérie en utilisant de l'imagerie calcique. Cela nous a permis d'adapter nos stimulations optogénétiques pour mimer l'entrée sensorielle en périphérie. Nous avons ensuite reproduit notre premier protocole de l'illusion classique en replaçant les stimulations tactiles de la patte par des stimulations directes corticales. Les résultats préliminaires de ces expériences montrent un effet similaire à ce qui a été observé auparavant avec l'illusion classique, montrant la possibilité d'induire l'appropriation d'une prothèse à travers un retour tactile cortical.

À terme, ces travaux ont permis de développer une plateforme de recherche chez le modèle souris pour le développement de neuroprothèses et permettront de développer de meilleures stratégies de retour sensoriel, pour un meilleur contrôle et une meilleure appropriation des prothèses chez des patients.

Title: Embodiment of a forelimb prosthesis in the mouse model

Keywords: embodiment, rubber hand illusion, optogenetics, direct cortical stimulation, mouse model

Abstract: Research on bodily embodiment is necessary for the development of prostheses. Indeed, the inability to embody a prosthesis is a source of discomfort and is accompanied by phantom pain in the residual limbs of many amputees.

The mouse model offers many advantages for this type of research due to its rich upper limb behaviours and the availability of optogenetic technologies in this model. These techniques allow for precise exploration of the role of tactile feedback in prosthesis embodiment and represent an innovative approach to studying this phenomenon.

As part of this thesis, I contributed to the construction of a motorized prosthesis prototype at the mouse scale, controllable by neuronal activity recorded using chronic electrodes implanted in the animals' motor cortex. The study of embodiment is particularly important in the context of developing a neuroprosthesis model to understand the interaction of various sensory or motor elements on the integration of an artificial limb. To investigate this question, my thesis focused on using behavioural methods, exploiting perceptual illusions to manipulate limb embodiment. For instance, in the rubber hand illusion, synchronous visual and tactile stimulations cause participants to perceive a fake hand placed in front of them as part of their body, while their real hand remains hidden.

We adapted this illusion in the mouse model to explore the role of tactile feedback in prosthesis embodiment. We exposed mice to this paradigm by placing them in front of a prosthesis resembling their paw while hiding their actual paw. After 2 minutes of stimulations, we threatened the paw and observed the animals' reactions to this threat using an automated analysis of various points of interest on the animal's face. The animals showed signs of embodiment towards the prosthesis, demonstrating that this sense can be studied at this level in mice.

In the context of neuroprosthesis development, it is necessary to provide artificial tactile feedback to patients when the peripheral limb is lost. With this goal in mind, we explored the possibility of inducing this illusion through cortical stimulations of the sensory regions of the paw using optogenetics. We first conducted an observational study of the cortical dynamics generated by peripheral paw stimulations using calcium imaging. This allowed us to adapt our optogenetic stimulations to mimic peripheral sensory input. We then replicated our initial classical illusion protocol by replacing the tactile stimulations of the paw with direct cortical stimulations. The preliminary results of these experiments showed a similar effect to what was previously observed with the classical illusion, indicating the possibility of inducing prosthesis embodiment through cortical tactile feedback.

Ultimately, this work led to the creation of a research platform using the mouse model for neuroprosthetic development which could help in providing better sensory feedback strategies for improved control and embodiment of prostheses in patients.

SYNTHESE EN FRANÇAIS

Les recherches sur l'appropriation corporelle jouent un rôle central dans le développement de neuroprothèses. L'incapacité à intégrer une prothèse comme une extension naturelle de son corps, en plus d'entraîner des sensations d'inconfort, est souvent accompagnée de douleurs fantômes, ressenties dans les membres amputés. Pour améliorer la qualité de vie des patients porteurs de prothèses, il est donc impératif de comprendre les mécanismes sous-jacents à l'appropriation et de pouvoir les influencer afin de faciliter une utilisation optimale des dispositifs prothétiques.

Le modèle animal, en particulier la souris, représente un outil puissant pour ce type de recherche. La souris présente des comportements de membres supérieurs complexes, et bénéficie d'un accès à des technologies optogénétiques, d'abord accessible dans ce modèle. Ces technologies, qui permettent un contrôle précis de l'activité neuronale à travers des photostimulations, constituent un atout unique pour étudier le rôle du retour sensoriel, et plus spécifiquement tactile, dans l'appropriation d'un membre artificiel. Ces technologies offrent une nouvelle approche pour étudier la manière dont les circuits neuronaux intègrent différents types d'informations motrices et sensorielles pour permettre l'appropriation d'un membre prothétique.

Pour répondre à ces questions, ma recherche s'est appuyée sur des méthodes comportementales reposant sur des illusions perceptives, telles que l'illusion de la main en caoutchouc. Dans cette illusion, des stimulations tactiles et visuelles synchrones amènent les participants à percevoir une fausse main comme étant la leur, tandis que leur véritable main reste cachée.

Nous avons transposé cette illusion au modèle souris, dans le but d'explorer plus en détail le rôle du retour tactile dans l'appropriation d'un membre artificiel. Pour cela, nous avons placé les souris face à une prothèse ressemblant à leur patte, leur patte réelle étant dissimulée. Après quelques minutes de stimulations synchrones, nous avons menacé la prothèse en observant les réactions des souris grâce à une analyse automatisée des mouvements de la face. Les résultats ont montré que les souris réagissaient comme si la prothèse faisait partie de leur corps. Spécifiquement, nous observons des dynamiques de mouvement de pupille, allant vers la menace, plus prononcés dans la condition test, confirmant que ce modèle animal

permet bien d'étudier l'appropriation à ce niveau.

Dans le contexte de la mise au point de neuroprothèses destinées aux patients amputés, une question cruciale est celle du retour sensoriel : comment fournir un retour tactile artificiel lorsque le membre périphérique a été perdu ? Afin de répondre à cette problématique, nous avons exploré l'idée d'induire cette illusion d'appropriation par des stimulations directes du cortex sensoriel via l'optogénétique.

Nous avons tout d'abord mené une étude observationnelle des dynamiques corticales déclenchées par des stimulations périphériques de la patte, en utilisant de l'imagerie calcique. Ces observations nous ont permis d'ajuster nos stimulations optogénétiques afin de reproduire de manière aussi fidèle que possible les entrées sensorielles d'origine périphérique.

Nous avons ensuite reproduit notre paradigme d'illusion en remplaçant les stimulations tactiles classiques par des stimulations corticales directes. Les premiers résultats ont montré des effets comparables à ceux observés avec l'illusion classique, ouvrant ainsi la voie à l'induction d'une appropriation d'un membre artificiel par le biais d'un retour sensoriel cortical.

Au cours de ma thèse, j'ai également eu l'occasion de participer à la création d'un prototype de prothèse motorisée adaptée à la souris. Ce dispositif peut être contrôlé par l'activité neuronale, enregistrée via des électrodes chroniques implantées dans le cortex moteur des animaux.

Ces travaux ont permis le développement d'une plateforme de recherche sur le modèle souris, dédiée à l'étude des neuroprothèses et à l'amélioration des stratégies de retour sensoriel. À terme, cette plateforme pourra contribuer à l'optimisation du contrôle des prothèses, en favorisant une meilleure appropriation corporelle de ces dispositifs par les patients. En effet, offrir aux utilisateurs de prothèses un retour sensoriel plus naturel et plus précis représente une avancée significative pour améliorer leur confort et leur qualité de vie. Grâce à ces recherches, des stratégies innovantes pourraient être élaborées pour renforcer l'intégration des prothèses, non seulement au niveau fonctionnel, mais aussi sur le plan perceptif, en permettant aux patients de véritablement s'approprier ces dispositifs comme une extension de leur corps.

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PART 1

INTRODUCTION

1 INTRODUCTION

1.1 LIMB EMBODIMENT

1.1.1 Embodiment phenomenology

How do we know that our bodies belong to us? We use our body to interact with the world, and we are constantly receiving a stream of sensory information from it. It is the element that we are most familiar with, to the extent that it is intimately linked to our sense of self. The idea that our body is ours seems to be a given, and most of us do not actively think about it unless it is disturbed through bodily illusions or in medical conditions such as asomatognosia (a neurological disorder characterized as loss of recognition or awareness of part of the body) or limb loss. We aim to better understand this sense of embodiment in this work.

Firstly, let us define the terms regarding embodiment and body ownership that we are aiming to study. Defining embodiment is a complex task, particularly in navigating the intricacies of how external objects are perceived in relation to one's bodily self. The challenge lies in establishing criteria for what constitutes an embodied object, while avoiding a recursive definition that relies on the very concept it seeks to define, as the embodied body is the reference point.

We consider the conception of body ownership as one of the components of embodiment (de Vignemont, 2011; Longo et al., 2008). Embodiment is the process by which individuals experience a sense of ownership, control, and emotional connection over their body parts. This encompasses many components including body ownership. De Vignemont proposes the following definition: "E is embodied if and only if some properties of E are processed in the same way as the properties of one's body". This definition allows us to encompass different nuances of embodiment that include tool usage, prosthetic limbs or transplant members. It is possible for an object to be embodied without being experienced as part of oneself. The properties of an object can be processed similarly to body properties without being felt as part of the body, as seen with tool embodiment for

example. One can make a judgment about an object being part of their body, however, feeling ownership over an object can only be experienced if said object is embodied. Embodiment is then an essential factor and pre-requisite for experiencing body ownership.

We can break down several aspects of embodiment that can allow us to analyse and measure different modalities of this sense. These can be grouped in three categories: spatial, motor and affective (Table 1.1).

Spatial
•If E is taken into account by the representation of the body space, by replacing a missing body part, by adding a body. part, or by stretching an existing body part. •If one is able to localize bodily sensations in E. •If the location of E within the external frame is processed in the same way as the location of a part of one's body. •If the space surrounding E is processed as peripersonal space.
Motor
•If one feels that E directly obeys one's will. •If one feels that a part of one's body is moving when E is moving. •If E is taken into account as an effector by the motor system in action planning.
Affective
•If E is protected from hazardous situations. •If one reacts in the same way when E is threatened or hurt and when a part of one's body is threatened or hurt.

Table 1.1: Different measures of embodiment (adapted from de Vignemont, 2011)

1.1.1.1 Spatial features:

An embodied object can be considered in the body's spatial representation by replacing, adding or stretching a body part:

It has been shown that prosthesis wear increases the perceived length of amputees' residual limb (Canzoneri et al., 2013; McDonnell, 1989). One can also provoke an embodiment towards two additional hands in healthy participants with supernumerary illusions, giving rise to an extension of the body schema to take account of an additional limb: in these experiments, participants report feelings of embodiment and referral of touch towards two prosthetic limbs (Ehrsson, 2009; Fan et

al. 2021). Moreover, systems like the MIRAGE illusion box (Newport et al., 2015; Preston & Newport, 2011) allow for a manipulation of perceived finger length by stretching the size of an existing body part: The participants' hand is recorded during the experiment and the subjects are exposed to this live video recording that is manipulated to stretch one finger to double its actual length. At the same time the experimenter tugs on the participant's real finger placed inside the box. This gives the illusion to the participants that their finger was really stretched, thus embodying the hand's representation shown by the video system. Here the participants are not embodying a new object, rather, the embodiment towards the representation of their hand shown via the system, warps the vision of their finger size. In similar experiments using virtual reality (VR) environments, one can also manipulate the perceived size of the body by exposing people to a recording of a small doll. When the doll body was embodied, the perception of object size and distance was warped. This reflected that the subjects perceived their body size as smaller in accordance to the doll's size (Hoort et al., 2011).

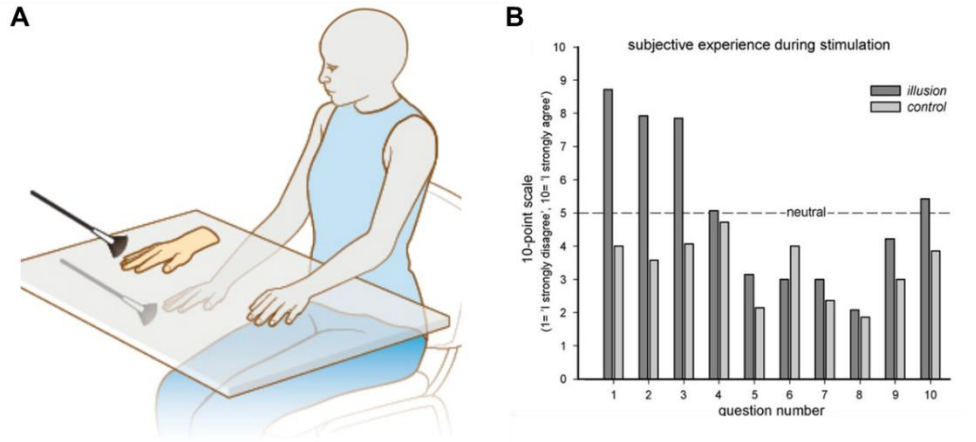


Figure 1.1: Rubber hand illusion paradigm

(A) Participants are exposed to an artificial limb while their own limb is hidden from their view. Tactile stimulations are applied to both limbs synchronously to create the illusion(The Scientist Magazine®, 2017).

(B) Responses to questionnaire items rating the illusory experience. The first 3 questions are test questions while the rest are controls (Kammers et al., 2008).

Another aspect of spatial representation is being able to perceive and localize a touch on the embodied object:

In the rubber hand illusion (Armel & Ramachandran, 2003; Botvinick & Cohen, 1998; Ehrsson et al., 2004), participants are exposed to an artificial limb while their hand is hidden from their view and stroked in synchrony with the prosthetic limb to generate the impression that the latter limb belongs to them (asynchronous stroking is used as a control where the illusion is not generated) (Figure 1.1A). Successful embodiment is often accompanied by reports of perceiving touch on the artificial limb rather than on one's own hand (Figure 1.1B) (Makin et al., 2008; Reader et al., 2021b; Tsakiris, 2010).

Localizing touch from the embodied object can also arise with prosthesis use: amputated patients can report tactile feelings on a prosthetic device when congruent tactile information is provided on their stump (Rosén et al., 2009). Similarly, in the case of tool use, it has also been reported that participants would localize touch on the tip of a stick being held as opposed to their hand (Yamamoto et al., 2005). Although the sticks being held do not give rise to ownership feelings, there is a different level of embodiment taking place as the tool is being treated as an extension of the body in regards of tactile localization (de Vignemont, 2011; Holmes et al., 2004).

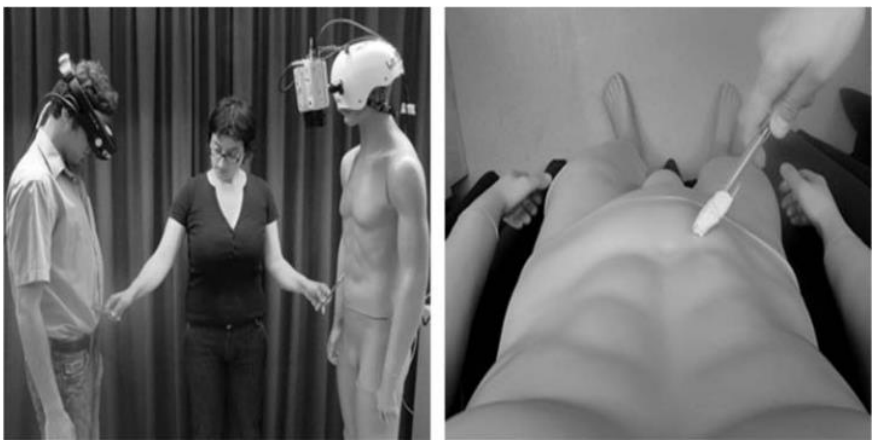


Figure 1.2: Experimental set-up to induce illusory ownership of an artificial body (Petkova & Ehrsson, 2008).

The participant could see the mannequin's body (left panel) from the perspective of the mannequin's head (right panel) using VR goggles that played the live camera feed placed on top of the mannequin's head.

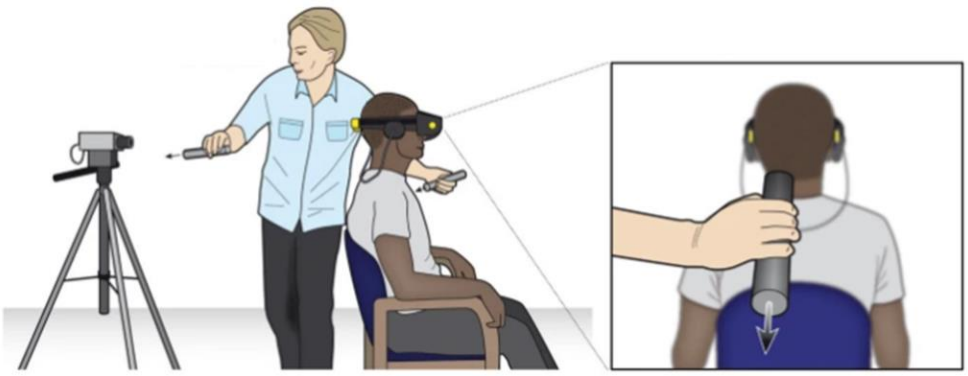


Figure 1.3: Paradigm for Out of body illusions (Yong, 2011).
(Left panel) A subject wears goggles showing him the view from a camera behind him. An experimenter prods the subject's chest at the same time as prodding the camera. (Right panel) The subject sees the hand prodding towards the camera as he feels his chest being prodded. He also sees his body from behind. This creates a vivid sense that his real body is floating behind the one he sees.

The next element regarding spatial features of embodiment is the location of the embodied object being sensed as one's own location:

Studies have showed that it is possible to induce body swapping illusions by using a combination of congruent tactile stimulations and VR systems to manipulate the visual perspective to be from the mannequin's point of view (Petkova et al., 2011; Petkova & Ehrsson, 2008). When the participants embody the mannequin, this also drives a shift in their perceived body location (Figure 1.2).

This type of paradigm can also take the form of out-of-body illusions. To achieve this illusion, a similar approach with VR is used to change the visual perspective. This is used, not to show the perspective of the mannequin, but to show an exterior vision of the participants themselves (Ehrsson, 2007; Guterstam & Ehrsson, 2012). In this experiment there is no external object being embodied per se. Instead, the synchronous tactile stimulation seen from a first-person point of view, while looking at one's self, produces a shift of perceived body location to fit the point of view shown by the VR environment (Figure 1.3). This can also be translated as neural correlates of self-localization: a brain imaging study using fMRI in the context of body swapping illusions showed that perceived self-location could be accurately decoded from patterns of activity in the hippocampus (Guterstam et

al., 2015).

Outside of full body illusions, shifts in self-localization can also be perceived in the rubber hand illusion. When participants are asked to close their eyes and asked to locate their own hand, the perceived location of their hand moves closer to the rubber hand, during test conditions: this is referred to as the proprioceptive drift (Abdulkarim et al., 2021; Botvinick & Cohen, 1998).

In a previous work published in 2021, I investigated in the laboratory of H. Ehrsson the temporal correlates of proprioceptive drift and the sense of ownership (Abdulkarim et al., 2021). We exposed 40 participants to the rubber hand illusion paradigm. After 60 seconds of stimulation we asked them to rate their illusory experience and perform a hand localization task while their eyes were closed. We repeated these measurements every 20 s for the first minute, and additionally 120 s and 300 s after the end of brushstrokes (Figure 1.4A). Half of the participants could see the rubber hand between the measurements (Exp1), the other half were instructed to close their eyes between measurements (Exp2) (Figure 1.4A).

No significant differences were seen between experiment 1 and 2 indicating that in our paradigm the effect of the visual input of the rubber hand did not play a role in the temporal evolution of the illusion.

Our results reveal that both the feeling of ownership and the proprioceptive drift can be sustained for up to 300 s and 40 s respectively. Moreover, we revealed that the decay of subjective ownership and proprioceptive drift follow similar time courses, suggesting that these processes depend at least in part on similar mechanisms (Figure 1.4B-C).

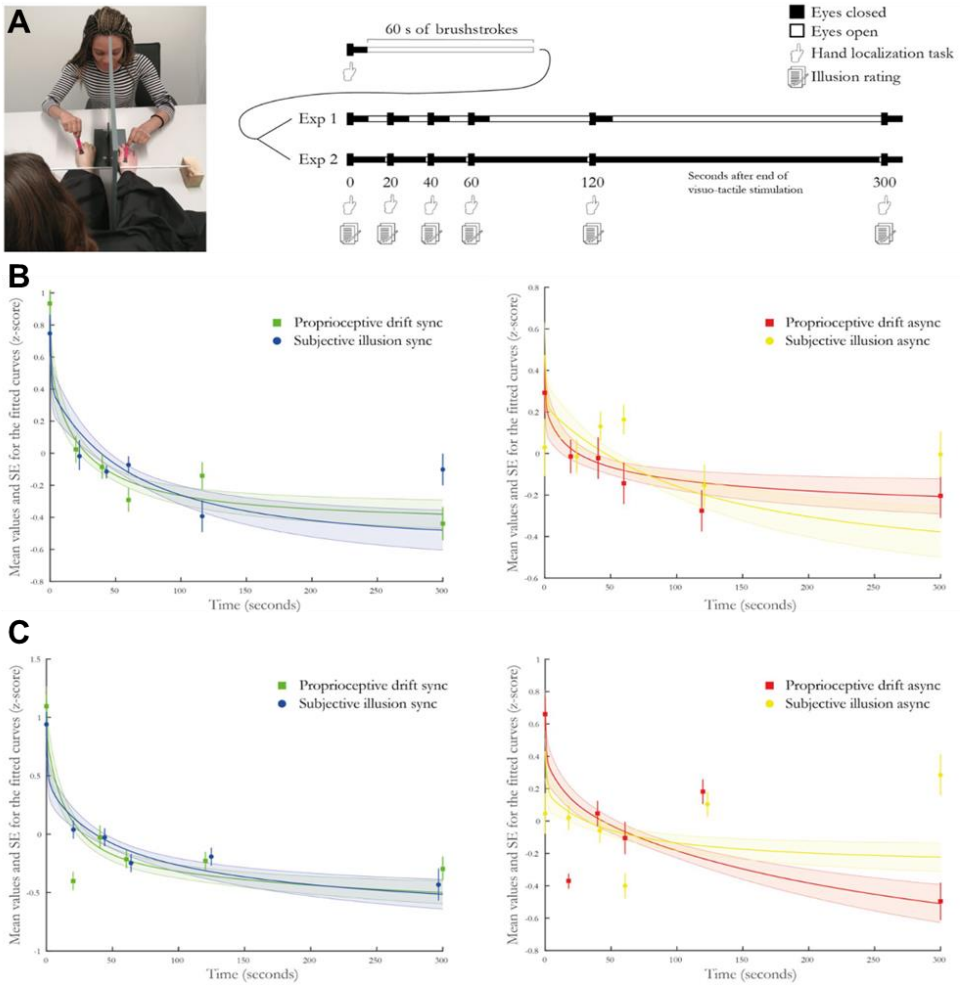


Figure 1.4: Subjective ownership and proprioceptive drift follow similar temporal decays at the end of visuo-tactile stimulation. Panels reproduced from (Abdulkarim et al., 2021).

(A) Experimental setup for the investigation of temporal evolution of ownership and proprioceptive drift. Participants receive brush strokes on their hidden hand while observing the rubber hand being stimulated in synchrony or asynchrony for 60 s. At the end of the experiment, participants rated the subjective feeling of ownership towards the prosthetic limb. They were also asked to localize their hand. This was repeated at different time points to evaluate the evolution of ownership and proprioceptive drift in time (0,20,40,60,120,300s after end of stimulations).
(B) Mean fitted curves of ownership scores and proprioceptive drift for experiment 1.
(C) Same as B for experiment 2.

Finally, the spatial dimension of embodiment also encompasses the representation of the space around the embodied object as peripersonal space (the area immediately surrounding the body where objects can be easily reached and interacted with):

Electrophysiological studies have pioneered the investigation the peripersonal space. During these experiments, visuo-tactile neurons in monkeys' premotor cortex are recorded while a visual stimulus approached the animal's limb. Results showed that neurons respond the most to the stimulus that approached the limb's tactile receptive field (Figure 1.5)(Graziano, 1999). When the animal's hand was moved, the neurons strongest response shifted to the new location regardless of eye position. This suggests that these neurons encoded visual information in peripersonal space, that is within a hand-centred coordinate system (Makin et al., 2008).

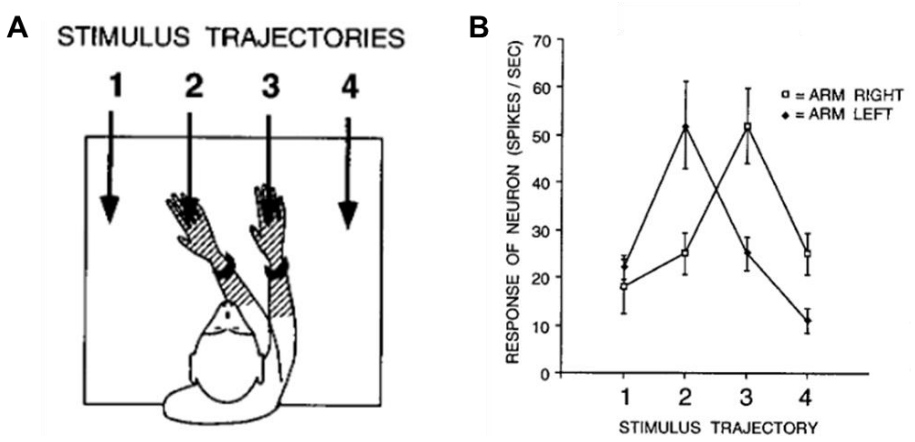


Figure 1.5: Visual responses of a typical premotor neuron with a tactile RF (Receptive field) (hatched) on the forearm and hand, and a visual RF within 10 cm of the tactile RF (Graziano, 1999).

(A) On each trial, the arm contralateral to the neuron was fixed in one of two positions and the visual stimulus was advanced along one of four trajectories (1–4). For this neuron, the two arm positions were chosen to align the visual RF near the hand and forearm with trajectories 2 and 3. For other neurons, the arm was moved to different extents depending on the location of the visual RF, to better capture the movement of the visual RF with the arm.

(B) Responses of the neuron to the four stimulus trajectories when the arm was visible to the monkey. When the arm was fixed on the right, the response was maximum at position 3. When the arm was fixed on the left, the maximum response moved to the left, to position 2.

To explore similar peripersonal mechanisms in humans, one of the paradigms that have been widely used, relies on cross-model congruency tasks where participants have to discriminate different vibrotactile targets while ignoring irrelevant visual distractors. These studies have shown how tools can get incorporated in visuotactile

representations of the body and peripersonal space (Holmes et al., 2004; Maravita et al., 2002; Spence et al., 2004). Similarly, when amputated patients are exposed to an auditory-tactile interaction task to measure the distance at which sounds interact with the processing of tactile information on their limbs, it has been shown that prosthesis wear affects their reaction time to tactile stimulus which indicates a modification of the patients' peripersonal space boundaries to include the prosthetic limb as well (Canzoneri et al., 2013).

In summary, spatial features of embodiment encompass several dimensions that have been extensively studied. These include integrating new or altered body parts into the body schema, such as through prosthetics, supernumerary limbs, or altered limb length or body size perceptions. The second aspect is the ability to localize sensations on embodied objects, demonstrated in experiments like the rubber hand illusion. Additionally, the spatial processing of embodied objects involves treating their location similarly to that of one's own body, as seen in body swapping illusions and out-of-body experiences. Lastly, the representation of peripersonal space involves understanding how the space around embodied objects is perceived, which is explored through visuo-tactile interactions and the impact of prosthetics on spatial boundaries.

1.1.1.2 Motor features:

The first aspects of motor measures of embodiment are the feeling that the embodied object directly obeys one's will. The subject needs to feel that their own body or body part is moving when the embodied object moves:

These aspects are tested in motor versions of the rubber hand illusion protocol. In the more frequent version of this paradigm, the participants' hidden index finger is mechanically connected to the index finger of the rubber hand. Thanks to this device, when the participants lift their index finger, the finger of the artificial limb moves as well (Figure 1.6A-D) (Abdulkarim et al., 2023; Kalckert & Ehrsson, 2014b, 2017). In this motor version, it is possible to measure a sense of agency towards the rubber limb. The sense of agency describes the feeling of control over the movements: one experiences the sense of

being the agent performing the action. Here the participants not only feel that the rubber hand is part of their body but also that they can intentionally move it (Figure 1.6E).

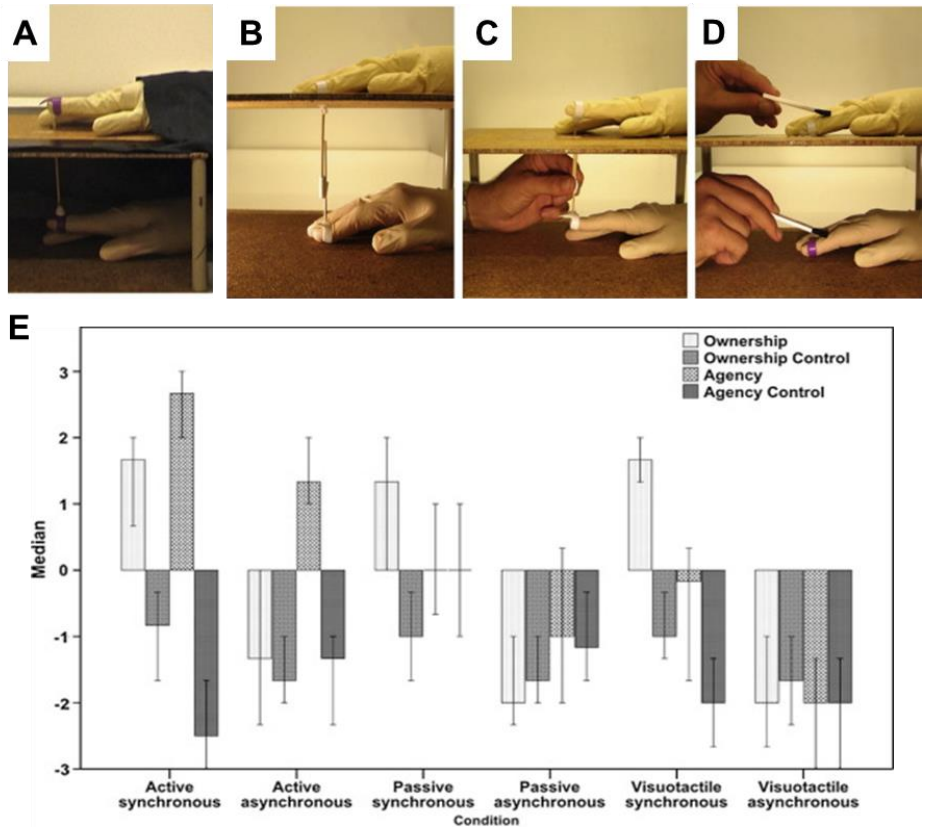


Figure 1.6: Setup of the moving rubber hand illusion (Kalckert & Ehrsson, 2014a)
(A) Setup with the occluding cloth as used in the experiment. (B–D) Illustration of the three conditions: (B) Active movement: the participant taps the finger. (C) Passive movement: the experimenter moves both the rubber hand's and participant's fingers by moving the connecting stick. (D) Visuotactile stimulation: the experimenter strokes both fingers with a small brush
(E) Results of the questionnaire data. mean score was computed for each category (Ownership, Ownership Control, Agency and Agency Control).

Embodiment related to the sense of agency can also be seen without requiring active movement. In brain machine interfaces, participants can directly control a motorized prosthesis or a virtual avatar, mostly through the use of electroencephalography recordings or more rarely through invasive recordings. Studies investigating embodiment have been conducted using brain machine interfaces based on motor imagery tasks. In these experiments, participants are instructed to

imagine performing certain movements to control avatars in VR systems or robotic arms (Tomás et al., 2023). These studies show that when the avatar arm or robotic hand moves in congruency with the motor imagery attempts, there is an increase in the sense of embodiment, including a higher sense of ownership and a higher sense of agency. Some studies have used electromagnetic sensors to record peripheral nerve activity on the residual limb of amputees to control avatar arms and legs in a VR environment. This has led to higher scores for the sense of agency as the subjects sensed the movement as coming from the embodied virtual limb (Cole et al., 2009).

The second aspect of motor embodiment is incorporating the embodied object into motor planning:

In tasks where participants are required to plan and execute reaching movements based on the integration of visual and proprioceptive information about their hand's position seen in a mirror, results show that the reaching accuracy is affected by the perceived location of the embodied limb, be it the hand of the participant or a rubber limb (Holmes & Spence, 2005). In contrast, the impact on reaching performances is not as strong when the object shown in the mirror is a wooden block (Holmes et al., 2006). Moreover, in this study this effect was independent of body ownership, showing that certain aspects of embodiment (like the sense of agency) can be studied independently of other modalities (like the sense of ownership).

1.1.1.3 Affective features:

When one's own body is threatened or is in danger, a series of responses to the threat is triggered to avoid harm to the body. This involves the activation of the sympathetic nervous system, which leads to measurable physiological reactions, such as an increase in heartbeat, pupil dilation, or the increase in skin conductance response These changes enable the body to react swiftly and effectively to potential threats, enhancing survival chances. When an object is perceived as being part of its body, this can lead the subject to protect the embodied object from the harmful situations, provoking a similar stress reaction to a threat on an actual body part. This is an important

element that could explain the lack of ownership usually ascribed to tools compared with limbs or prosthetic limbs. Although, recent research showed that tools can also give rise to ownership (including an affective response). In this study, the tool used was a grabber which looks scarcely like a hand. This type of affective response has not been reported with more simple tools, like sticks, to our knowledge. These results indicate that tools can get incorporated in a way that provokes an affective response but only when they share functional similarity to the limb (Cardinali et al., 2021).

Affective features can be measured in rubber hand illusion protocols: when the subjects report that the rubber hand feels like their own hand, these verbal reports are also accompanied with overt behaviours of the subject during the experiments. In particular, the subjects report an anxiety-like response when the artificial limb is visibly threatened or "injured" by the experimenter. This is seen through higher scores of skin conductance responses (Figure 1.7) and participants showing signs of anxiety and pain anticipation before the injury or threat of the prosthetic limb (Armel & Ramachandran, 2003). This also translates in increased activity in the insula and anterior cingulate cortex, regions that are associated with anxiety and interoceptive awareness (Ehrsson et al., 2007).

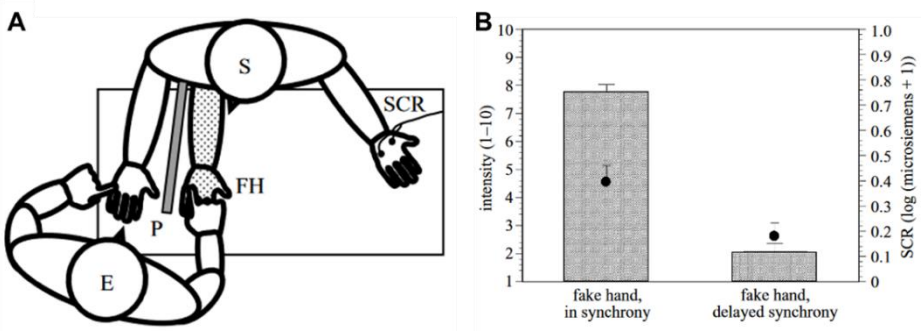


Figure 1.7: Threatening a rubber hand after synchronous stimulations provokes stress reaction (Armel & Ramachandran, 2003).
(A) Rubber hand illusion setup with skin conductance response measurement.
(B) Questionnaire ratings (grey bars) and skin conductance response (SCR) (black circles) during the rubber hand illusion. Synchronous touch to the real hand was delayed by 1 s in the control condition.

Similar effects have been reported in studies using virtual reality (VR) environments, with participants showing signs of stress by retracting their hand after the avatar hand is threatened. They also report strong feelings of fear when the hand is threatened by a malfunctioning crusher (Fribourg et al., 2021).

The position in which the virtual avatar is placed can also provoke feelings of discomfort: participants that embody a virtual avatar sitting in an uncomfortable position show signs consistent with discomfort, such as a higher variability of heart rate and mistakes in cognitive tasks, signs they do not show when embodying an avatar sitting in a comfortable position (Bergström et al., 2016).

1.1.1.4 Neural features of embodiment:

Embodiment can also be assessed through neural features. Indeed, neuroimaging studies, highlight the involvement of several key brain regions:

Most of these studies used fMRI to investigate the neural correlates of embodiment during rubber hand illusion paradigms. Prominently, the posterior parietal cortex (PPC) and the ventral premotor cortex (PMv) have been consistently implicated, as well as the inferior parietal sulcus (IPS) (Bekrater-Bodmann et al., 2014; Brozzoli et al., 2012; Ehrsson et al., 2004). Parietal and premotor areas integrate visual and somatosensory information through multimodal neurons that continuously update the body's spatial representation. The parietal cortex is a key region for multisensory integration, mapping limb positions and orientations within a body-centered reference frame. Additionally, the ventral premotor cortex (PMv) has neurons that respond to touch on specific body parts or approaching stimuli (Castro et al., 2023; Graziano, 1999).

In a study published in 2023, we aimed to further the understanding of neural correlates of embodiment (Abdulkarim et al., 2023). Specifically, we were interested in separating the neural substrates of body ownership (feeling that one owns a body/body part) and the sense of agency (feeling that one's actions are controlling the body/body part). In 30 healthy participants, we elicited the moving

rubber hand illusion with passive and active movements and recorded brain activity during this task using fMRI. This allowed us to separate different aspects of the illusion related to agency and illusory ownership.

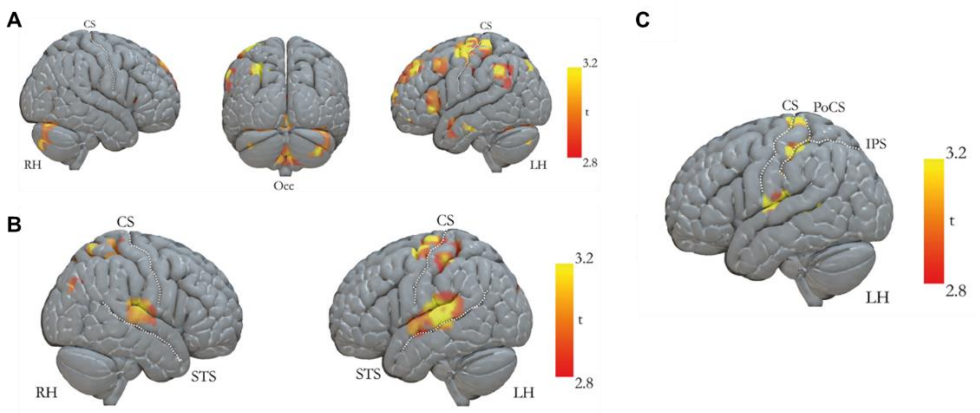


Figure 1.8: Neural substrates of ownership and agency during the moving rubber hand illusion (Abdulkarim et al., 2023).

(A) Body ownership was associated with higher activations in the premotor cortex, posterior parietal, and cerebellar regions.
(B) The sense of agency was associated with higher activations in the superior temporal cortex and dorsal premotor cortex.
(C) Brain regions that display activation reflecting the unique combination of agency and body ownership (dorsal premotor cortex and somatosensory cortex).

Our findings reveal that feelings of ownership towards the rubber hand during this paradigm was associated with premotor and posterior parietal activity, similarly to what has been observed in previous literature (Figure 1.8A). We also saw cerebellar activation associated with ownership (Figure 1.8A). The sense of agency on the other hand was associated with activity in the dorsal premotor cortex and superior temporal cortex (Figure 1.8B). We also saw an overlapping of both senses in the dorsal premotor cortex (Figure 1.8C). Moreover, our findings revealed a higher activation of the somatosensory area when both ownership and agency were experienced (Figure 1.8C). This was accompanied by higher ownership scores, suggesting an agency-induced ownership enhancement of somatosensory cortical activity specific for voluntary movement (Abdulkarim et al., 2023).

Other functional connectivity studies have further explored the role of

different neural networks during embodiment illusions. During the rubber hand illusion, enhanced connectivity is observed between the PMv, inferior parietal sulcus (IPS), secondary somatosensory cortex (S2), and the extrastriate body area (EBA). This network supports the idea of integration and reconciliation of conflicting sensory inputs conveyed to parietal integrative regions, reinforcing the perception of ownership over the rubber hand (Castro et al., 2023).

To assess the temporal dynamics of brain activity during induced changes in embodiment, several studies have used electroencephalography (EEG) during the rubber hand illusion. Studies using somatosensory evoked potentials (SEPs) reveal a modulation of both early and late brain potentials. Early SEPs indicate decreased amplitude around 50 ms in the illusion condition, suggesting early attenuation of sensory inputs in S1 (Zeller et al., 2015). Later SEPs show increased amplitude at 150 ms and decreased at 460 ms, indicating involvement of sensorimotor and associative regions (Peled et al., 2003). Collecting SEPs during the illusion's induction is challenging as brush strokes are not a short-lasting discrete event. These electrophysiological results paint a complex picture of signal modulation during body illusions, and further studies are needed to elucidate the role of early and late SEPs in illusory perception. Electrophysiological evidence also shows modulation across frequency bands, with increased gamma activity in parietal areas and alpha and beta modulation in central and parietal regions, linked to illusory embodiment (Castro et al., 2023).

Additionally, non-invasive brain stimulation techniques like transcranial magnetic stimulation (TMS) have also investigated the neural mechanisms underlying the rubber hand illusion. Studies using TMS to stimulate M1 have shown mixed results regarding corticospinal excitability during the illusion. While some studies report decreased motor-evoked potentials during synchronous stimulation, reflecting a suppression linked to illusory embodiment (Schütz-Bosbach et al., 2009), others fail to replicate these findings, possibly due to methodological variability (Reader et al., 2021a). Additional studies indicate that TMS over the premotor cortex or parietal areas can either increase or decrease measures of body ownership and agency during the rubber hand illusion. Specifically, stimulation over PMv tends to

attenuate the conscious detection of visuo-tactile congruence (Peviani et al., 2018), while inferior parietal lobe stimulation may attenuate proprioceptive drift (Kammers et al., 2009). These findings highlight the nuanced effects of stimulation protocols on cortical regions implicated in body representation and multisensory integration.

1.1.1.5 Conclusion

In conclusion embodiment is a multi-faceted phenomenon that is revealed through different features.

Spatially, we can say that an object is embodied if it is taken into account in the subject's perceived body representation as evidenced by illusions where an artificial limb replaces or adds to the body schema, or alters the size of a body part. This also includes changes in the processing of peripersonal space and localization of the body. Moreover, subjects would report the ability of being able to localize somatosensory inputs from the embodied limb.

Motor embodiment is indicated if one perceives direct control over the embodied object's movements, senses it as an extension of one's own body in action planning, and includes it as an effector within the motor system.

Affective features of embodiment allow us to reveal an emotional attachment to the embodied object as the threat of harming it elicits similar emotional and physiological responses as would be expected if one's own body were at risk.

Finally, neural correlates of embodiment, explored through fMRI, EEG, and non-invasive brain stimulation techniques, converge on several key brain regions central in embodiment. Parietal and premotor areas, crucial for integrating visual and somatosensory signals, consistently emerge across studies of body ownership. Functional connectivity studies underscore the coordinated activity between premotor cortex, inferior parietal sulcus, and somatosensory areas during illusion induction, suggesting their role in reconciling conflicting sensory inputs critical for illusory embodiment. Moreover, investigating neural substrates of embodiment in specific conditions also reveals activations related to perceived changes in spatial location, as well as

stress responses after the threat of an embodied object.

In essence, embodiment is a complex phenomenon rooted in multisensory integration, where a distributed network of brain areas integrates sensory inputs to construct a coherent representation of one's body.

1.1.2 Disruption of embodiment

With this understanding of the multiple facets of embodiment in mind, we can now examine cases where these elements are disrupted. Such disruptions are evident in neurological disorders, and in disruptions to body integrity, such as amputations. They highlight the importance of embodiment in maintaining a coherent sense of self. Understanding these disruptions is crucial for gaining insights into disorders of embodiment, providing a foundation for developing effective interventions and therapies.

Embodiment can be impacted by a variety of phenomena. We can classify them in three general categories: Neurological disorders, psychiatric disorders, and transient disruptions.

1.1.2.1 Neurological disorders

Embodiment is disrupted in a number of neurological disorders, mostly stemming from brain damage, that can affect different facets of this sense.

One of these disorders is asomatognosia, which is defined as a lack of recognition of the body, with patients reporting an inability to experience ownership towards their body or body parts. This includes disruption in their sense of existence, visual self-recognition and sense of belonging (Jenkinson et al., 2018). This disorder arises after brain lesions (right hemisphere strokes typically) with a reported involvement of the tempoparietal and medial frontal brain regions. In some cases of asomatognosia, patients seem to be confused and make errors about the ownership of their limbs, that can be corrected by someone pointing out the identity of the limb to them.

However, in some cases, the non-recognition of the limb is

accompanied by delusions and confabulatory attributions of the limb to another person. This subset of asomatognosia is called somatoparaphrenia. In these cases, the misattribution of one's own limb to another person still persists even after correction attempts by exterior sources or caregivers, or after showing clear evidence that the limb, does in fact, belong to them. Brain lesions similar to those related to asomatognosia have been linked to somatoparaphrenia. However, in addition it has also been linked to larger brain lesions and more orbitofrontal damage compared to asomatognosia (Feinberg et al., 2010). It is important to note that these disorders do not stem from impairments of other senses like vision or touch, as these patients can still see their limbs and often still have tactile perception from them. This strengthens the idea that the regions affected by these lesions are involved in the multi-integration processes giving rise to embodiment and more specifically to the sense of ownership.

Alien limb syndrome is another example of a disruption of the sense of body ownership as well as the sense of agency. In this affliction patients experience involuntary movements of a limb, also accompanied by feelings of disownership towards said limb. This disorder is mostly seen as a consequence of cortical basal syndrome, a parkinsonian syndrome caused by an overproduction of tau protein. However, it has been linked to other etiologies such as strokes or tumours. Reports show that this condition is linked to brain damage in the parietal cortex, mostly on the right hemisphere. Some of the symptoms include limb rigidity, disruption of motor execution (apraxia), disturbance of directional cutaneous kinaesthesia, resulting in difficulty recognizing letters or numbers traced (agraphesthesia) and an inability to recognize objects using touch alone (astereognosis) (Graff-Radford et al., 2013).

Disorders such as asomatognosia or alien limb disorders affect specific limbs, usually upper limbs, but medical reports exist of disruptions of embodiment affecting patients' whole bodies. In "The man who mistook his wife for a hat" — a collection of case reports — clinical neurologist Oliver Sacks gives an account of one of his patients that has lost all sensation of touch and proprioception and reports feeling 'disembodied' after suffering a polyneuritis that mostly affected the sensory roots of her spinal and cranial nerves. This made the patient

unable to stand unless she looked at her feet: she was unable to coordinate basic movements and even lost her facial expressions and vocal posture. The patient Christina reports: "I may "lose" my arms. I think they're in one place and I find they're another" and "I feel my body is blind and deaf to itself… It has no sense of itself". Additional reports of "disembodied" patients have followed that of Christina. These additional patients — who suffered from severe sensory neuritis likely stemming from an overconsumption of vitamin B6 — also reported disturbances in body image and body ownership (Sacks, 1985).

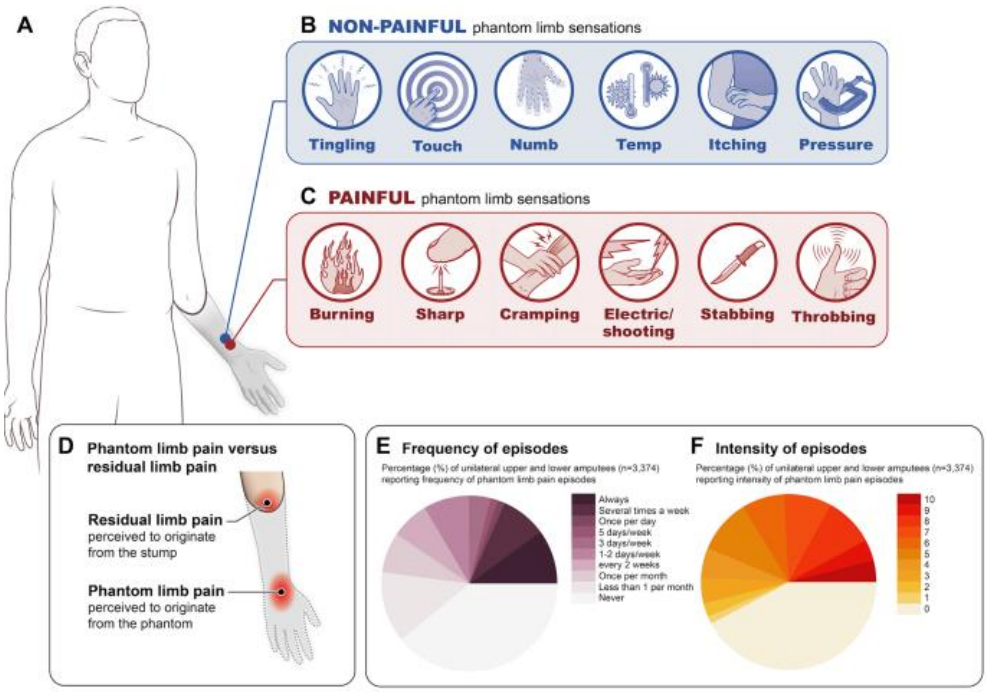


Figure 1.9: Phantom limb sensations (Schone et al., 2022).
(A) Diagram of upper limb amputee with a phantom arm. Spectrum of phantom sensations from non-painful (B) to painful (C).
(D) Residual limb pain (RLP) and PLP illustrated in an upper limb amputee. RLP is perceived to originate from the stump/residual limb, whereas PLP is pain perceived to originate from the phantom limb.
(E) Cross-sectional surveys on PLP frequency and (F) average intensity.

These disruptions of embodiment describe cases of patients not being able to feel embodiment towards their own limbs or bodies, but the opposite problem can also occur with amputees. Patients that have lost a limb often still experience ownership of a non-existing limb. This

is referred to as phantom limb syndrome. It has been reported in a majority of post amputation cases (86 to 91%)(Bailey & Moersch, 1941; Blumberg & Dooley, 2017).

Some of the symptoms of this syndrome include the sensation of feeling the lost limb, hypersensitivity, spasms on the stump, tingling, numbness and itching. Some of these phantom sensations can be quite painful, including burning or cramping sensations which can become very difficult to bear for patients (Bailey & Moersch, 1941; Schone et al., 2022). The loss of a limb leads to a cortical reorganization of primary motor and somatosensory areas, which has been linked to phantom limb pain as a form of maladaptive plasticity (Flor et al., 1995; Serino et al., 2017).

1.1.2.2 Psychiatric disorders

Several psychiatric disorders can cause a disruption in the sense of embodiment which can be a disturbing experience and cause distress for patients dealing with these symptoms.

Disruption in the sense of body ownership is most commonly seen in dissociative disorders. Depersonalization/derealization disorder is defined in the DSM-V as a "clinically significant persistent or recurrent depersonalization (i.e., experiences of unreality or detachment from one's mind, self, or body) and/or derealization (i.e., experiences of unreality or detachment from one's surroundings). When it comes to depersonalization, which affects embodiment amongst other things, this includes patients reporting the impression that they are not really there or that their hands do not belong to them. The sense of agency can also be affected in this disorder with reports of "feeling robotic" or lacking control over speech and movements (American Psychiatric Association, 2012). This disorder does not affect the ability to evaluate reality (reality testing), and patients are well aware of the abnormality of their experience. Yet they remain convinced that an ineffable change has occurred in the way they perceive their bodies and surroundings (Sierra & David, 2011). Depersonalization symptoms can be observed in comorbidity with neurological disorders such as temporal lobe epilepsy or certain mental health conditions notably schizophrenia and post-traumatic stress disorder, as well as in non-pathological transient

states like extreme fatigue (Orrù et al., 2021).

Disturbances in embodiment can also be observed in body Integrity identity disorder, a rare condition where patients feel that a body part does not belong to them, resulting in wishing for the amputation of said body part. Patients suffering from this disorder desire to become physically disabled, and report wishing they were born without the "strange" limb that they wish to be removed. They often also display simulating behaviour like binding their legs to simulate amputation or using a wheel chair or crutches. In extreme cases, this disorder can even lead to mutilation or self-harm (World Health Organization, 2018). This condition has been linked to reduced grey matter volume in the left dorsal and ventral premotor cortices and larger grey matter volume in the cerebellum (lobule VIIa) (Blom et al., 2016).

1.1.2.3 Transient disruptions

Disturbances of embodiment are not necessarily pathological and can be induced temporarily through bodily illusions or transient states.

As mentioned previously, the sense of embodiment can be manipulated through the rubber hand illusion and its many variants, also including full body illusions using VR systems. As already discussed, participants in this paradigm experience embodiment towards an artificial limb or mannequin body (Ehrsson, 2007; Ehrsson et al., 2007; Hoort et al., 2011). The participants not only report feelings of ownership and/or agency towards the embodied object, but they can also report a lack of ownership towards their own limb as if the embodiment were 'transferred' from their real body towards the rubber limb or virtual avatar. These illusions and experiences in immersive environments are temporary and this disruption of embodiment only lasts during the experiment and in certain cases tens of seconds after the end of the tactile stimulations. Often, participants would feel as if their hand were numb when they embody the prosthetic limb. They may need to shake their hand at the end of the paradigm, and their movements may appear strange to them for a couple of seconds.

Beyond body illusions, healthy subjects can also experience out-of-

body experiences where they perceive themselves as existing outside of their physical bodies, often observing their own body from an external perspective. These phenomena can occur while sleeping, dozing, or relaxing. Some studies have found that out of body experiences can start during sleep and may occur as hypnagogic (transitional state from wakefulness to sleep) and hypnopompic (transitional state from sleep into wakefulness) hallucinations among many other experiences (Blanke et al., 2004; Bünning & Blanke, 2005).

Another type of temporary disruption of embodiment can be caused by transient paraesthesia, which describes the temporary numbness or tingling that may appear on a limb or the body. This is caused by a disturbance in the transmission of sensory information that can occur after prolonged pressure on peripheral nerves. The medical term "obdormition" is used to describe this phenomenon but, more commonly, we know this sensation as a limb 'falling asleep'. This experience triggers a momentary dissociation or disembodiment from the affected limb. During this period, individuals may feel disconnected from the limb, experiencing it as numb or tingling, until normal sensation and control are restored once the pressure on the nerves is relieved (Imbelloni & Gouveia, 2012).

Understanding the disruptions of embodiment can help us shed light on the challenges faced by patients suffering from disturbances of this sense. We are particularly interested in the realm of prosthetic use, where achieving true embodiment of a prosthetic limb remains difficult for many.

1.1.3 Embodiment in prosthesis use

Prosthetic limbs play a crucial role in enhancing life quality of individuals who have undergone amputation, offering greater autonomy and integration. Despite advancements in prosthetic technology, a significant number of upper-limb amputees choose not to use their prothesis, indicating challenges that need to be addressed in improving acceptance rates. Here we explore the challenges associated with prosthetic use and discuss embodiment issues experienced by prosthetic users.

The loss of a limb comes with many challenges for the patients. Amputees suffer from a loss of mobility and autonomy, they may have limited access to rehabilitation services, and 69% become unemployed or have to change their workplace after amputation (Jang et al., 2011). Amputees often complain about the inability to perform certain daily tasks such as lacing shoes, opening bottle tops, and using scissors which many prostheses can't achieve (Jang et al., 2011). Reports indicate that around 20% of upper limb amputees do not use a prosthetic limb, and around 30 to 50% do not use their prosthetic limb regularly (Espinosa & Nathan-Roberts, 2019; Maimon-Mor & Makin, 2020).

In the existing literature on prosthesis use, there is no systematic measurement of the sense of embodiment (Aflalo et al., 2015; Collinger et al., 2013; Flesher et al., 2021). Studies would usually report the general satisfaction with the prosthesis and the performance of the patient in achieving certain tasks using their prosthetic limb. However, there are several aspects related to different modalities of embodiment that have been considered to explain the abandonment of prosthetic limbs in amputees.

The first element is related to the sense of agency and the precise control of a prosthesis (Espinosa & Nathan-Roberts, 2019). In open-loop myoelectric devices, patients can control an active prosthesis through the recording of nerve signals on the residual limb. In these systems, misreading of the recorded electromyography (EMG) signals can cause the user's intent to be misinterpreted creating an unpredictability and/or a delay of the movement of the prosthesis. This is particularly noticeable when the hand opens and closes without the intent of the patient (Chadwell et al., 2016). In this situation, the patient loses some of the control they have towards the limb, disrupting the potential sense of agency they have towards it. This type of mismatch between the intent of the patients and the movement being executed by the prosthesis can be detrimental to integrating the limb in the body schema.

The second element that can explain prosthesis abandonment is related to the lack of somatosensory feedback and the necessity to rely on visual feedback instead: As mentioned previously, neurological

disorders affecting the transmission of sensory input can create disruption in the sense of embodiment and motor impairments, such as the need of relaying on constant visual feedback to be able to stand for example (Sacks, 1985). Amputated patients suffer from the same issue in regards to their prosthetic limbs, most of which do not offer somatosensory feedback: we rely on our sense of touch and proprioception to execute different reaching and grasping tasks, so when that feedback is not present, these tasks are much harder to perform.

This poses two main issues : the first one is related to the fact that visual feedback is not the most informative in terms of fine movements and interactions with more fragile objects, as it is difficult to evaluate necessary grasp force using vision alone (Peerdeman et al., 2011).

The second issue is related to mental workload: it has been shown that, during grasping tasks, patients will typically allocate about 90% of their visual attention to monitoring their prosthetic device or focusing on the immediate area around the object they intend to grasp (Sobuh et al., 2014). This heightened visual focus imposes an extra cognitive load necessary to successfully complete activities. In routine activities, where the main focus is on completing the task at hand, the requirement to continually monitor the prosthetic device can result in significant interference. When operating the prosthesis demands more attention than the task itself, the manipulation of the prosthetic becomes the primary focus, thereby relegating the task to a secondary role (Espinosa & Nathan-Roberts, 2019).

It is important to note that both these elements are fundamental to the experience of embodiment. First, the ability to control the prosthesis and feeling like it obeys one's will which affect the smooth control of the limb. Second, the ability to refer touch and localize sensory input coming from the prosthesis. This can partly explain why some of the most commonly reported causes for prosthesis abandonment are awkward control over the prothesis that does not feel organic, lack of predictability and lack of somatosensory feedback (Maimon-Mor & Makin, 2020).

1.2 THE RUBBER ILLUSION TO STUDY EMBODIMENT

1.2.1 Methods to induce and measure ownership of a rubber hand

The challenges faced by prosthetic users highlight the need for innovative approaches to enhance prosthetic integration. One interesting paradigm that allows us to explore these concepts is the rubber hand illusion. This experimental setup investigates multisensory integration in the context of establishing a sense of ownership over an artificial limb. By manipulating sensory inputs and observing perceptual changes, we can gain insights into the mechanisms underlying embodiment and how they can be leveraged in clinical use.

Studying embodiment was traditionally limited to case studies of patients suffering from disturbances of that sense. These studies faced constraints due to the small number of affected patients and the wide variety of underlying pathologies, which included conditions from brain lesions to amputations. However, the discovery of body illusions that can be tested on large numbers of subjects has enabled researchers to investigate the sense of embodiment in a controlled laboratory environment using healthy subjects.

In 1998, psychologists Matthew Botvinick and Jonathan Cohen published the first study reporting "The rubber hand illusion" (Botvinick & Cohen, 1998). They exposed 10 participants to an artificial left hand while their real hand was hidden, and they used two paint brushes to apply synchronous stimulations to both hands for 10 minutes. At the end of these stimulations, the participants were instructed to answer a questionnaire rating several statements about their experience such as 'I felt as if the rubber hand were my hand'. The answers reveal that the participants experienced an illusion where they felt the touch of the viewed brush but not the touch of the hidden brush, as if the rubber hand that they were embodying was the one experiencing the touch. In a second experiment they also reported the existence of a 'proprioceptive drift' which refers to the displacement of self-localization of the hand towards the rubber hand. This study also added a control group where a small asynchrony was introduced between the stimulations on the real hand and the rubber hand. This experiment revealed a lower subjective ownership of the artificial limb

and lower proprioceptive drift (shift of the hand's perceived location towards the rubber hand).

Since the publication of this original study, this paradigm has been replicated in various conditions, and a variety of its perceptual rules have been studied, revealing insights into the mechanisms of embodiment and the factors that influence it. Here we will focus on the different modalities that allow the embodiment of a rubber hand that have been elucidated in the literature. They can be divided in the 4 categories: spatial congruency, anatomical congruency, temporal congruency, tactile congruency (Abdulkarim, 2020).

1.2.1.1 Spatial congruency

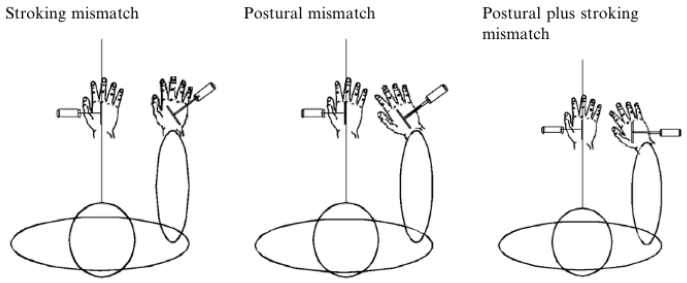
The spatial congruency rule states that, for the rubber hand to be embodied, the visuo-tactile stimulations need to be spatially congruent.

The first element concerns the tactile stimulations applied on the rubber hand, which need to be at a place that matches the location on the real hand. A study (Costantini & Haggard, 2007) investigated the effect of spatial mismatch between the visual and tactile stimulation: the authors manipulated the angular position of the rubber hand and the hand of the participants, as well as the angular position of the tactile stimulation (Figure 1.10). This revealed that the illusion persisted despite small changes in the subject's hand posture but vanished when similar changes were made to the rubber hand. The illusion also disappeared when the stimulation of the subject's hand and the rubber hand did not match. Interestingly, the illusion remained when the subject's hand posture differed slightly from the rubber hand, as long as the stimulation was congruent in a hand-cantered spatial reference frame, even if it was incongruent in external space. Conversely, the illusion diminished when the stimulation was incongruent in hand-centred space but congruent in external space. These findings suggest that the visual-tactile correlation causing the rubber hand illusion is computed within a hand-centred frame of reference.

In addition to the hand-centred spatial congruency rule, embodiment is also affected by whether the rubber hand is representing a right or

left limb: when there is a mismatch between the two, the strength of the illusion is diminished. Moreover, this study also reveals that embodiment can be seen at the level of fingers on the hand: when participants are asked to locate their fingers to measure proprioceptive drift, they tend to exhibit this drift only for the finger that was stroked in synchrony during the experiment. Even when two fingers received an equal amount of tactile stimulations, it was only the felt position of a synchronously stimulated finger that drifted significantly toward the rubber hand (Tsakiris & Haggard, 2005).

Finally, the rubber hand must also be located in a certain spatial radius to be embodied. Studies investigated the spatial limits affecting the strength of the rubber hand illusion by manipulating the distance between the rubber hand and the real hand of the participants. These findings reveal that the rubber hand is generally embodied in a radius of about 30 cm, with the strength of the illusion being reduced significantly after that. The strongest scores are seen at 17.5 cm, although the illusion can still be experienced at a distance of 27.5 cm. This has been shown in both horizontal and vertical settings of the rubber hand illusion (Kalckert & Ehrsson, 2014b; Lloyd, 2007).



Spatial reference frame for stimulus matching	Hand space	Viewer-centred space	Hand space	Viewer-centred space	Hand space	Viewer-centred space
Stimulation match	Mismatch	Mismatch	Match	Mismatch	Mismatch	Match
Postural representation match	Match	Match	Mismatch	Mismatch	Mismatch	Mismatch

Figure 1.10: Investigation of the effect of postural and tactile mismatch on the RHI (Costantini & Haggard, 2007).
Hypothesised interactions between variation of stimulus angle in regards to hand-centred representation and subject's postural representation.
(Stroking mismatch) The subject's hand receives a stimulation with a 10° angle compared to seen stimulation on the rubber hand.
(Postural mismatch) The subject's hand is placed at an angle.
(Postural plus stroking mismatch) Combines both conditions. Mismatch in both stimulation and postural representation would reduce the illusion more than mismatches in stimulation alone and in postural representation alone.

1.2.1.2 Anatomical congruency

The anatomical congruency rule explores the limits of artificial limb embodiment by studying the impact of the similarity between the artificial and the physiological hands in the rubber hand illusion.

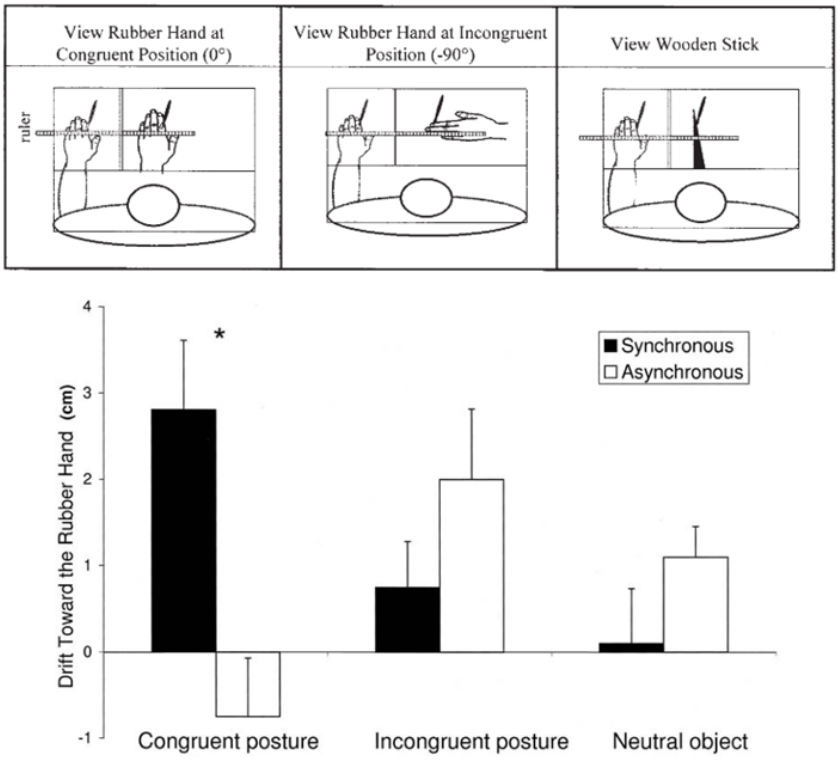


Figure 1.11: Effect of posture and anatomical congruency on proprioceptive drift (Tsakiris & Haggard, 2005).
(Top) Experimental setup. Participants saw in different conditions: a rubber hand in a congruent posture, a rubber hand in an incongruent posture, or a wooden stick. The participant's left hand was out of sight for the whole duration of the experiment. The rubber hand or the wooden stick appeared, aligned with the participant's midline, only during the stimulation and disappeared during the judgment period.
(Bottom) Proprioceptive drift is stronger during synchronous stimulations of a rubber hand in a congruent posture compared to incongruent posture or the stimulations of a wooden stick.

A classic way this is studied is by replacing the rubber hand by a wooden block or stick and observing the strength of the illusion

produced. These studies show higher embodiment scores for a rubber hand resembling a human hand compared to the wooden block/stick (Figure 1.11). This demonstrates the importance of the shape of the object being embodied which must fit with a reference model of the body (Finotti et al., 2023; Tsakiris, 2010; Tsakiris & Haggard, 2005).

Although the general shape of the hand seems to be important to elicit ownership towards the artificial limb, other aspects such as the material composition of the hand seem to have a lower impact. This illusion has been reproduced with rubber hands; metallic robotic hands (Rosén et al., 2009); wooden hands (Abdulkarim et al., 2023) and different hand designs in VR environments (Zhang et al., 2023).

1.2.1.3 Temporal congruency

The temporal congruency rule stipulates that there needs to be synchronous timing between the stimulation of the rubber hand and the real hand to create the illusion.

Indeed, applying asynchronous stimulations to both limbs is a classic control used in the RHI that was already introduced in the original paper but with no precision about the delay introduced (Botvinick & Cohen, 1998).

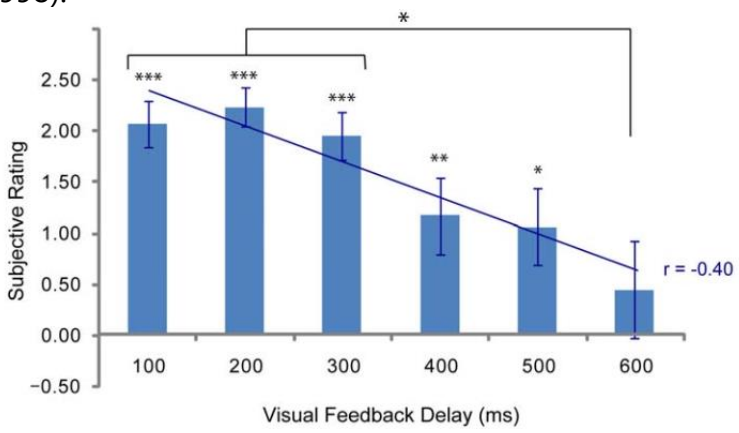


Figure 1.12: Rubber hand illusion strength decays with temporal delays (Shimada et al., 2009).
Subjective rating of the body ownership questionnaire for each temporal delay shows a decrease of illusion strength when the temporal delays between touch and visual input increase.

Additional studies have elucidated the delays necessary to provoke an interruption of the illusion. A study investigating the effect of increasing delays between the tactile stimulations of the rubber hand and real hand on the strength of the illusion, showed that subjective ratings of ownership remain significantly higher than a control, up to 300 ms delays, and decrease drastically after (Figure 1.12) (Shimada et al., 2009).

1.2.1.4 Tactile congruency:

The tactile congruency rule stipulates that the rubber hand illusion occurs when there is a correspondence between the texture of the object stimulating the hand and the one applying the visual stimulus on the rubber hand. Although this rule was not extensively explored, studies suggest that participants have expectations about the type of tactile stimulations they expect to feel when watching the rubber hand being stimulated by a pencil vs a soft brush.; And so when these two stimulations do not match, the embodiment is diminished (Filippetti et al., 2019; Ward et al., 2015).

1.2.2 Embodiment in non-human animals

The rubber hand illusion has proven to be a powerful tool in exploring various modalities of embodiment and understanding the intricate processes by which our brains integrate sensory information to produce the experience of owning a body part. However, while the rubber hand illusion has provided valuable insights, translating this paradigm to animal research offers an exciting opportunity to expand our understanding of embodiment using the experimental opportunities available only in animal studies. Translating the principles of this illusion to animal research not only broadens the scope of inquiry but also offers unique opportunities to explore neural and behavioural correlates of embodiment in different contexts in ways that are not feasible in human studies.

The first study exploring this phenomenon in non-human animals exposed two rhesus macaque monkeys to a visual stimulus of a virtual avatar arm resembling a monkey's upper limb (Shokur et al., 2013a). The virtual avatar was touched by a ball while the monkeys' arms were

stimulated by a brush at the corresponding area on their arm either synchronously or asynchronously, or only exposed to the visual stimulus of the avatar being touched. The monkeys were implanted with microwire arrays in M1 and S1 cortical neuronal ensembles. These findings show an increase of neuronal firing in S1 and M1 following synchronous visual and physical touch. This also included a response to the visual stimulation with no physical touch but only after synchronous physical stimulations. It is important to note that this is unlikely to be a mirror response as the visual only stimulation with no synchronous physical touch does not produce this activity. We have also observed similar correlates in a human fMRI study of the rubber hand illusion where we found an increased activity in S1 when participants reported ownership and agency towards the artificial hand (Abdulkarim et al., 2023). The monkeys' experiences demonstrate that synchronous visual and tactile stimulation can induce specific sensory-motor correlates that parallel the embodiment expected in the rubber hand illusion paradigms.

The sense of embodiment has also been explored by a few studies in the mouse model. In the first study reporting this "Rubber tail illusion" phenomenon (Wada et al., 2016), authors exposed mice to a fake tail while their real tail was hidden from their view. Tactile stimulations were then applied to both tails with the classic synchronous or asynchronous conditions usually used in this paradigm. At the end of the stimulations, the experimenter grabbed the fake tail to measure an affective response to the threat of this artificial limb, which would indicate the degree of embodiment towards this object. More specifically the authors observed the head movements and retractions of mice and established scores based on the intensity of these movements. They found that mice have a stronger response to the grasp of the fake tail after synchronous stimulations compared to the asynchronous control (Figure 1.13). This is similar to what is observed in human experiments where subjects show greater skin conductance responses and anxiety-like behaviour to the threat of an embodied rubber hand after synchronous stimulations (Armel & Ramachandran, 2003). This paradigm was the first evidence of the possibility to study an embodiment-like behaviour in mice.

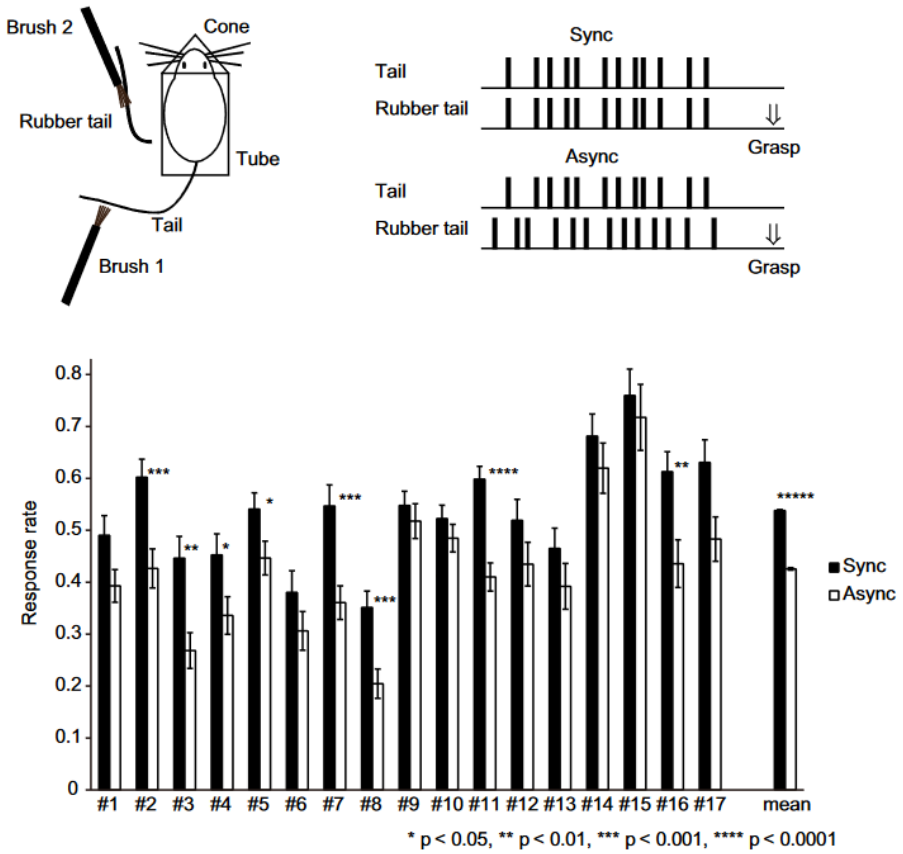


Figure 1.13: The rubber tail illusion: Fake limb embodiment can be elicited in mice (Wada et al., 2016).

(Top left) The rubber tail task. The mice were placed in a tube. An operator applied brush trokes both on the real tail that the mouse could not see, and on a fake, rubber tail that was visible to the mouse. After this pairing step, the rubber tail was grasped and the mouse reaction was assessed by the operator.

(Top right) The mice performed daily tests under two conditions: real tails and rubber tails were stroked synchronously or asynchronously.

(Bottom) Response rates (head movement) when the rubber tail was grasped. The bars indicate the mean response rate under each condition from the responses obtained over 10 d of testing (mice 1–17, synchronous condition, 71.7 6.0 trials; asynchronous condition, 70.6 5.5 trials). Error bars indicate SEM.

In a subsequent study, the same authors reused the Rubber tail Illusion protocol described above, and explored how embodiment is affected in an animal model for autism (Wada et al., 2019). In this study the authors compare the rubber tail illusion response in wild type mice and Caps2-KO mice which exhibit autistic-like phenotypes. They found the response rates to the threat of the rubber tail to be significantly lower

in the Caps2-KO mice compared to the wild-type mice. These findings mirror the results from human experiments that have explored how the sense of body ownership is modified in autism. Studies exposing individuals with autism spectrum disorder to the rubber hand illusion protocol showed that these participants experience certain aspects of the illusion differently such as less sensitivity to visuotactile-proprioceptive discrepancy, more accurate proprioception, or delayed effect of the visuo-tactile stimulation (Cascio et al., 2012; Paton et al., 2012).

Finally, the rubber tail illusion protocol has also been used in another study by different authors (Buckmaster et al., 2020). In this particular study, mice were exposed to the same protocol as the Wada papers, with the exception of a different control than the asynchronous stimulations: here instead, the control used was a condition where the tactile stimulation on the rubber tail was mimicked, without actual contact, while the real tail of the animal did not receive any stimulation, which is a similar condition to the visual only stimulation of the Shokur et al. 2013 study introduced in the first paragraph of this chapter. In this last "rubber tail" study, the authors found a significant reaction to the pinch of the rubber tail after synchronous stimulations compared to the mimicked condition.

Moreover, they also reported sex differences: indeed, when comparing the effect of slow and fast stroking on the strength of the illusion they found that slow stroking enhanced the rubber tail illusion in female mice specifically. Although the control condition used in this publication differs from the standard asynchronous condition, these results extend on the findings in Wada's research which was only on male mice, by showing that both sexes in mice can respond to this paradigm. The results on sex differences for slow stroking reflects tactile manipulation associated with affective touch. These findings remain exploratory as no similar effects have been reported on the human literature of the rubber hand illusion. Note however, that consistent with this finding, women tend to rate slow stroking as more affectively pleasant than men (Jönsson et al., 2017).

1.2.3 The rubber hand illusion to study prosthesis integration

The rubber hand illusion provides a relevant platform for the study of embodiment in both humans and certain non-human animals. This paradigm is particularly interesting in the field of prosthetic development where researchers are trying to understand how we can best integrate a prosthetic limb to the body schema of amputated patients. Indeed, several studies have explored the possibility of reproducing this paradigm in amputees and further our understanding of prosthetic embodiment after limb loss (Castro et al., 2023).

One of the studies that investigated this exposed 18 upper limb amputees to a modified version of the rubber hand illusion (Ehrsson et al., 2008). Participants were exposed to a prosthetic limb while their residual limb was hidden from their view. The experimenter then applied brush strokes to the rubber hand and to the stump of the subject. In the first experiment (Figure 1.14A,D) the participants had to rate the subjective ownership of the limb in 3 different conditions: First, in the "stump condition," they brushed the stump where the person feels the missing index finger. Second, in the "arm condition" that serves as a control, they brushed the back of the lower arm on the opposite side of the body at a spot corresponding to the missing finger's location. Finally, in an additional control, the "finger condition," they brushed the index finger of the rubber hand and the non-amputated hidden hand to see if the amputees could experience the classic rubber hand illusion. This experiment reveals that amputees can experience the rubber hand illusion after congruent stimulations of the residual limb as they report feeling ownership towards the prosthetic hand in the stump condition more strongly than in the control condition (Figure 1.14D). Although, it is important to note that the “finger” condition where participants are exposed to the classic rubber hand illusion is the one where they report the highest illusion scores. A second and third experiment were used to measure other elements of the illusory experience, mainly the proprioceptive drift and the skin conductance response after threat of the rubber hand (Figure 1.14E-F). Both these experiments revealed higher responses after synchronous stroking of the stump along the rubber hand when compared to the asynchronous control used in these subsequent experiments.

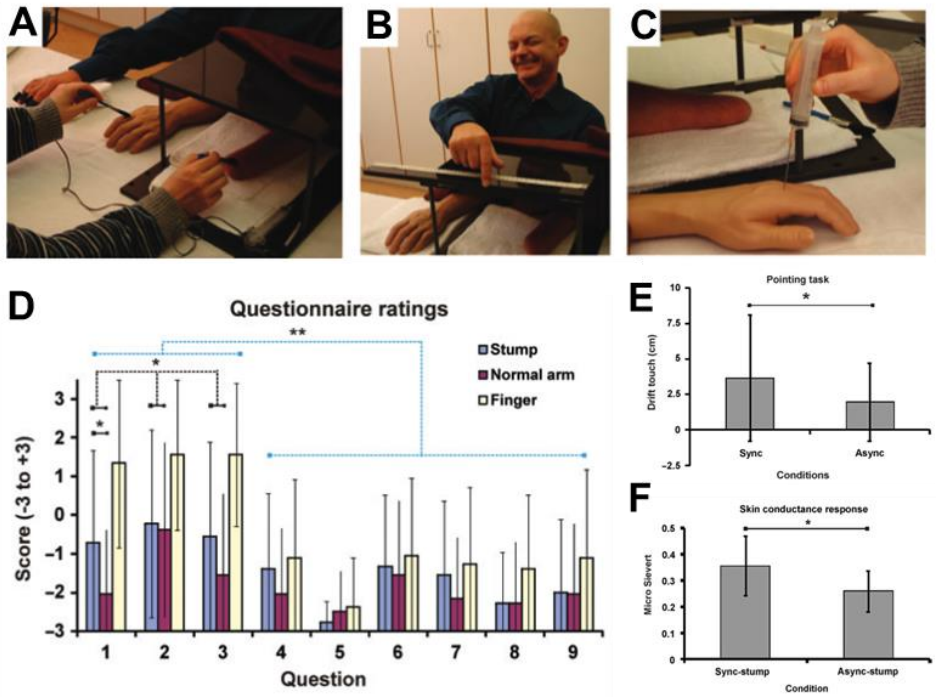


Figure 1.14: **The rubber hand illusion can be elicited in amputees by stimulating their stump (Ehrsson et al., 2008).**
(A) Rubber hand illusion protocol in amputees: the residual limb is stimulated in synchrony with the rubber hand.
(B) Participants performing a self-localization task of their residual limb to measure proprioceptive drift.
(C) Rubber hand is threatened by a needle at the end of stimulations and skin conductance response is measured.
(D) Subjective ownership of the rubber hand is higher during synchronous stimulations of the stump: The responses to Questions 1-3 reflect the experiences of the illusion. The responses to Questions 4-9 served as controls for suggestibility and task compliance.
(E) Higher proprioceptive drift during synchronous condition.
(F) Higher skin conductance response during synchronous condition as a response to the threat of the prosthesis.

In a similar experiment, this paradigm has also been reproduced with a robotic arm in place of a rubber hand, with 5 participants giving high ratings of embodiment of the robotic limb after the synchronous brushstrokes were applied compared to the asynchronous brushstrokes. The results also showed higher proprioceptive drift towards the prosthetic arm. In this study, the authors performed an additional experiment. A subset of participants could control the robotic arm through EMG signals recorded from surface electrodes on

the stump. In this condition, participants report higher illusion ratings compared to the condition that only provides somatosensory information (Rosén et al., 2009).

This paradigm has also been reproduced in an fMRI study where 2 amputees were exposed to the same protocol with synchronous or asynchronous brush strokes on the stump and rubber hand (Schmalzl et al., 2014). Similar reports of subjective ownership towards the rubber hand have been observed in this experiment. Additionally, these findings reveal a stronger activation of dorsal and ventral premotor cortex, as well as intraparietal sulcus during the synchronous condition.

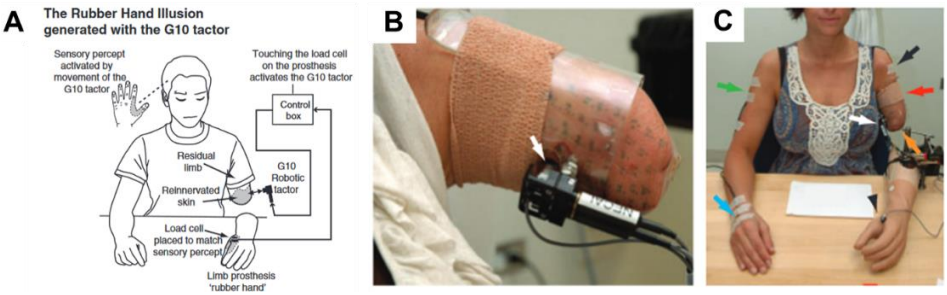


Figure 1.15: The rubber hand illusion recreated through pressure sensors giving an artificial tactile information on the stump of amputees (Marasco et al., 2011).

(A) Schematic diagram of the experimental setup for the Rubber Hand Illusion where the G10 tactor was used to provide a physiologically relevant artificial sense of touch for a prosthesis. The illusion was generated on the amputated side of the subjects by having them watch the investigator touch the prosthetic hand and load cell while the G10 tactor pressed into the reinnervated target skin of the residual limb.
(B) The placement of the G10 tactor on the reinnervated skin of Subject S-2. The plunger (white arrow) pushes into a region of skin where she feels sensation projected to the dorsal skin between digits 1 and 2 of her missing hand.
(C) The placement of the prosthetic limb on the table in front of Subject S-2. The amputee fine-positioned the arm where it felt most natural. The G10 tactor can be seen on the inner aspect of her residual limb (white arrow) and the load cell that provides touch input to the G10 tactor can be seen placed in the centre of the projected field of sensation (black arrowhead). Coloured arrows mark the location of each thermistor: proximal residual limb (black arrow), mid residual limb (red arrow), distal residual limb (orange arrow), proximal intact limb (green arrow), intact hand (blue arrow).

These results align with the literature exploring neural corelates of the rubber hand illusion in healthy subjects where the same brain regions have been reported to be involved (Ehrsson et al., 2004).

Other methods have been used to generate embodiment of a rubber hand in amputees. Marasco et al have used pressure sensors to provide an artificial sensory input to 2 targeted reinnervation amputees. The authors used a load cell allowing the captures of touch input on the rubber hand that is then transmitted to a G10 tactor placed on the patients' stump. The G10 tactor then displayed this touch input as a proportional pressure on the reinnervated residual limb. The results from both subjective questionnaires as well as temporal order judgements and residual limb temperature measurements indicate that congruent tactile feedback from a prosthetic limb drives a perceptual shift towards embodiment of the device for these amputees (Figure 1.15)(Marasco et al., 2011).

Vibrotactile stimulation of the phantom finger on the stump can also be used to generate embodiment towards a prosthetic limb. In a study with 9 upper limb amputees, authors applied tactile stimulations on the rubber hand in synchrony with the vibrotactile stimulations. This paradigm also succeeded in provoking the illusion in these participants as seen with higher ownership ratings, and higher skin conductance response and proprioceptive drift (D'Alonzo et al., 2015).

Finally, more invasive methods have also been used to generate the rubber hand illusion in amputees: Rognini et al used nerve stimulations in patients implanted with transverse intrafascicular multichannel electrodes. Both patients reported higher ownership towards the rubber hand during the synchronous test condition and even report a reduction of the abnormal phantom limb experience of telescoping (Rognini et al., 2019). In a similar protocol, Page et al used microelectrode arrays implanted in the residual median and ulnar arm nerves and intramuscular electromyography recording leads implanted in residual limb muscles, to allow sensory feedback and motor control. The participants show signs of embodying the prosthesis in an open-loop motor, open-loop sensory and closed-loop conditions. They also report a reduction of phantom pain symptoms (Page et al., 2018). Similarly, a study by Collins et al 2017, the authors used electrocorticography (ECoG) to demonstrate that electrical stimulation of the somatosensory cortex can induce a sense of ownership of an artificial hand in two human subjects. By synchronizing the electrical stimulations with tactile input on a rubber

hand, they created the illusion of ownership. The effect did not occur when stimulation was asynchronous or targeted a different body part. This suggests that this type of brain stimulation can be used in some cases to induce temporary ownership over an artificial limb (Collins et al., 2017).

In conclusion, different methods can be used to induce the illusion of ownership in amputees via peripheral stimulations of the residual stump.

However it appears that the classic rubber hand illusion caused by stimulation, triggered by touch on an intact limb, is stronger than the illusion generated in the stump (Ehrsson et al., 2008). This may be due to the difficulties in accurately mapping sensations on the residual limb and the neural reorganization that occurs after amputation. This limitation on the prosthesis embodiment that was obtained by targeting the stump suggests that, while these methods can partially recreate the illusion of limb ownership, more invasive approaches may be necessary to achieve a stronger and more consistent sense of embodiment in amputees. For instance, techniques involving direct neural interfaces or advanced sensory feedback systems have shown promise in enhancing the illusion's strength but also in having the potential to alleviate phantom limb pain and improve overall prosthetic integration.

By focusing on these invasive strategies, future research and development on embodiment may better address the unique challenges faced by amputees, leading to improved outcomes in prosthetic use and integration.

1.3 STRATEGIES FOR NEUROPROSTHESIS DEVELOPMENT

1.3.1 Introducing Promice

Research in the field of neuroprosthesis development calls for the study of embodiment to help in the integration of external devices into one's body schema. Understanding how to achieve seamless control and acceptance of these devices is crucial to improve patients' life quality and comfort.

Prosthetic limb development is a diverse and evolving field, encompassing a wide range of technologies and approaches to restore functionality for individuals with limb loss or paralysis. Upper limb prostheses vary significantly, from simple mechanical to sophisticated myoelectric devices and systems controlled by direct cortical readout of neuronal activity (Bensmaia & Miller, 2014). Such invasive brain-machine interfaces based on chronic recordings of neuronal activity are a promising technology that opens new possibilities for tetraplegic patients to regain mobility and autonomy.

However, the development and refinement of invasive neuronal recording technology requires comprehensive fundamental research to ensure safety, efficacy, and functionality. So far, this type of research is carried out on humans or non-human primates as, despite the advances in this domain, there are virtually no non-primate animal models that comprehensively mimic human prosthetic use, making research in this area particularly challenging, especially for the development of invasive interfaces. Thus, developing a research model in mice in this field can be crucial for several reasons:

Firstly, the use of mice allows for high-throughput studies and the application of advanced genetic tools first available in mice, such as optogenetics and transgenic models for brain imaging, to precisely manipulate and monitor neuronal activity (Kim et al., 2017; Madisen et al., 2012; Piatkevich & Boyden, 2024).

Moreover, mice models enable the study of various neurological conditions and injuries. In humans, there is an important variability in these medical conditions, such as differences in the extent and location

of neural damage (Feinberg et al., 2010). This variability can complicate the interpretation of results and the assessment of treatment efficacy. By using mouse models, we can minimize these variables and create more consistent and reproducible experimental conditions, therefore allowing for a more controlled environment to study the mechanisms underlaying the successful use and integration of a prosthetic limb.

Additionally, mice are a particularly interesting model for upper limb neuroprosthetic research as they display a rich array of forelimb behaviours, allowing for detailed analysis of motor control and dexterity (Whishaw et al., 2017).

And, as mentioned previously, we can also translate human rubber hand illusion experiments to the mouse model which allows us to study the embodiment of an artificial limb in mice using the diverse behavioural and genetic tools offered by this model (Buckmaster et al., 2020; Wada et al., 2016, 2019).

Brain-machine interfaces developed in rodents typically focus on controlling robotic devices to deliver water rewards (Arduin et al., 2013, 2014; Chapin et al., 1999) or on controlling virtual cursors (Lassagne et al., 2022). We wanted to develop a prosthetic limb that closely resembles the paw of the animal, allowing us to explore questions related to more naturalistic limb control and embodiment. It is in this context that we have developed "Promice" a motorised neuroprosthesis for the mouse model (Figure 1.16A).

During my PhD, I supported the design and prototyping of this robotic arm. This involved extensive 3D printing to create and refine various models of the prosthesis. I explored different skin options, including rubber, real fur, and artificial fur, to enhance the similarity of the prosthesis to a physiological mouse limb. Additionally, I redesigned 3D models to optimize their functionality and tested various types of cables to enhance the movement of the limb. Description of the work I carried is provided in the Methods and Results part of the thesis, section *2.3 Motorized prosthesis*.

Edouard Ferrand (PhD student in the laboratory) has taken over this effort and fully developed the mouse scale prosthesis, which features

a 2 cm forearm length. The device has 3 bones that allow movements with 4 degrees of freedom and is covered with 3D printed rubber skin. Mice are implanted with chronic electrodes in the M1 cortex in the region associated to the forelimb. The neuron's firing rate is used to make a normalized command that is then translated to the motors controlling the prothesis. Each neuron controls the speed of the prosthesis on one axis: for a 3D control, 3 neurons are recorded to make the command (Figure 1. 16B), 1D and 2D controls are also possible with the corresponding number of neurons used for each.

Mice were then trained to modulate the activity of these neurons to control the prosthesis and use it to solve a task. In this task, the water restricted mouse has to move the prosthesis from a water cup to its tongue to be able to obtain a water reward (Figure 1.16B). Preliminary results show that mice can learn to solve this task as shown by an increase of the lick frequency, and more importantly, the increase of the number of back and forth movements between the water cup and tongue (Figure 1.16B-D).

Publication of the design of the prosthesis and its control will be soon submitted for publication (manuscript in preparation, Ferrand, Hayatou et al.).

This device built in our lab allows us to tackle different issues related to the development of prostheses controlled by brain machine interfaces with the flexibility of the mouse model. In particular, it allows to explore different strategies for upper limb motor control strategies. It can also be used to explore the modalities of somatosensory feedback and develop a bidirectional upper limb control strategy by connecting the prosthesis to the closed loop brain machine interface that has been developed by the team in the last years (Abbasi et al. 2018, 2023). These strategies are actively pursued in the team by several PhD students.

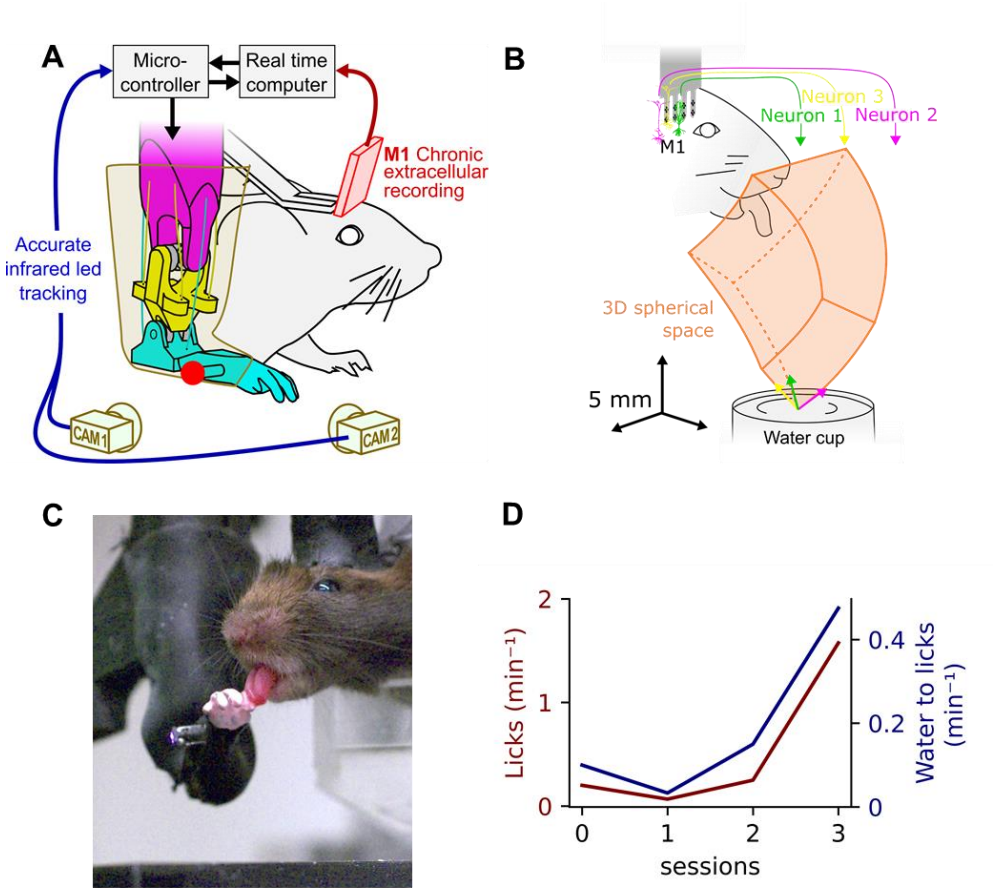


Figure 1.16: ProMice: a forelimb neuroprosthesis for mice (Adapted from a Poster for the joint Chen Institute and NeuroPsi conference: (E. Ferrand, z. Hayatou et al. 2024).

(A) Schematic of the BMI-controlled prosthesis. The prosthesis has 4 degrees of freedom. Infrared led allow for accurate tracking of the device.

(B) A mouse trained to control the prosthesis learns to recuperate a water reward using ProMice.

(C) Behavioural task requiring the motor control of the prosthesis: neuronal activity is recorded via chronic electrodes implanted in the M1 cortex of the mouse and used to control the speed of the prosthesis on each axe of a 3D subspace. The mouse has to move the prosthesis in this space to obtain a water reward.

(D) Example of a mouse trained to control the prosthesis in a 3D subspace improving their performance on the task.

1.3.2 The need for somatosensory feedback

The term somatosensation typically refers to the senses of touch, proprioception, pain, and temperature (Goodman & Bensmaia, 2020). It provides vital information that allows an individual to interact with their environment and plays a crucial role in the control and coordination of movement. Indeed, it enables the central nervous system to receive information about the body's position and movements in space, facilitating precise motor control.

1.3.2.1 Effects of lack of somatosensory feedback

As mentioned previously, disruption of somatosensory pathways can have an array of negative impacts on patients. Damage to sensory roots can provoke a complete loss of proprioceptive information, making it impossible for patients to locate their limbs or perform basic tasks such as walking, without having to constantly look at their feet (Sacks, 1985). Report cases have also explored how motor control is affected after acute central deafferentation following resection of S1. In this case, the patient was also incapable of maintaining a constant grip force without continuous visual feedback (Richardson et al., 2016).

Researchers also studied the effect that the lack of somatosensory input can have on fine motor control in healthy subjects. A well-known example is from Johansson's lab, who showed a subject performing a match lighting task in normal conditions and under digital anaesthesia (which does not affect the motor system): we can see in that example that this simple task takes almost 8 times longer for the subject to perform when touch input is disrupted (Johansson, 2005).

Another study exploring the effect of digital anaesthesia on force control revealed some of the mechanisms affected by the loss of somatosensory input (Monzée et al., 2003) (Figure 1.17). The subjects were asked to lift and hold the object stationary for 4 s without and under digital anaesthesia. The goal was to understand how internal models established from previous familiarity can compensate for the loss of somatosensory information. The results showed that, indeed, participants applied a stronger grip force under anaesthesia compared to the control condition (Figure 1.17A). These findings also reveal how

the loss of sensory input causes a delay in the initiation of the lifting and load force by more than 100 ms (Figure 1.17B).

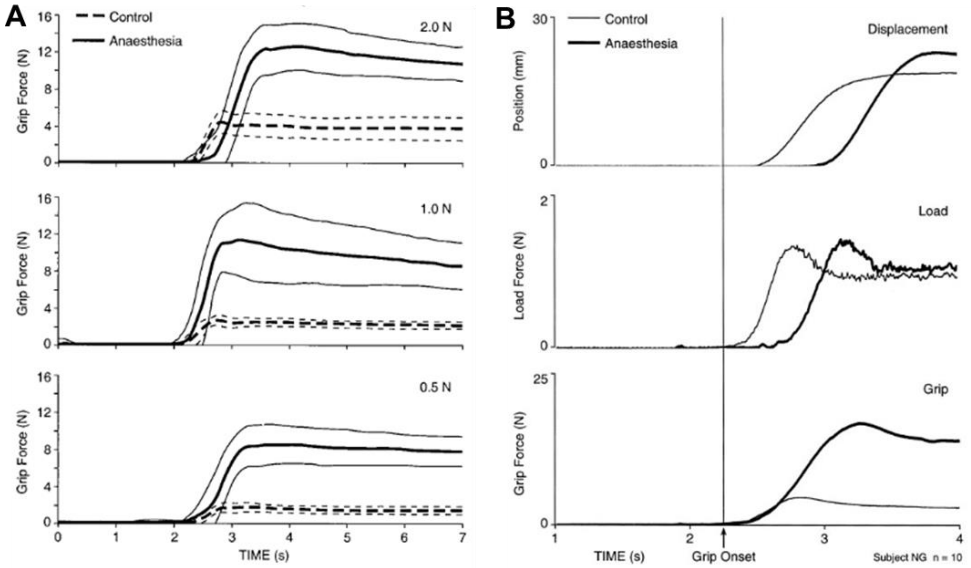


Figure 1.17: Digital anaesthesia disrupts motor control (Monzée et al., 2003).
(A) Effect of digital anaesthesia on the manipulation of 3 different resistive forces simulating object weight for a single subject.
(B) Effect of digital anaesthesia on the synchronization of grasping and lifting.

Similarly, most neuroprosthetic limbs do not provide somatosensory feedback. This limits the quality of the movements that these devices can make. Indeed, patients that use a prosthetic limb often report a lack of somatosensory feedback as one of the reasons they abandon their prosthesis (Maimon-Mor & Makin, 2020).

1.3.2.2 Providing somatosensory feedback via non-invasive strategies

We have seen in a previous chapter that it is possible to provide amputees with non-invasive and invasive somatosensory feedback to provoke the embodiment of a rubber hand. Studies have also explored the effect that this feedback has on different aspects of prosthesis control and integration.

Non-invasive methods of transmitting artificial somatosensory information often include the use of vibrotactile feedback, sometimes electrotactile feedback, to provide touch information and improve prosthesis control (Buratti et al., 2023; Raveh et al., 2018; H. J.

Witteveen et al., 2015; H. J. B. Witteveen et al., 2012). Advancements in haptic technology can also allow the combination of several sensory modalities to further improve feedback accuracy. An example of this, is a haptic device developed by Campanelli et al to provide sensory-motor feedback by combining skin stretching and contact cues. Results indicated that this combined feedback method more effectively conveyed size and stiffness information without visual input and improved fine motor task performance, with visual input, compared with traditional vibration-based force feedback (Campanelli et al., 2024) .

1.3.2.3 Invasive feedback at the periphery can reduce phantom pain and boost prosthesis embodiment

More invasive methods can be used to transmit tactile information from the prosthesis directly to the user's nervous system. By directly interfacing with the nervous system, these methods can provide a more seamless and natural experience, closely mimicking the sensations of a real limb (Bensmaia & Miller, 2014).

A study by Page et al used intramuscular electromyographic implants in the median and ulnar nerve of a patient that was amputated midway along the left forearm to provide tactile information from a prosthetic hand (Figure 1.18) (Page et al., 2018). In this study, neural and electromyography recordings enabled real-time control of a virtual or physical prosthetic hand based on the subject's phantom hand movements (motor-only condition). Sensory feedback was provided through electrical stimulation to evoke percepts corresponding to sensor locations on the prosthetic hand (sensory-only condition). The combined closed-loop condition integrated both motor control and sensory feedback. A control was added where the patient was only required to visually fixate the prosthetic limb. All three test conditions showed an increased embodiment towards the prosthesis especially in the sensory-only condition (Figure 1.18A). The authors also found a significant reduction in phantom limb pain in the test conditions (Figure 1.18B). This data provides insights on the interplay between motor control, somatosensory feedback and the role they can have on prosthetic embodiment and on alleviating phantom pain.

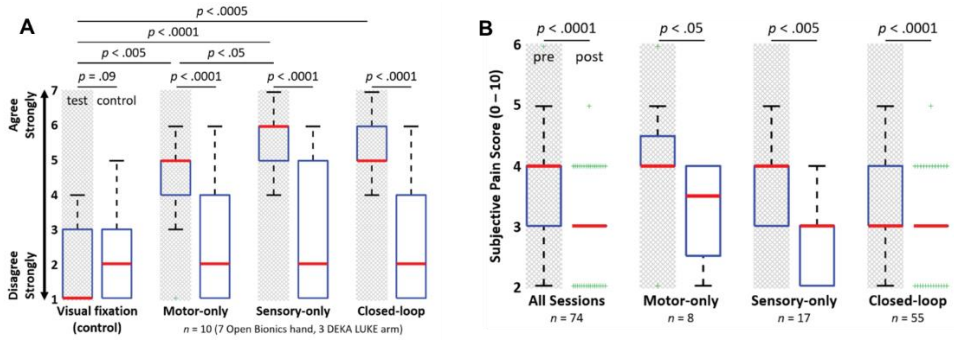


Figure 1.18: *Somatosensory feedback via nerve stimulation allows the embodiment of a prosthetic limb and reduces phantom pain (Serino et al., 2017).*

(A) Survey questions indicate level of embodiment for different conditions. The motor-only, sensory-only, and closed-loop test conditions each exhibited a significantly higher response on the test questions compared with the control questions. Each of the three test conditions (open-loop motor control, open-loop sensory feedback and closed-loop control) showed a greater level of embodiment compared with the visual fixation control condition. The open-loop sensory feedback condition also showed a significantly higher level of embodiment relative to the open-loop motor control condition.

(B) Reduction in phantom limb pain. A significant reduction in phantom pain was observed sessions involving only motor control of a virtual or physical prosthesis, open-loop nerve microstimulation and closed-loop sessions involving motor control and sensory feedback.

Similarly, a study by Petrini et al. has shown similar effects of phantom pain reduction by adding somatosensory feedback to a neuroprosthetic leg. Moreover, the addition of this feedback also increased walking speed and patient's confidence in the prosthesis, and reduced metabolic consumption during outdoor walking (Petrini et al., 2019).

Additional data suggests that somatosensory feedback may play a role in mitigating phantom pain by counteracting maladaptive cortical plasticity typically observed after limb loss (Flor et al., 1995). A study by Serino et al aimed to investigate the effect of motor control and redirected somatosensory stimulation on M1 and S1 remapping which have also been linked to phantom pain (Serino et al., 2017). For this, the authors used fMRI to investigate brain reorganization in amputated patients that have undergone targeted muscle and sensory reinnervation (TMSR). Movement of the prosthesis was enabled via

decoded electromyography activity from reinnervated muscles and touch sensation was enabled by stimulation of the reinnervated skin areas. The study found that M1 maps of the amputated limb in TMSR patients were more similar to those of healthy controls, and differed significantly from non-TMSR patients. S1 maps of TMSR patients also showed a more normal topographical organization compared to non-TMSR patients. However, functional connectivity between S1 and fronto-parietal regions remained reduced compared to healthy controls, underscoring the ongoing challenge in achieving comprehensive sensory integration in prosthetic limbs.

More invasive interfaces that directly target cortical sensory representations could help in achieving better somatosensory feedback, especially when peripheral nerves are not accessible and have been severely damaged.

1.3.2.4 Providing feedback through direct cortical stimulation

Somatosensory feedback can be provided using cortical stimulations. This type of feedback usually relies on intracortical microstimulation which involves direct electrical stimulation of the brain's cortical neurons through implanted microelectrode arrays. This method aims to restore sensory and motor functions by eliciting specific percepts, often in individuals with severe paralysis or sensory loss.

A study by Flesher et al. applied intracortical stimulations to the hand area of the somatosensory cortex in a person with long-term spinal cord injury. This successfully evoked tactile sensations corresponding to specific hand locations (Figure1.19). These sensations, which adhered to somatotopic organization, were reported as naturalistic, were stable over months, and their intensity could be modulated by varying the stimulation amplitude (Flesher et al., 2016).

In another study involving a tetraplegic subject with microelectrode arrays implanted in S1, researchers achieved replicable sensations in both cutaneous and proprioceptive modalities localized to the contralateral arm. The sensations were dependent on the amplitude and frequency of the stimulation, with higher amplitudes consistently eliciting tactile and proprioceptive percepts (Armenta Salas et al.,

2018). Together, these studies show the possibility to provide nuanced, naturalistic sensory feedback by directly targeting the cortical regions associated with the areas of interest.

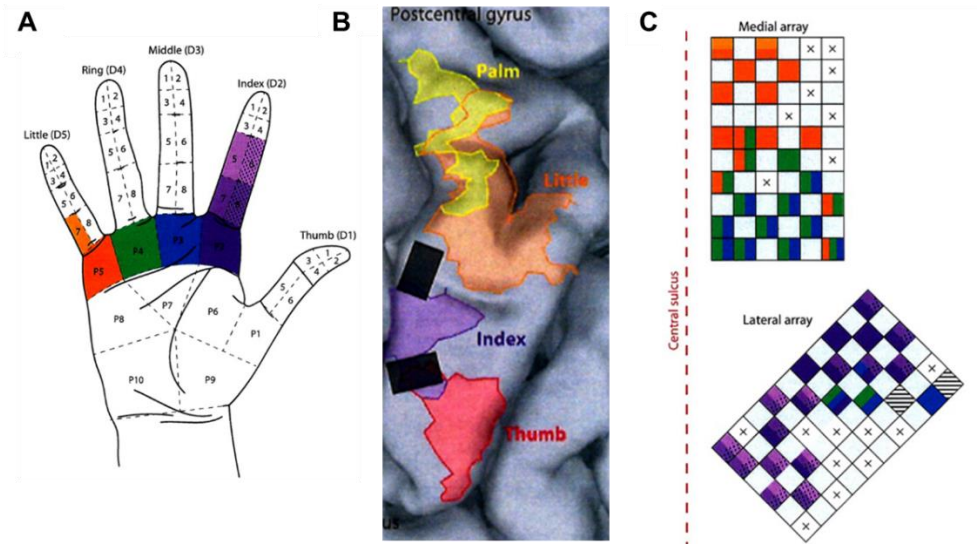


Figure 1.19: Projected field maps of the hand in response to intracortical microstimulation of S1 (Flesher et al., 2016).
(A) Hand diagram shown to the participant during the experiment to point out the regions where they feel the stimulation.
(B) MEG mapping was used to identify the cortical regions that responded to imagined or real somatosensory stimulus.
(C) The two electrode arrays implanted in S1.

Providing this type of feedback through intracortical stimulation has been shown to elicit detailed sensory percepts, allowing texture discrimination, which can be effectively integrated into motor tasks to enhance control and functionality of neuroprosthetic devices. For instance, in a study with monkeys, a brain-machine-brain interface was developed to control the exploratory movements of an actuator and provide artificial tactile feedback via cortical stimulation of S1 (O'Doherty et al., 2011). Monkeys used this system to discriminate between visually indistinguishable virtual objects by identifying unique artificial textures associated with specific temporal patterns of electrical stimulation. This setup demonstrates the potential for motor neuroprostheses to incorporate cortical somatosensory feedback, creating artificial somatic perceptions for more intuitive control of prosthetic devices. Another study by the same authors, further

explored the capacity to convey texture information (O'Doherty et al., 2019). Monkeys scanned virtual gratings with an avatar controlled by a brain-machine interface, receiving pulses each time the avatar's fingertip crossed a virtual ridge. This active tactile exploration allowed monkeys to discriminate between objects, with their accuracy reflecting normal cutaneous sensation principles.

Similarly in humans, Flesher et al. showed in their study, an improvement of robotic arm control by providing tactile sensation through intracortical microstimulation of the somatosensory cortex (Flesher et al., 2021) (Figure 1.20). In this study the tetraplegic patient was implanted with microelectrode arrays in the hand and arm regions of the motor cortex and somatosensory cortex. An observation-based paradigm was used to train a decoder to control the robotic arm in 5 degrees of freedom. The somatosensory stimulation was driven by sensors on the robotic arm that responded to object contact and grasp force. The authors showed that the patient was able to improve his performance in action research arm test (ARAT) where he had to move an object from one starting area to a platform as fast as possible (Figure 1.20A). The subject also solved the task much faster, as seen through a decrease of the time spent for reaching, transport and particularly for grasping which was much easier with tactile input (Figure 1.20B-C).

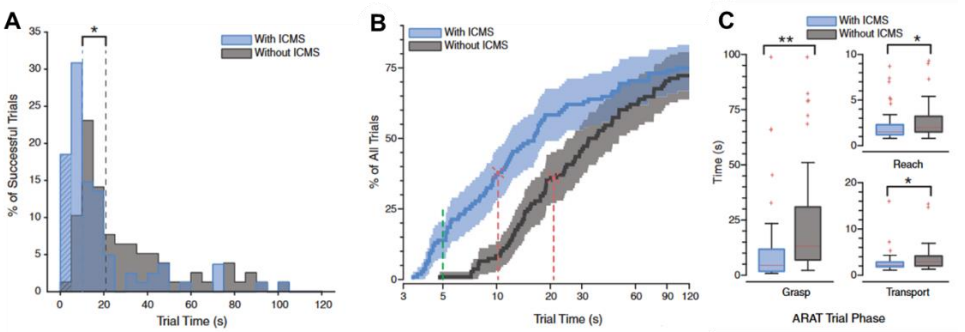


Figure 1.20: Effects of somatosensory feedback on motor performance (Flesher et al., 2021)

(A) Histogram of successful trial times completed with and without tactile feedback. Median trial times (dashed lines) were significantly faster with ICMS.

(B) Empirical cumulative distribution of individual trial times, including failed trials, shown on a log-normalized axis. Vertical red dashed lines indicate when 50% of successful trials were completed. Data to the left of the vertical green dashed line represent trials completed in less than 5 s.

(C) Amount of time spent in each phase of the ARAT task.

In conclusion, the methods outlined demonstrate the importance of integrating somatosensory feedback into prosthetic devices. Studies employing non-invasive approaches like vibrotactile and electrotactile feedback have shown promising results in enhancing motor control and user experience. More invasive methods, such as nerve or intracortical stimulations, have further pushed the boundaries by enabling direct neural interfaces to convey tactile information. These methods have not only improved the patients' performances while using prosthetic limbs but also address issues like phantom pain and maladaptive brain remapping that has been linked to the latter. Incorporating this feedback remains a crucial next step to further enhance the efficacy of prosthetic devices and their acceptance in the body schema of patients.

However, while brain machine interfaces based on intracortical microsimulation have shown great promise and suggest that sophisticated control and detailed sensory feedback is achievable, they can face some significant challenges. The area that can be stimulated is limited, as electrodes cannot be implanted everywhere, and sensory discrimination is not perfect especially in areas close to one another (Flesher et al., 2016). Another significant limitation is the inability to record electrophysiological activity and stimulate electrically simultaneously, necessitating an alternating approach for delivering pulses, which could reduce the efficiency and effectiveness of the system (O'Doherty et al., 2011). Most importantly, the effect of topographic stimulations on sensorimotor tasks hasn't been extensively studied. This is crucial, as a recent study by Abbassi et al. (2023) suggests that topographical targeting to provide somatosensory information may not be sufficient to provide efficient feedback due to the necessity of maintaining spatiotemporal continuity for effective sensorimotor integration. These challenges highlight the need to explore novel technologies, such as optogenetics, which may offer new solutions for advancing brain-machine interfaces.

1.3.3 Optogenetics as a feedback strategy to restore touch input

Studies on the rubber hand illusion enable the manipulation and study of embodiment, and data suggesting its feasibility in mice has been published in recent years. Mice are a valuable animal model to explore the role of somatosensory feedback in embodiment and motor control. Traditional methods to provide somatosensory feedback, such as non-invasive techniques, lack precision for complex tasks, while invasive methods like intracortical electrode arrays are highly invasive and pose challenges with stability and durability. This is where optogenetics presents a promising alternative, allowing precise control of somatosensory inputs with high spatial and temporal resolution, and providing the possibility to target specific neuronal populations, thus overcoming many limitations of other techniques.

1.3.3.1 Potential of optogenetic technology

A key tool in this field is channelrhodopsin-2 (ChR2), a neural-activating cation channel that allows precise activation of neurons with excellent temporal precision on a millisecond scale. This technology enables highly specific control of neural circuits and is widely used in neuroscience research (Madisen et al., 2012).

Studies have used these optogenetic technologies to explore the neural circuits underlying somatosensory perception. One such study demonstrated that photostimulating a subset of layer 4 neurons in the somatosensory cortex can evoke robust sensations of illusory touch and perceptions of object location (O'Connor et al., 2013). To target layer 4 neurons, the researchers used transgenic mice expressing Cre recombinase selectively in layer 4 neurons (Six3Cre34 and Scnn1a-Tg3-Cre), combined with adeno-associated virus expressing ChR2 in a Cre-dependent manner injected in the C2 whisker column which mimicked touch-evoked spiking in L4 neurons (Figure 1.21A-B). The overlap between neurons activated by natural touch and those activated by light suggests that optogenetic stimulation can effectively mimic natural sensory inputs and mice can learn to discriminate object's location using this feedback (Figure 1.21C-D). These findings also revealed that inhibiting activity in these neurons led to a decreased perception of touch.

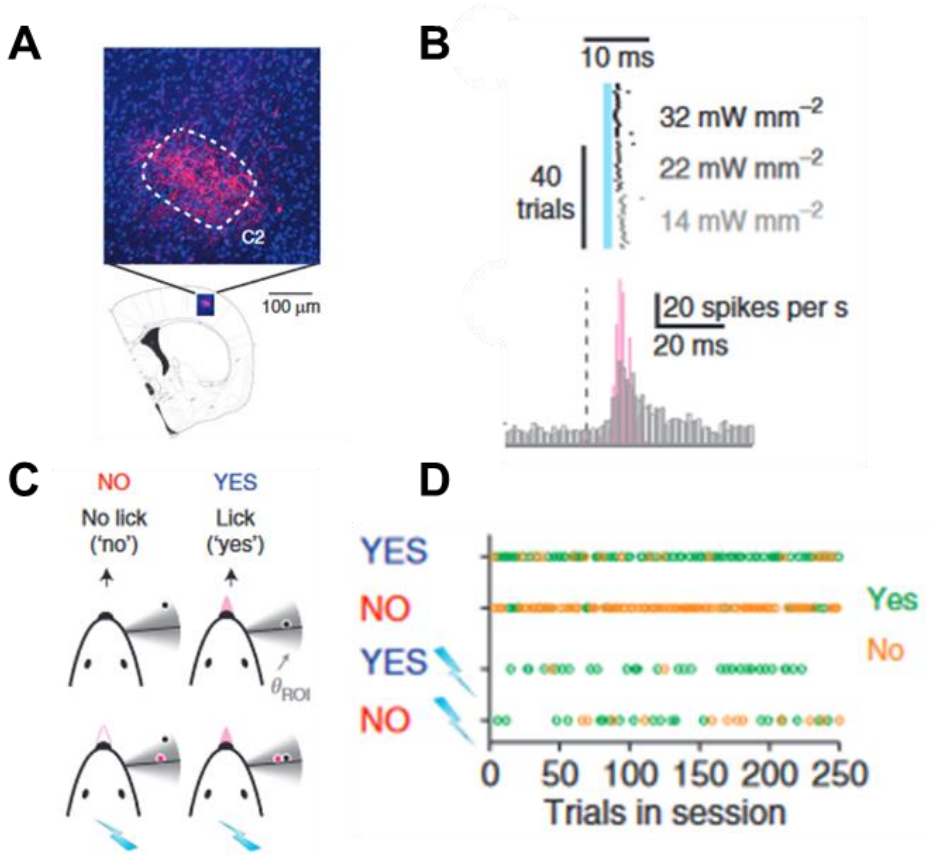


Figure 1.21: Closed-loop photostimulation mimics touch-evoked spiking in L4 neurons and causes illusory perception of object location (O'Connor et al., 2013).

(A) Targeting ChR2 to L4 neurons. ChR2 expression (magenta) in one barrel.

(B) Single example of a neuron responding to different light intensities. Cyan, photostimulus

(C) Four trial types during a photostimulation behavior session depending on pole location and photostimulation (cyan lightning bolts). The virtual pole (magenta) was in the θROI. Mice reported object location by licking or not licking.

(D) Responses in the four trial types across one behavioral session. Green, yes responses; gold, no responses. Responses consistent with illusory touch in the YES location were evoked in about half of the stimulated NO trials starting in the first behavioral session with photostimulation.

There are several strategies to trigger optogenetic activation of neurons. Firstly, two-photon excitation, while highly precise, is limited in its effectiveness, to be integrated into this type of task, due to its capacity to stimulate only limited numbers of single neurons (Kim et al., 2017). This method does not align well with the brain's natural

topography, where groups of neurons work together to produce sensory and motor responses that tend to encompass large areas, as illustrated by the need to implant multiple arrays of electrodes to cover the topographical representations across the cortical region in studies carried in humans (Flesher et al., 2016; Penfield & Boldrey, 1937). In contrast, distributed 1-photon optogenetic stimulations target larger cortical areas, aligning better with the topographic organization of the cortex. This approach can activate entire regions that correspond to specific body parts, providing more naturalistic and comprehensive sensory feedback. By stimulating multiple neurons across a topographically organized area, distributed stimulation can better mimic the natural activation patterns of the brain.

Patterned photostimulations at the mesoscale level have been used to deliver other types of sensory feedbacks such as audition. The authors demonstrated using patterned stimulations that it is possible to elicit precise neural responses in the auditory cortex, crucial for discriminating complex auditory stimuli such as frequency-modulated versus pure tones (Ceballo et al., 2019).

1.3.3.2 A bidirectional brain-machine interface in the mouse model

Similar strategies of patterned photostimulations have been used in our research team to study how the structure of the somatosensory feedback in S1 influences the formation of motor commands.

This interface is based on electrophysiological recordings of whisker M1 neurons (vM1). A craniotomy is performed on mice to expose the somatosensory area associated with the barrel cortex. An optical window is glued to have constant access to the regions of interest for photostimulations. The somatosensory feedback was delivered through optogenetic photostimulations in whisker S1 (vS1) (Figure 1.22A). Blue light patterns are projected (~50 µm resolution) to the mouse's cortex accessible through the optical window using our mesoscale patterning system. This system uses a digital light module and lenses to focus the light. The setup allows for the light patterns to be updated every millisecond (Figure 1.22B).

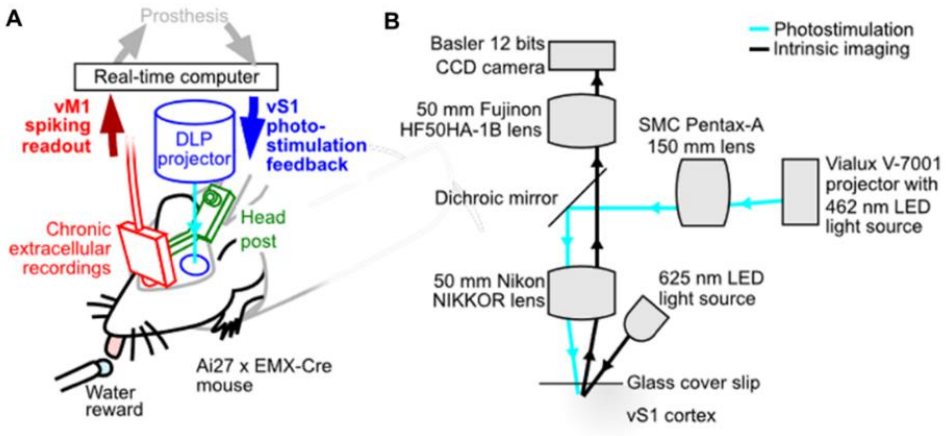


Figure 1.22: **A BMI design including a mesoscopic sensory feedback in vS1 (Abbasi et al., 2018).**
(A) Overall principle of the BMI. Red path: the readout of vM1 spikes. Blue path: Feed-back synthesis and projection in vS1 of an Ai27 x EMX-Cre mouse. Channelrhodopsin is expressed in pyramidal neurons, which are therefore light-triggered.
(B) Optical path of the macroscope used for both vS1 patterned photoactivation and intrinsic imaging.

Lassagne et al. 2022 in our laboratory demonstrated that mice can effectively learn a mesoscale tactile discrimination task using the cortical photostimulation generated by this device over S1, in an open-loop configuration. This study utilized Emx-CreAI27 mice expressing ChR2 in pyramidal neurons across accessible cortical regions, rather than restricting expression to specific whisker columns (O'Connor et al., 2013). This approach allowed mice to track the trajectory of a rotating photostimulation bar within the somatosensory cortex, revealing their ability to integrate optogenetic tactile inputs based on the structure of the photostimulation and topographical consistency (Figure 1.23). These findings underscore the potential of optogenetic somatosensory feedback to be integrated into closed-loop configurations to enhance motor control performances.

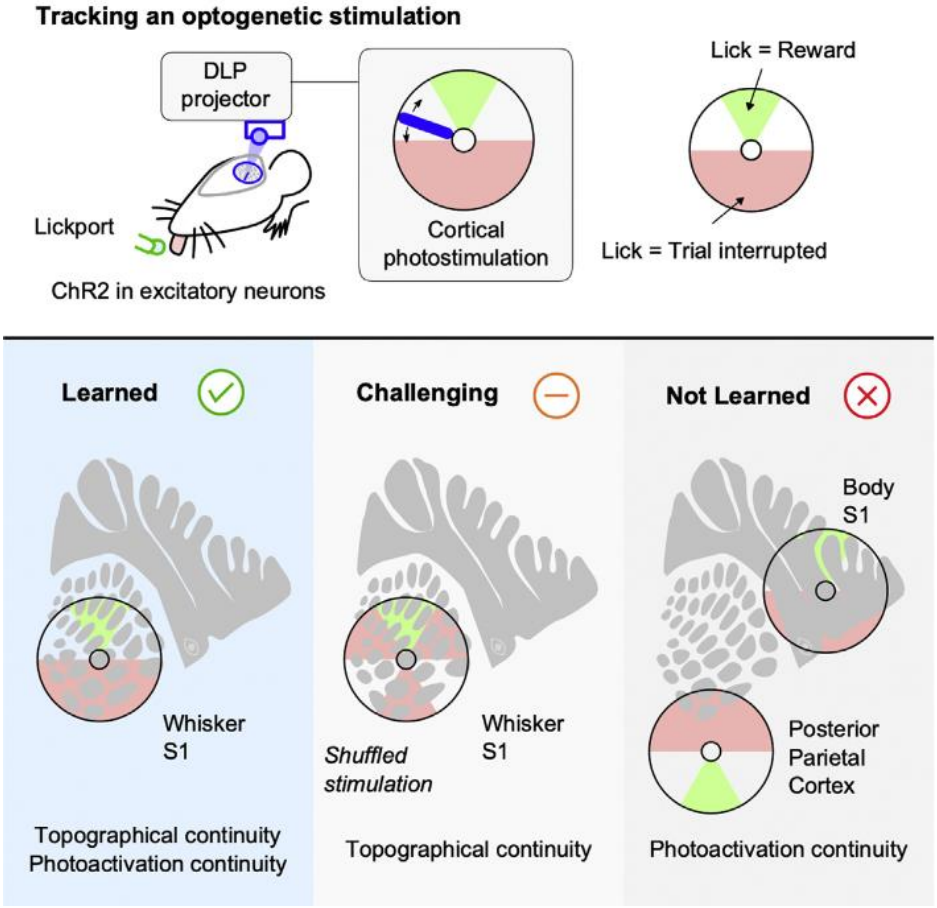


Figure 1.23: Mice learn how to track a cortical optogenetic stimulation (Lassagne et al., 2022).
(Top) Photostimulations are applied to the whisker region of S1 in the form of a mesoscale rotating bar (500 µm long) that rotates in a circular space. Mice get a water reward when licking in the green rewarded area, while the trial is reset if the mice lick in the red area.
(Bottom) The mice manage to learn this task when the photostimulation is applied in the barrel cortex, a cortical somatosensory region that features a continuous topography (left). In contrast, the mice failed to learn efficiently the task when the spatiotemporal continuity of thefeedback was disrupted (center). They also failed the task when the feedback was provided on a non-topographical area of the cortex — the Posterior Parietal Cortex.

To this aim, in a related study, Abbasi et al trained mice to modulate M1 activity to control a virtual cursor (Abbasi et al., 2023). The results revealed that mice could learn to modulate their vM1 firing rates to manipulate the virtual bar effectively, thereby activating targeted photostimulation in vS1 (Figure 1.24A-D).

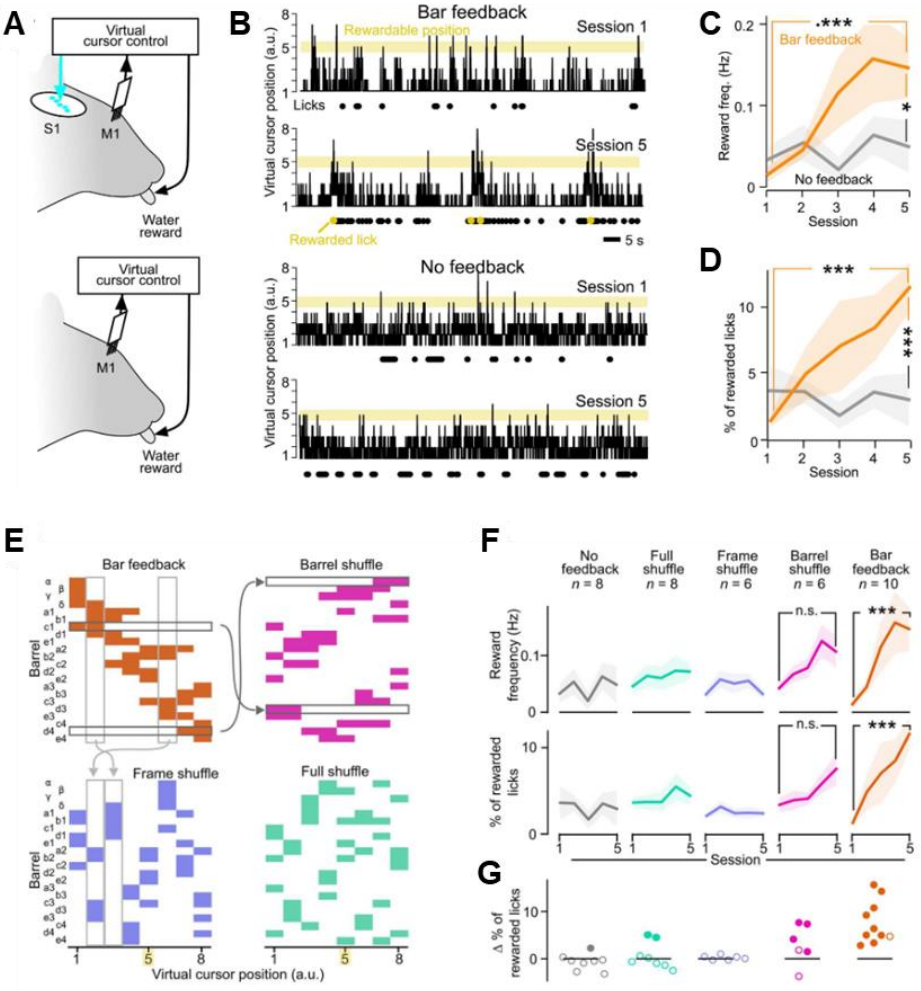


Figure 1.24: Sensory feedback of S1 enhances task performance and disrupting the spatiotemporal structure of the bar feedback impairs learning (Abbasi et al., 2023).

(A) Schematic of the Bar feedback and No feedback conditions.

(B) Position of the virtual cursor computed from the merged activity of the Master neurons, in the first versus the fifth training session of one mouse, in the Bar feedback condition (top) and in the No feedback condition (bottom) (100 s displayed). Yellow background, rewardable position; black dots, lick times; yellow dots, rewarded lick times.

*(C) Performance quantified by the average frequency of rewards per session across training, comparing the Bar feedback condition (orange, 10 mice) and the No feedback condition (gray, 8 mice). Shaded backgrounds: ±SEM. *P < 0.05 and ***P < 0.001, nonparametric Mann-Whitney tests.*

(D) Same as (C) for the specificity of licking, quantified as the proportion of rewarded licks among all licks, across behavioural sessions.

(E) Spatial and temporal structure of the feedback across frames in the four tested conditions. Horizontal arrows, barrel identity permutation to generate the Barrel

shuffle from the Bar feedback; vertical arrows, frame identity permutation to generate the Frame shuffle from the Bar feedback; yellow highlight, rewardable virtual cursor position.
*(F) Reward frequency (top) and percentage of rewarded licks (bottom) of the mice over five training sessions. ***P < 0.001, nonparametric Mann-Whitney tests. n.s., not significant. Shaded backgrounds: ±SEM. Bar feedback and No feedback data are the same as in Fig. 2.*
(G) Difference between the proportion of rewarded licks of the mice between the first versus fifth training session. Each point represents a mouse (arbitrary order). Filled point: bootstrap significance test, P < 0.05. Colors refer to the feedback conditions defined in (E).

This study found that performance was highest when feedback provided the cursor's position as a bar-like photostimulation across the cortical surface. Disrupting the spatial contiguity of simultaneously stimulated barrels or the continuity of the bar in time, significantly reduced learning and performance (Figure 1.24E-G). This not only highlights the importance of somatosensory feedback in shaping motor behaviour. It also suggests that topographical targeting alone may not be sufficient to provide effective feedback in sensori-motor tasks, emphasizing the importance of alternative feedback strategies, which can only be identified through flexible research methods like optogenetics.

Finally, early findings indicate that a mouse can integrate rotary-type optogenetic stimuli into its motor control, akin to proprioception. Our team has developed a similar paradigm to explore the efficacy of a closed-loop optogenetic brain-machine interface for encoding 360° movements of robotic actuators in a prosthesis simulation (Goueytes et al., 2022). Authors tested mice's ability to control a virtual prosthesis joint to solve a rewarded reaching task by modulating M1 neuron activity to adjust joint speed. Continuous optogenetic feedback projected onto S1 provided real-time positional information about the joint's location (Figure 1.25A-B). The results demonstrated that mice took advantage of this rotating cortical feedback, and enhanced their ability to detect reward opportunities, move the joint faster towards rewarded positions, and maintain it within those zones longer compared to conditions without feedback (Figure 1.25C-D). These findings suggest that optimized patterns of cortical feedback can

significantly improve control in brain-machine interfaces, particularly in prosthetic applications involving rotary joints.

During my work in the laboratory, I have been taking advantage of this setup to explore optogenetic strategies to induce embodiment. This work is presented in part *2.2 Embodiment of an artificial limb through direct cortical optogenetic stimulations*.

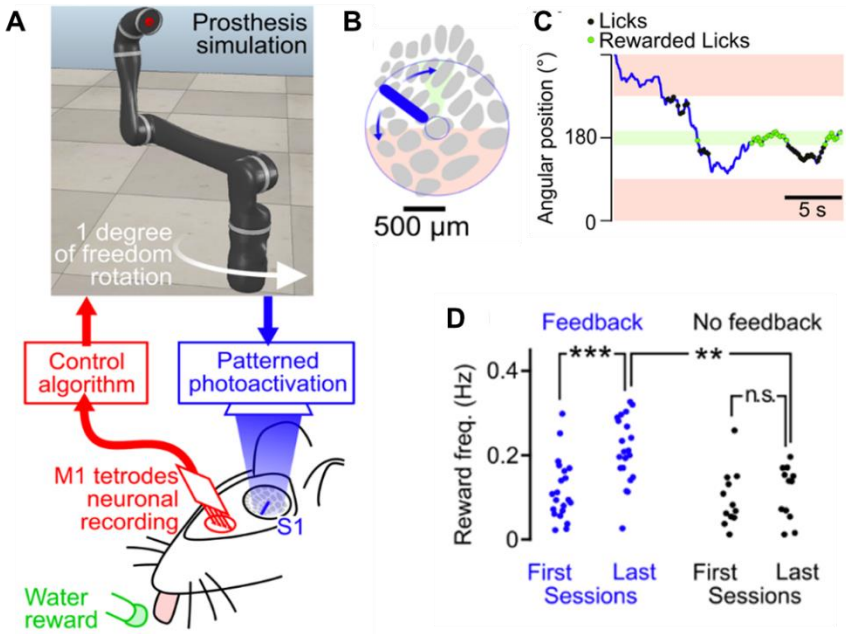


Figure 1.25: Closed-loop control of a virtual prosthesis with patterned optogenetic somatosensory feedback (Goueytes et al., 2022).
(A) Schematic representation of the closed-loop setup, including a snapshot of the prosthesis simulation in V-REP. The most proximal joint of the prosthesis is controlled by neuronal activity recorded in M1. The angular position of the joint is fed back to the mouse through spatio-temporally patterned photoactivation of S1.
(B) Location of the targeted cortical surface with respect to the barrels of the whisker primary somatosensory cortex. The photostimulation bar rotates in synchrony with the proximal joint in prosthesis space. The Start position of the photostimulation corresponded to the most posterior position of the stimuli in brain coordinates.
(C) Example trajectory of the rotating prosthesis, and associated licks (black dots) and rewards (green dots), during the 20 s of one uninterrupted trial.
(D) Reward frequency averaged across each session, plotted for the first three and the last three training sessions of each mouse. Blue: Optogenetic feedback (7 mice, 21 sessions). Black: Control, no feedback (subset of 4 mice, 12 sessions.

PART 2

METHODS AND RESULTS

2 METHODS AND RESULTS

2.1 EMBODIMENT OF AN ARTIFICIAL LIMB THROUGH PERIPHERAL STIMULATIONS

2.1.1 Participation statement

In this work, I built the setup and coded the hardware for the rubber paw illusion experiment. I designed and carried all the experiments, and performed all the surgeries. I analysed the data and generated all the figures, and wrote the pre-print presented, with input from all the authors.

2.1.2 Developing a protocol to study embodiment-like behaviour in mice

Based on our knowledge of the rubber hand illusion in human subjects, we aimed to develop a similar test to probe embodiment-like behaviour in mice. This would allow us to explore artificial limb integration into the body schema, as part of our project to build a neuroprosthetic research platform in the mouse model, using the tools that have been developed in our lab.

To this aim, we first developed a head fixed setup, where the animals were exposed to the rubber paw, while their right forepaw was hidden from their view, resting on a capacitive sensor (Figure 2.1A). The pod holding the mouse didn't have a flat surface for animals to rest their paw. In the middle of this empty space, we placed a sensor so that it was the only place where animals could rest their right forelimb. The goal was to be able to detect when mice correctly placed their paw in the area where the tactile stimulations would be applied. This proved rather challenging as animals would often move their paw away from the platform, or even leave it hanging in the empty space (Figure 2.1B). This was a critical point since we know that tactile and motor congruency are important factors to generate the illusion.

This led to the development of a hand cuff system that allowed us to stabilize the mouse's right forepaw in place, so that tactile stimulations could be applied in a precise and consistent manner (Figure 2.1C). With

this new system, we were able to restrain the paw and keep it in the same position successfully for full trials.

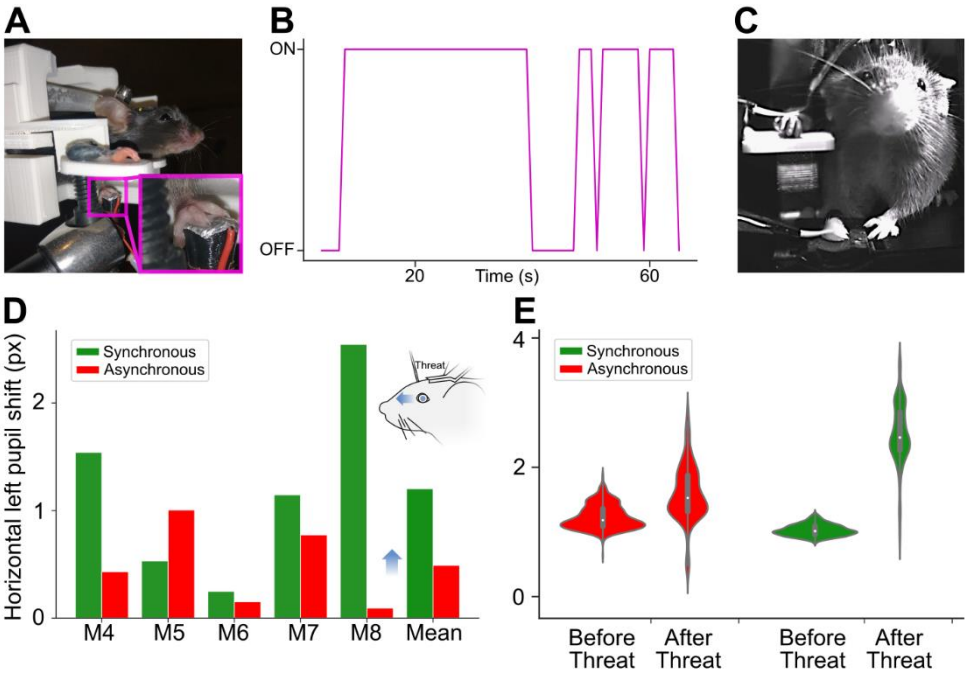


Figure 2.1: Development of the Rubber Paw illusion protocol
(A) Head fixed mouse with right paw placed on top of a platform with a capacitive sensor. The animal's paw is hidden from view while the artificial limb is visible to them.
(B) Capacitive sensor data showing that mice tend to move their paw from the platform.
(C) Frontal image of a mouse with the handcuff system that allows for paw restriction.
(D) Difference in horizontal left pupil position of 5 handcuffed mice, 5 s before and after the threat. Mouse data is averaged on 3 trials, Mean is the average of all trials for all mice.
(E) Average of horizontal left pupil position normalized relative to the pupil's resting position 5s before and after threat (n = 5).

With this forelimb restriction system, we performed a pilot study where we exposed 5 wild type mice to the rubber paw illusion protocol. For 1 session per day during 3 days, mice were exposed to two conditions. A synchronous condition where their paw and the artificial limb were stroked in synchrony for 60 seconds, and an asynchronous condition where these two stimulations were desynchronized. At the end of the stimulations, a fast object threatened the prosthetic limb by falling above it (see paper for more details). Although not significant because

of the limited data, these preliminary results revealed a clear tendency for animals to shift their left pupil forward after the threat (Figure D-E). This movement was stronger during synchronous stimulation conditions, suggesting an increased interest. We also suspected that these movements of the left pupil might be correlated with the right pupil shifting towards the threatened area during the test condition.

This initial pilot study enabled us to improve the rubber paw illusion setup. Firstly, we recorded the mouse during an additional baseline period before the start of the stimulations to allow for a better normalization of the tracking data. Secondly, as we were aiming to explore general facial and body movements, we initially recorded the frontal side and the left side of the animal's face (contralateral side to the artificial limb) as it was easier to access due to the right side being crowded by stimulation motors. However, in our pilot experiments, the frontal camera proved to not be very informative. In contrast, the left side of the animals' face yielded interesting preliminary results. Therefore, we modified the setup by placing a mirror next to the animal, allowing us to place our second camera facing this mirror and successfully recording the right side of the mice's face which we previously didn't have access to. Finally, we also increased the tactile pairing time to enhance the chances of the illusion being experienced.

The results of the rubber paw illusion experiments that followed this this preliminary work is presented in the pre-print presented in the following pages. It has been published in BioRxiv on the 28/05/2024, at DOI link https://doi.org/10.1101/2024.04.25.591084

Embodiment of an artificial limb in the mouse model

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Abstract

Having the ability to probe the strength of limb embodiment is a requirement to better understand body ownership disorders that are triggered both by disease and by accidental body damage. It is also an essential tool towards the development of neuro-prostheses that better integrate into the user's body representation in the brain.

One key way to probe limb embodiment is through the rubber hand illusion. Here we adapted this paradigm to the mouse forelimb, which is a relevant model for upper limb research thanks to its diverse and rich behavioural repertoire and unparalleled access to genetic and optogenetic research tools.

We exposed head-fixed mice to a visible, static 3D-printed replica of the right forelimb, while their own forelimb was hidden from their sight and stimulated by brush strokes in synchrony with the replica. Following these visuo-tactile associations, the replica was visually threatened, and we probed the mice's reaction using automated tracking of pupils and facial expression. We found that mice focused significantly more their gaze towards the forelimb replica when they received congruent tactile and visual information, compared to control conditions in which tactile and visual information were desynchronized. This observation is consistent with the human overt response to the rubber hand illusion. In summary, these findings indicate that mice exhibit behavioural signs of embodiment of an artificial forelimb, and this phenomenon can be quantified using behavioural measurements.

Introduction

When we observe and use our limbs to engage with the world, we perceive that they are part of ourselves. This sense of embodiment can be disrupted by brain lesions leading to a loss of recognition of physiological body parts (asomatognosia) and even their active rejection from the body representation — somatoparaphrenia (Feinberg et al., 2010). In the case of amputated patients, efficient use of the prosthesis can be hampered by a lack of prosthesis embodiment, thereby causing a decrease in daily prosthesis use and abandonment of the prosthetic limb (Espinosa & Nathan-Roberts, 2019; Maimon-Mor & Makin, 2020). Further, lack of embodiment of prosthetic substitutes is associated with a build-up of sensations that are perceived as arising from the "phantom" of the missing limb, including painful perceptions (Bekrater-Bodmann et al., 2021).

In an experimental setting, it is possible to either build or disrupt the sense of embodiment of an artificial limb by manipulating the temporal consistency of the tactile and visual stimulation. This multisensory strategy has been widely used to study the sense of body ownership and embodiment (Botvinick & Cohen, 1998; Ehrsson et al., 2004). In particular, in the "rubber hand illusion" experiment, participants are placed next to a prosthetic limb that they can see, while their corresponding real hand is hidden from their view. Both the hidden real hand, and the visible artificial hand are stimulated in synchrony with a brush (Botvinick & Cohen, 1998). A large share of the subjects in these experiments report that the rubber hand they are seeing is their real hand (Kalckert & Ehrsson, 2017; Reader et al., 2021b). These verbal reports are consistent with overt behaviours of the subject during the experiment, and in particular with a fear-like reaction when the artificial hand is visibly threatened or "injured" by the experimenter.

This has been assessed through high skin conductance responses and reports of participants showing signs of anxiety or pain anticipation before the injury of the prosthesis (Armel & Ramachandran, 2003). In addition, an increased activity in the insula and anterior cingulate cortex was observed : these regions are associated with anxiety and interoceptive awareness (Ehrsson et al., 2007).

By building on the ability of visuo-tactile synchronized inputs to generate embodiment, it is even possible to induce the embodiment of an artificial device, including robotic human prostheses, by stimulating the stump of amputees (Castro et al., 2023; Ehrsson et al., 2008; Rosén et al., 2009). This demonstrates the flexibility of this body-pairing mechanism, which may be key to embodied neuroprosthetics (Makin & Micera 2020). This however does not extend to any object, as studies show that the object needs to have the shape of a hand for successful embodiment. When the hand-shaped object is replaced by another object, reports show a significantly weaker embodiment (Finotti et al., 2023; Tsakiris et al., 2010).

So far however, the physiological basis of this sensory-based forelimb embodiment remains unclear. This is partly due to the lack of an animal model for studying embodiment. To address this, embodiment experiments have been carried in macaques and mice (Buckmaster et al., 2020; Fang et al., 2019; Shokur et al., 2013b; Wada et al., 2016, 2019). In mice, this was studied by applying tactile stimulations to a rubber tail and to the tail of the mouse and by observing the animal's reaction to an experimenter grasping the tail after the end of the stimulations (Wada et al., 2016, 2019). These particular studies suggest that it is possible to study embodiment in mice. In this study the authors focused on the embodiment of a body part that is specific to rodents. We wanted to expand on this model and explore the possibility of using this illusion in a way that can be translated to humans in the context of research on prosthetic limbs.

Mice can use their upper limb for rich and complex behaviours, including cortically dependent reaching (Estebanez et al., 2017), adjustment of a joystick position (M. W. Mathis et al., 2017), as well as manipulation of food with complex shapes (Whishaw et al., 2017). This makes the mouse upper limb, a relevant model for the study of the human upper limb function. Here, we aim to expand the mouse embodiment model and find if embodiment of an upper limb prosthesis can be achieved in the context of a non-invasive embodiment test.

Automated face tracking of rodents has emerged as a powerful strategy to probe the internal state of mice. Face tracking reveals ear/eye and snout mimics that are related to basic emotions of the mice and have been linked to different brain states (Dolensek et al., 2020). In particular, pupil movements have been related to many behavioural aspects such as fear learning, arousal and attempted movement (Langford et al., 2010; Meyer et al., 2020; Salay et al., 2018; Vinck et al., 2015).

In our paradigm, head-fixed mice were presented with an artificial, static replica of their right forelimb at a plausible physiological forelimb location. Meanwhile, their forelimb was hidden from sight, and held in place below the platform where the artificial limb was located. Next, during two minutes, we applied synchronized brush strokes to the real and to the artificial forelimbs. Finally, we presented a rapidly approaching object towards the artificial forelimb. Along this experimental test, the gaze of the mice was tracked by high-speed videography. Consistent with the human rubber hand illusion, the mice focused significantly more their gaze on the incoming — and potentially threatening — object when brush strokes were synchronous (versus asynchronous) and when the artificial forelimb looked similar to an actual biological limb (versus a white cuboid object).

Materials and Methods

All animal experiments were performed according to European and French law as well as CNRS guidelines and were approved by the French Ministry for Research (Ethical Committee 59, authorization 25932-2020060813556163v7). In order to reduce the number of mice involved in research experiments, we carried our experiment on EMX-Cre mice (Jax #005628) that were raised towards the maintenance of transgenic lines in the institute animal house, but were not directly used in other experiments. We could take advantage of these mice as their genotype is normal (Jax #005628) and we found no noticeable difference in behaviour when compared to baseline C57BL/6 mice. Mice were housed in cages of

5 in a non-inverted light cycle. They had access to enrichment (a wheel, wooden and plastic toys, as well as nesting material). The mice used were aged 60 to 100 days at the time of the experiment.

Surgery

During the experiments, the mice were head fixed. This allowed us to maintain the mouse and its paw immobile to ensure the stability of the tactile stimulations during the experiment. It also allowed for a direct translation of our paradigm into experimental setups for brain imaging and optogenetic stimulation. Implantation surgeries were carried under Isoflurane anaesthesia (4% for induction and 1-1.5% for maintenance). Surgeries were performed on a heated pad, while the mouse was held by a nose clamp. After a subcutaneous injection of lidocaine (4mg/kg) the scalp was removed, the conjunctive tissue resected and the skull was cleaned. A titanium head-fixation plate was then bonded to the skull using a cyanoacrylate glue primer topped with dental cement. Finally, the mice received a subcutaneous injection of anti-inflammatory medication (Meloxicam, 1-8mg/kg) and were monitored during their recovery in a temperature-regulated cage.

Recovery and habituation

The 10 tested mice (5 males, 5 females) were placed in the experimental setup for the first time after a 5-day recovery phase in their home cage. During this habituation phase, the mice were placed in a pod and head-restrained. They were given water with sugar to associate habituation with a positive reward. After an initial 10 min session with head fixation only, the next 4 head-fixation sessions lasted 20 min. They were coupled with movement restriction of the right forelimb. The paw was restricted using a custom-made handcuff that was adjusted secured on a dedicated, foam cushioned location on the pod (Figure 1A).

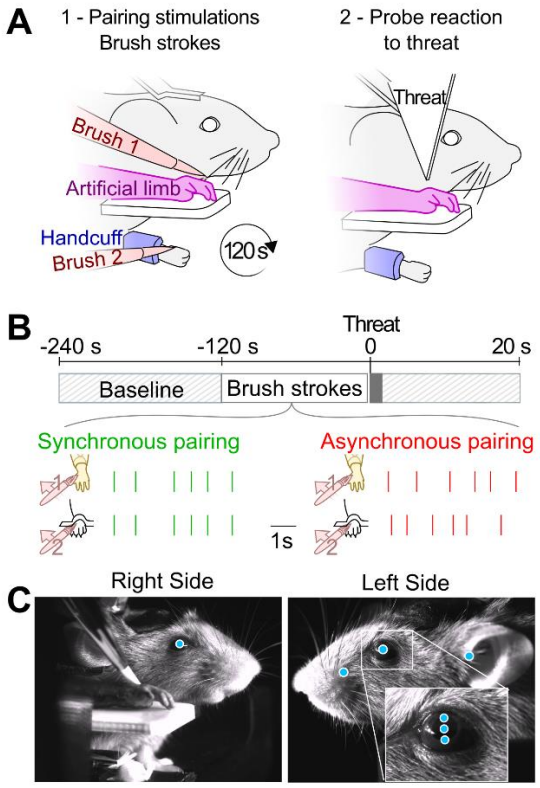

Figure 1. A forelimb embodiment test in the mouse model.

(**A**) Protocol scheme. During 120 seconds, brush stimulations were applied with brush 1 to the artificial limb (visual input) and with brush 2 to the corresponding forelimb, which was hidden from the mouse's sight (touch input). Embodiment of the artificial limb was then tested by showing a rapidly incoming threat-like object (sharp object in panel A2) that targeted the artificial limb, and by probing the intensity of the reaction of the mouse.

(**B**) Top: timeline of the pairing and test sessions. 10 mice were exposed to the protocol: 5 sessions were run for each experiment (1 session per day). During each session, the mice were exposed to 2 trials (synchronous and asynchronous pairing). The presentation order of these two trials changed on each session. Bottom: example brush stroke times for the synchronous (green) and asynchronous (red) strokes.

(**C**) Views from the right and left side of the mouse acquired by high-speed cameras during the pairing stage. Magenta dots: points of interest that are tracked, including the pupil position and diameter (measured on 2 points in the vertical axis, see close-up) for both eyes, the left whiskerpad and the left ear.

Design of the rubber paw illusion

A 3D model of a right mouse forelimb was designed, based on a 3D atlas of adult C57BL/6 mice derived from micro-CT sections (Wang et al., 2013). It was printed using a resin 3D printer (FormLabs Form3B, Grey Flexible Resin) and painted using acrylic paint to match the colour of the fur of the black mice. It was placed aside the head-fixed mice, and illuminated by a ray of visible, white light (while the rest of the setup was only illuminated by infrared lighting for imaging). Meanwhile, the actual right paw of the mouse was restricted and hidden below the platform holding the artificial limb.

During the 120 s pairing time (Figure 1A left), which followed a 120 s waiting period (Figure 1B), the mouse was exposed to a series of soft strokes applied by paint brushes mounted on servo motors (Make Block Smart Servo). One “brush stroke” event was achieved by the brush making a (6 mm * 2) back and forth movement on the paw that lasts for 300 ms between touch onset and offset (Figure 1B). During the pairing time, the brush strokes were applied both on the mouse right forelimb and on the artificial limb, at random intervals, between 600 and 2000 ms (derived from a Poisson-distribution). In the synchronous condition the two brushes apply the stimulation at the same time, while in the asynchronous condition, each brush was activated at a different, randomized interval, such that the visual input from the artificial limb did not match the tactile input applied to the physiological forelimb (Figure 1B).

Finally, 240 s after the beginning of the trial and right after the end of the brush stimulations, a threat to the fake forelimb was presented to the mouse (Figure 1A, right). This was achieved by using a stepper motor (17HS15-0404S, OSM Technology) to rapidly move an arrowhead-like white plastic object towards the fake limb, at a speed of 1 degree per ms, for a total travel time of 400 ms stopping less than 1 cm away from the rubber paw and staying at that position for 10 s (Figure 1C-D).

Brush stroke pairing conditions

The embodiment experiments using the prosthesis resembling the mice’s limb lasted one week. Five sessions were run for each experiment (1 session per day). During each session, the mice were exposed to 2 trials (synchronous and asynchronous) whose order changed at each session. We chose this multi-day design to minimize mouse fatigue and reduce the effect of the habituation curve that would have been potentially more prominent if all the trials were run on the same day.

40 days later, we performed an additional, control experiment. The goal of this experiment was first to probe the animals baseline reaction to the threat alone. Secondly, we wanted to explore if the mice would react to the threat of any object stimulated in synchrony with their paw, or if this was limited to artificial limbs resembling their own. Mouse 20 was not part of this experiment as the animal had to be removed from the experimental pool for veterinary reasons. The third trials for mouse 19 were also removed due to a technical issue during this session. This control experiment was otherwise identical to the initial series of experiments, but this time we exposed the animals to the threat alone followed by two trials (synchronous and asynchronous) where the fake paw was replaced by a white plastic block of the same size as the prosthesis (See Supplementary Table 1 for trial order).

Face imaging and tracking

The mice were imaged at 200 Hz with two 1440x1080 px monochrome cameras capturing the right and left facial expressions (Figure 1C) using a custom high-speed imaging system (RD Vision, France). The reactions of the mice to the stimulations and the threat were recorded and the videos analysed with DeepLabCut version 2.3 (A. Mathis et al., 2018). We trained two networks (one network for each side of the mouse’s face) on 120 labelled images of 10 different mice to track a series of points of interest on the animals face (blue dots in Figure 1C), including the centre of the pupil position, 2 points of the pupil for the diameter, ear, and either the C1 or B1 whisker (depending on which one was more visible on camera) .

Statistical analysis

To correct for baseline shifts, we subtracted the mean position of the tracked position measured during the 120 s baseline that proceeded the brush stimulations. When looking at the effect after the threat on pupil shifts and diameter, we subtracted the mean values 1 s before the threat so we could normalize to pre-threat positions that may not be the same from trial to trial.

All statistical tests were non-parametric Wilcoxon (paired) tests with the exception of the Threat Only vs synchronous/asynchronous prosthesis comparisons that were Mann-Whitney tests. We performed the statistical tests on five 1 s time windows, 1 to 6 s after the threat’s arrival. Independent tests were run on each window. To address the multiple repeated test bias that stems from this testing strategy, we corrected the p value with the Benjamini-Hochberg procedure.

Results

Longer gaze in the direction of the threatened artificial limb following synchronous stimulations
Following habituation to the experimental settings, we carried out behavioural sessions during which head fixed mice were positioned next to an artificial right forelimb that was within their field of view. Meanwhile, their own right forelimb was hidden from their sight. During these behavioural sessions, we performed high-speed imaging of their face to track several features— and in particular pupil positions.

During the pairing stage of the behaviour (Figure 1A left), both the artificial forelimb and the physiological forelimb received brush strokes, either simultaneous (synchronous stimulation), or randomly time shifted (asynchronous stimulation). We tracked pupil position during the trial for each mouse, the vertical pupil position stays at the same During baseline and during brush stimulations (Case study in Figure 2A, average in Figure 2B), we found no significant difference in the behaviour of the mice between the synchronous and asynchronous conditions.

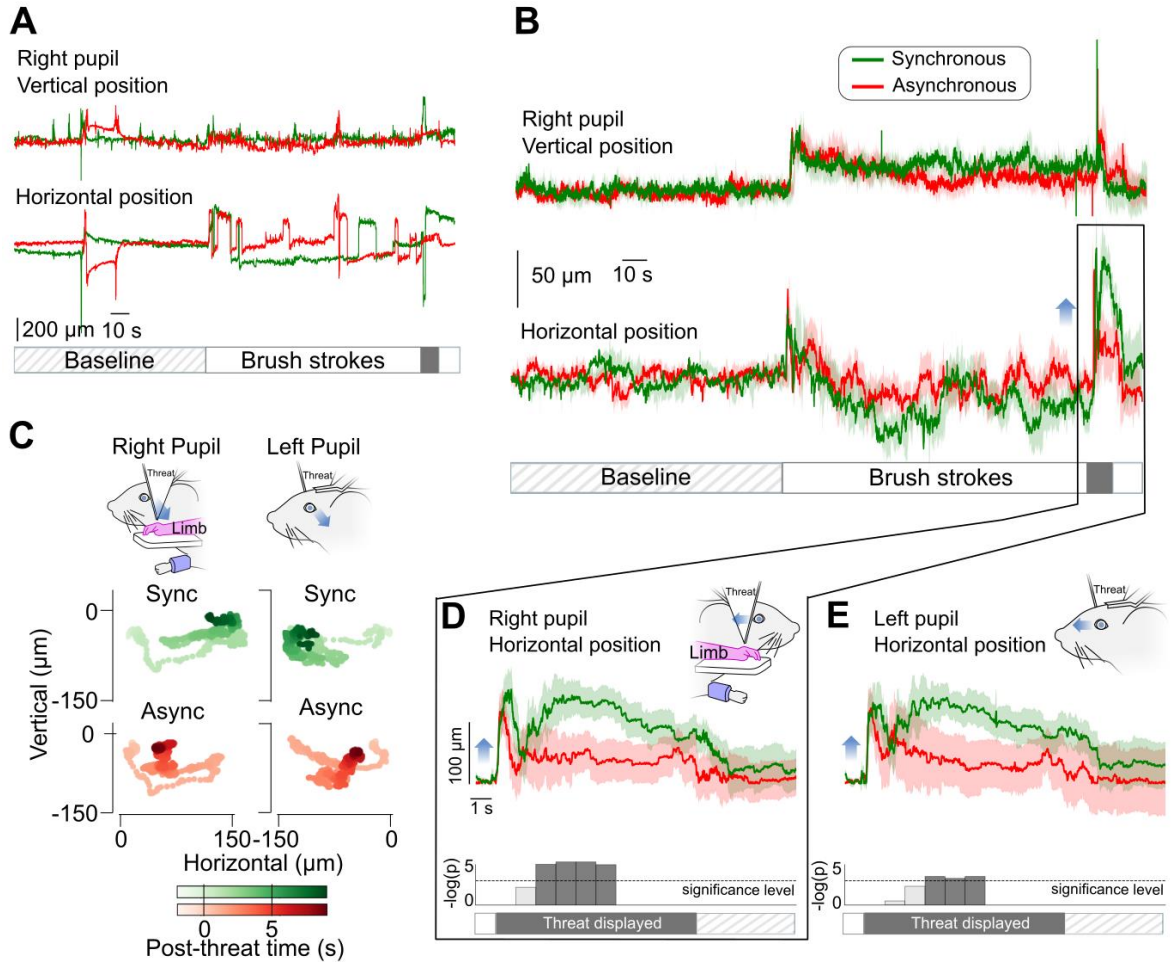

Figure 2. Pupil shifts in the direction of the threatened prosthesis are longer after synchronous stimulation
(**A**) Example session of vertical (top) and horizontal (bottom) movements of the right pupil during a synchronous (green) and an asynchronous (red) trial.
(**B**) Average vertical (top) and horizontal (bottom) movements of the right pupil during synchronous and asynchronous condition trials, normalized to the average position during the 120 s baseline (n = 10). The sequence includes a Baseline, Brush strokes pairing, and a threat to the artificial forelimb.
(**C**) Spatial distribution of the pupil position 1 s before and 7 s after the threat starts to be displayed. Top: synchronous pairing. Bottom: asynchronous pairing.
(**D**) Top: Average horizontal movements of the right pupil following the threat onset, normalized relative to the average position 1 s before the threat (n = 10). Light background: SEM. Blue arrow: direction of pupil movement. Bottom: statistical comparison of right pupil positions averages, 1-6 sec after threat (1 s bins). Bars show -log of the Wilcoxon p-values with Benjamini-Hochberg correction. p = 0.11, 0.0065, 0.0049, 0.0049, 0.0068. Dark gray: time bins reaching p = 0.05 threshold.
(**E**) Same as D for the left pupil. p = 0.6, 0.098, 0.029, 0.037, 0.029.

After pairing, the artificial forelimb was almost hit by a rapidly falling sharp arrow-like object (Figure 1A, right). In both the synchronous and asynchronous pairing conditions, the mice responded to this event with a rapid pupil movement towards the artificial limb and threatening object.

One second after this first response, the mice behavior diverged between the two conditions. In the synchronous condition, on average, the mice looked again in the direction of the menace and artificial limb, while in the asynchronous condition, the mice stopped looking in this direction and moved back their pupil to the resting position (Figure 2C). Overall, most of the pupil movements following the menace took place in the horizontal axis.

We carried multiple significance tests over 1 s windows ranging from 1 s to 6 s after the incoming threat (see methods for repeated test correction). This revealed that horizontal pupil positions were significantly different in the synchronous versus asynchronous conditions (Figure 2D-E). These differences were significant for 4 consecutive bins for the right pupil position 2-6 s post threat (Figure 2D) and for 3 consecutive bins for the left pupil position 3-6 s post threat (Figure 2E).

We then asked if mice would react similarly to the threat of an object that does not resemble their forelimb. Therefore, in a second series of experiments, we exposed the same mice (n = 9, see methods) to the same protocol as in Figure 2, but this time we replaced the prosthesis with a white rectangular block (Figure 3). In these experiments, we did find that there was a significant difference in average pupil shift. However, in contrast to the forelimb prosthesis condition, it lasted only for one bin 1-2 post threat for the right pupil (Figure 3A) but not the left pupil (Figure 3C).

To further compare the reaction to the two prosthesis shapes, we compared the amount of differences in average pupil shift between synchronous and asynchronous condition across the two shapes. We found that it was not significantly different for the first 3 time bins between the artificial limb and white rectangle condition for both pupils (Figure 3C-E). In contrast, we found a significant difference for the last 2 bins, indicating that the pupil shifts in the direction of the threatened area lasted longer during the artificial limb condition compared to the white rectangle control (Figure 3C-E).

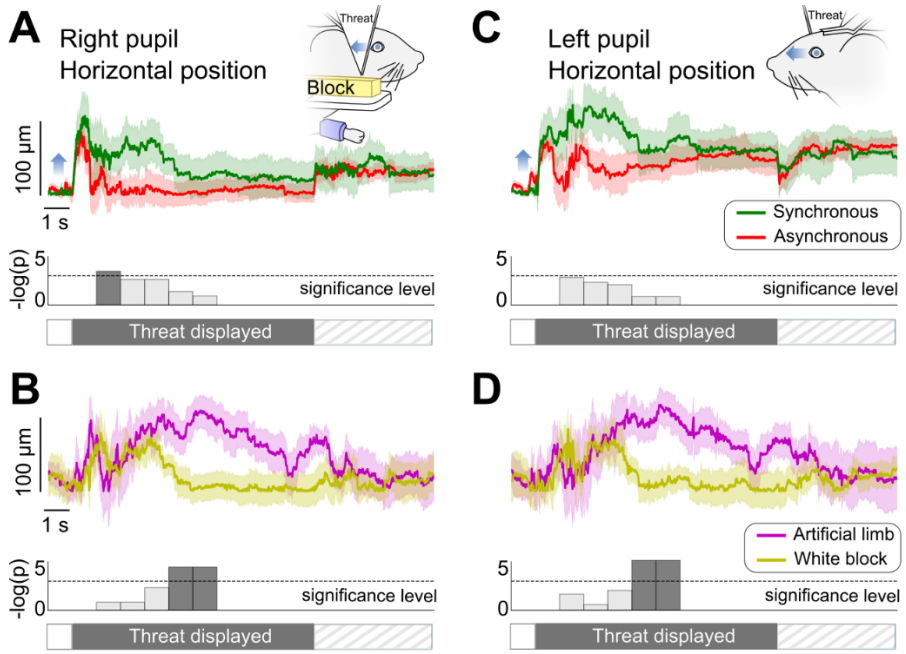

Figure 3. Pupil shifts are shorter when the prosthesis is replaced by a white rectangle
(**A**) Top: Average horizontal movements of the right pupil after the threat, normalized relative to the average position 1 s before the threat (n = 9). Green line: synchronous pairing. Red: asynchronous pairing. Light background: SEM. Blue arrow: direction of pupil movement.
Bottom: statistical comparison of right pupil positions averages, 1-6 sec after threat (1 s bins). Bar plot: -log(p-value). p = 0.031, 0.073, 0.073, 0.25, 0.40. Dark gray: significant time bins. Horizontal line: p = 0.05 threshold.
(**B**) Top: Amplitude of the synchronous/asynchronous difference in right pupil position. (n=9). Magenta line: prosthesis. Yellow: white rectangle. Bottom: Wilcoxon test with Benjamini-Hochberg correction, 1 to 6 sec after threat. Bar plot: -log(p-value). p = 0.44, 0.44, 0.098, 0.012, 0.012.

(**D**) Same as B for the left pupil. p = 0.059, 0.098, 0.12, 0.43, 0.43.
(E) Same as C for the left pupil. p = 0.19, 0.55, 0.13, 0.0059, 0.0059.
A further control condition was added during the second experiment to probe the baseline reaction of the animals to the threat's arrival, independent of the presence of an artificial limb or white block, and without any tactile stimulations (Figure S1). The pupil shifts observed in this *Threat only* condition were lower compared to the movements observed during the other conditions. In particular, this condition showed significantly less right pupil movements compared to our test condition where the mice were exposed to synchronous stimulations of an artificial limb (Figure S1A,C). While the left pupil showed a similar tendency, these shifts were less prominent and did not appear to significantly differ from the Threat only condition (Figure S1B,D). Finally, when now comparing the Threat only condition to the White block condition, we did not find any significant differences of right or left pupil position between the threat only and synchronous or asynchronous stimulations (Figure S1E-H).

Reduced pupil dilation during synchronous stimulations
In addition to the pupil position, we also looked at the evolution of pupil diameter after the threat. We found a consistent increase in both right and left pupil diameter in all condition after the arrival of the threat (Figure 4A-D).

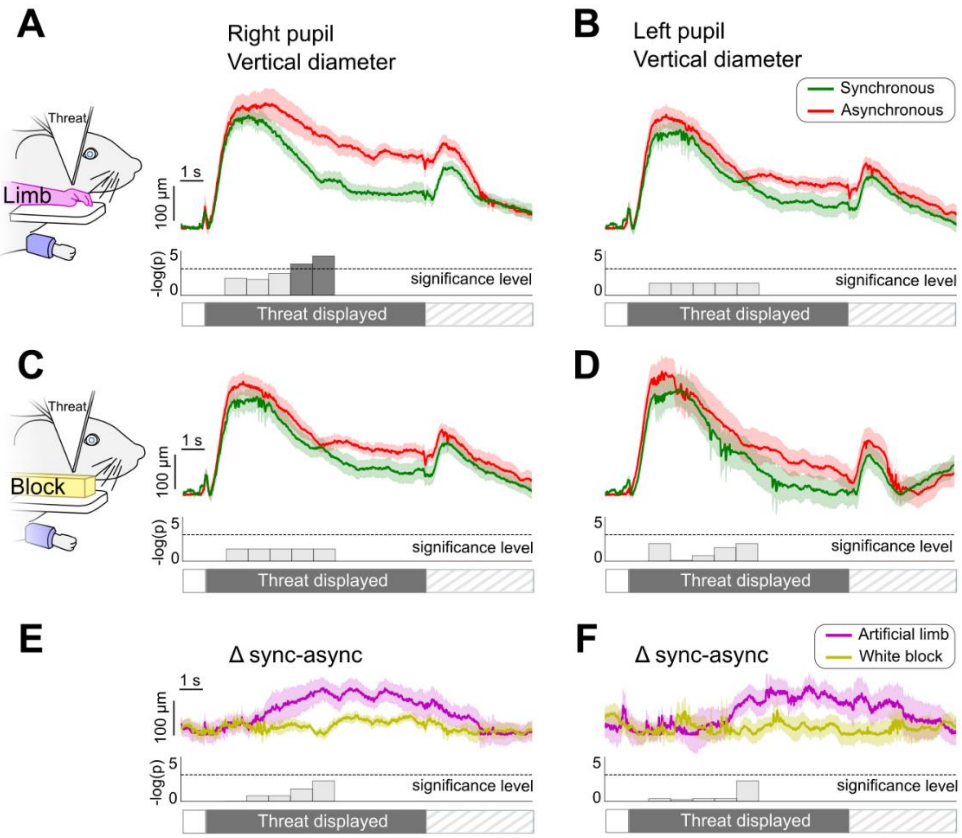

Figure 4. Pupils are less dilated in response to the threat in the synchronous condition
(A) Top: Average vertical diameter of the right pupil after the threat in the artificial limb condition, normalized relative to the mean position of the second before the threat (n = 10). Light background: SEM.
Bottom: Significance of sync/async difference in 1 s bins, measured with a Wilcoxon test with Benjamini-Hochberg correction, 1 to 6 sec after threat. Bar plot: -log(p-value). p = 0.15, 0.17, 0.084, 0.029, 0.012. Dark gray: significant time bins (P = 0.05 threshold).
(B) Same as A for the left pupil. p = 0.85, 0.85, 0.85, 0.48, 0.24.
(C) Same as A for the white block condition p = 0.25, 0.25, 0.25, 0.25, 0.25.
(D) Same as B for the white block condition p = 0.14, 0.91, 0.53, 0.21, 0.14.
(E) Top: Amplitude of the synchronous /asynchronous difference in right pupil diameter. Magenta line: prosthesis. Yellow: white rectangle. Bottom: Wilcoxon test with Benjamini-Hochberg correction, 1 to 6 sec after threat. Bar plot: -log(p-value). p = 1.0, 0.53, 0.53, 0.24, 0.098.
(F) Same as E for the left pupil. Wilcoxon p = 0.71, 0.82, 0.71, 0.71, 0.098.

Additionally, we found that the pupil diameter differed between visuo-tactile pairing conditions after this initial increase. When we probed difference between the right pupil diameter averages across 1 s bins 1-6 s after the application of the threat, we found a significant difference between synchronous and asynchronous stimulations for two consecutive time bins 4 to 6 s post-threat (Figure 4A). This difference was not observed in the white block condition for the right pupil diameter (Figure 4C). The left pupil did not show any significant effects during the artificial limb or the white block condition (Figure 4B,D).

When comparing the amount of synchronous/asynchronous differences in right and left pupil diameter between the prosthesis versus white block conditions (Figure 4E-F), we found that there were no significant differences.

Overall, we found that the right pupil of the mice seemed to be less open during the synchronous stimulation of a prosthesis resembling the animal's paw, although this effect does not seem to be different between the artificial limb and white block conditions. No differences were observed on the left pupil.

Increased changes in mice facial movements speed after the threat of the rubber paw

In addition to the pupil-related variable, when looking at the average horizontal ear movements speed on the same time window 1-6 s after the prosthesis threat (Figure 5A), we found a significant difference between the synchronous and asynchronous pairing conditions for the first bin 1-2s post threat (Figure 5A), and this difference was not significant in the control condition when we replace the prosthesis with a white block. Note however that, when comparing the amount of the synchronous/asynchronous difference in ear speed between the artificial limb and white block condition, we found no significance (Figure 5B).

An additional investigation of horizontal whisking speed in a 0-2 s window after the threat onset (Figure S2), showed a difference between synchronous and asynchronous conditions, which was only significant when mice were exposed to the artificial limb (Wilcoxon test: p= 0.049) but not when they were exposed to a control object (Wilcoxon test: p= 0.57, Figure S2B). Again, we saw no differences when directly comparing the prosthesis and white rectangle conditions (Wilcoxon test: p= 0.16, Figure S2C).

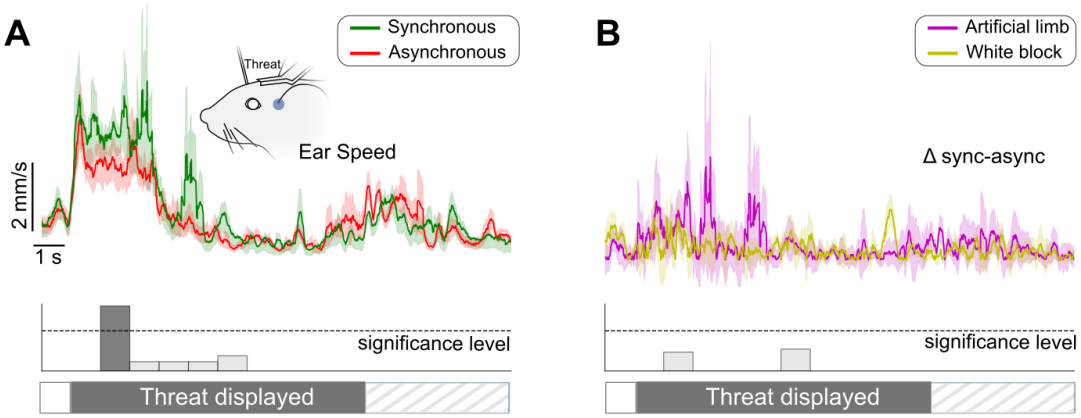

Figure 5. Increased changes in mice ear speed after the synchronous stimulation of the prosthesis
(A) Top: Average speed of the left ear after the threat (n = 9), convolved with a 200 ms box filter. Light background: SEM. Blue dot on mouse: area being tracked.
Bottom: Statistical comparison of ear movement speed averages 1-6 sec after threat. Every bar plot represents the -log(p-value) for every 1 s bin. P-values were corrected using the false detection rate Benjamini-hochberg correction. Wilcoxon p= 0.0078, 0.5, 0.5, 0.5, 0.32. Lighter gray: time bins not reaching significance, Darker gray: time bins reaching significance.
(**B**) **Top:** Difference between the average ear movement speed between synchronous and asynchronous conditions (n=9). Magenta line: Δsync-async during the prosthesis condition. Yellow: Δsync-async during the white rectangle condition.
Bottom: Statistical comparison of ear movement speed averages 1-6 sec after threat. Every bar plot represents the -log(p-value) for every 1 s bin. P-values were corrected using the false detection rate Benjamini-hochberg correction. Wilcoxon p= 0.24, 1.0, 1.0, 1.0, 0.20. Lighter gray: time bins not reaching significance, Darker gray: time bins reaching significance.

Discussion

Similarity of the rubber paw and rubber hand experimental design

In our experiment we exposed mice to a protocol that was directly derived from the design of the human rubber hand illusion. Our protocol was most similar to the vertical setups of the rubber hand illusion (Kalckert & Ehrsson, 2017). Consistent with these experiments, the artificial forelimb was placed on a platform 1.2 cm on top of the mouse's real paw. In our case, in order to adapt to the mouse anatomy, we also placed the prothesis 0.8 cm away from the mouse horizontally. These distances were the minimum achievable shift to ensure that the prosthesis would be seen by the mouse while remaining at a congruent position anatomically. We stayed in a distance radius of less than 1.5 cm which corresponds at the mouse scale to the 30 cm radius where the illusion can still be experienced in human experiments (Lloyd, 2007).

In the classic rubber hand illusion, participants are asked to remain static and focus their attention on the prosthesis (Abdulkarim et al., 2021; Botvinick & Cohen, 1998). To be able to achieve this in mice, we head-fixed the animals and restrained their paw which is a considerable change from the conditions in human experiments. This required habituating the animals to this condition which took around 1 to 2 weeks. This change in the set up was important as it allowed us to stimulate the same regions on the paw without any disturbances caused by movement as we know tactile and motor congruency is important for the emergence and maintenance of the illusion (Abdulkarim et al., 2023; Shimada et al., 2009). Our brush stimulations arrived at a frequency ranging between 0.6 and 2 Hz for 2 minutes which is what is usually used is human experiments (Bekrater-Bodmann et al., 2012; Crucianelli et al., 2013; Rohde et al., 2011).

Human rubber hand illusion experiments rely mostly on subjective questionnaires to assess the induced response. However other measures, not relying on questionnaires, have been developed to quantify more objectively the strength of the responses. This includes the proprioceptive drift reported in the initial rubber-hand study (Botvinick & Cohen, 1998) where the self-localization of the hand involved in the experiment shifted towards the rubber limb during synchronous conditions. Another alternative to questionnaires is the bodily response to a threat to the rubber hand: when the rubber limb in embodied in the test conditions, the threatening provokes an anxiety-like response that can be seen cortically as an increased activity in the insula and anterior cingulate cortex (Ehrsson et al., 2007) as well as through skin conductance response (SCR). These responses are also accompanied by participants' reports indicating an anticipation of pain, as well as facial, verbal and motor signs of surprise or nervousness upon the threat or injury of the fake limb (Armel & Ramachandran, 2003).

With no access to subjective questionnaires in the mouse model, we therefore based our analysis on the detection of a reaction to the threat of a prosthesis, which was already validated in the mouse model in the context of the rubber tail illusion (Wada et al., 2016). In human rubber hand experiments, threats include exhibition of a sharp object like a knife or a needle (Ehrsson et al., 2007). However, their threatening action stems from preestablished cognitive framework recognizing these objects as potentially causing harm which is why we could not use them in rodent experiments. Previous studies investigating this phenomenon in mice used a strong grasp of the tail as a threat (Wada et al., 2016), we wanted our setup to be experimenter-independent so our approach is based on a fast-moving object approaching the prosthesis. We found this to be efficient in eliciting a strong response in mice while being similar to impact based approaches in human paradigms that elicit SCR responses (K. Ma & Hommel, 2013).

Pupil position is a key observable

In our experiments, the mice showed signs of embodiment that were consistent with the human observation of a difference in the reaction to a menace in synchronous versus asynchronous pairing conditions. In particular, our videography of the mice's face revealed a coupled right and left pupil shifts towards the prosthesis and threat, which lasted longer and was significantly more prominent in the synchronous pairing condition (Figure 2) and very low when only the threat was applied (Figure S1). In two previous studies that investigated tail embodiment in mice through a rubber hand illusion (Wada et al., 2016, 2019), head movements were reported as the reaction of the mice to a menace to the tail. However, in our experiments, the mice were head fixed, and therefore pupil movements were likely used by the mice to rotate their gaze despite the head fixation (Meyer et al., 2020).

Impact of the shape of the stimulated object on embodiment

In our experiments, we have asked if, in the mouse model, there was an impact of the rubber limb shape on the observed pupil shifts (Figure 3). Human experiments have explored the limits of artificial limb embodiment by studying the impact of the visual similarity of an artificial hand to a physiological limb in the rubber hand illusion (Finotti et al., 2023; Tsakiris et al., 2010) and virtual environments (Zhang et al., 2023). These experiments tend to show higher scores of embodiment for hand-shaped objects compared to non-limb objects. Zhang et al. show that participants in VR environments tend to look less at hands with simple shapes as well. To take into account these findings, we designed our rubber forelimb shape to be faithful to mouse forelimb anatomy (Wang 2015) and we coloured it to match the mouse's fur and skin colour. To test for the specificity of the shape of the object being embodied, we replaced the artificial limb shape by a white rectangular block (Figure 3). We found that, in this condition, the differences in pupil shift between synchronous and asynchronous stimulations lasted for a shorter time. Although we did not find a total collapse of the differences between the synchronous and asynchronous conditions, these results suggest that, consistent with human results, mice are more capable to embody of a limb-like object rather than an arbitrary shape.

Contributions to the fluctuations of pupil diameter

Beyond the position of the pupil, we also found significant differences in the dynamics of the pupil diameter (Figure 4). After the threat of the rubber paw, we observed pupil dilation that can also be observed when only the threat is applied without prior exposition to the prosthesis and tactile stimulations. Pupil dilation has been shown to correlate with different arousal states (Reimer et al., 2014; Turner et al., 2023), attention (Abdolrahmani et al., 2021), as well as the processing of startling stimuli (Leuchs et al., 2019) and fear conditioning (Poli et al., 2023). We interpret this increase in pupil diameter as a sign that mice were strongly engaged by the arrival of the threat in all conditions in a similar manner. Beyond this overall trend, we noticed that the right pupil diameter was significantly larger in the asynchronous condition in a 1-2 s window after the threat following the initial pupil dilation. The dynamics of pupil size are known to reflect cognitive processes, including memorization. For instance, studies have shown that pupil constriction is stronger when individuals are exposed to images that they later recall (Naber et al., 2013), or when encountering novel stimuli (Kafkas & Montaldi, 2015). Additionally, pupil size adjustments are linked to the rapid switching between rod-driven and cone-driven vision systems, which allows animals to adapt their visual perception to specific environmental cues (Franke et al., 2022; Qiu et al., 2021). These insights may explain the delayed reduction in pupil size observed after the initial dilation in our test condition. Although, this delay did not seem to differ between the artificial limb and white block condition.

Towards grimace tracking of embodiment

The tracking of mice's facial expression has become a relevant strategy to probe different emotional states of the animal. When provoking a negative reaction such as fear, studies show that the animals grimace differs significantly from a neutral emotion (Dolensek et al., 2020; Langford et al., 2010), in particular by modulating the ear and whisker pad areas of the face. Facial tracking techniques (A. Mathis et al., 2018) allowed us to quantify the reactivity of mice to the menace presented on the prosthesis and compare this reaction across different conditions. Given that pain or anxiety can manifest through various facial expression changes (Langford et al., 2010; Wada et al., 2016) we evaluated mice grimace changes by quantifying the speed of the ear and vibrissae movements. We found that they could be indicators of the reaction to the threat of the artificial limb. Consistent with the main features of previously reported mouse grimaces, we found that beyond the pupil, the ear and whisker pad moved faster in response to the threat of the prosthesis-like object after synchronous stimulations compared to the asynchronous control although these effects were less prominent than the ones observed for the pupils (Figure 5, S2).

Perspective

Our work adds to the existing literature on body ownership in rodents and demonstrates behavioural correlates of forelimb embodiment in mice by reproducing key features of the embodiment in the rubber hand experiment context, including the reduction of embodiment following the degradation of the rubber hand shape. These experiments suggest that limb embodiment is shared across multiple mammalian species and could therefore be investigated in the rodent model using a broad array of experimental strategies. The methodology used in the experiments constitute a non-invasive videography strategy to probe forelimb embodiment in mice. In the future, this assay could be combined with invasive neuronal recordings and brain manipulations to explore the neuronal basis of embodiment and probe novel strategies to induce prosthesis embodiment in humans.

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Supplementary Material :

		Session 1	Session 2	Session 3	Session 4	Session 5
Prosthesis	Group A (19,21,23,25,27)	Synchronous Asynchronous	Asynchronous Synchronous	Synchronous Asynchronous	Asynchronous Synchronous	Synchronous Asynchronous
	Group B (20,22,24,26,28)	Asynchronous Synchronous	Synchronous Asynchronous	Asynchronous Synchronous	Synchronous Asynchronous	Asynchronous Synchronous
White block and threat only	Group A (19,21,23,25,27	Threat Only Synchronous Asynchronous	Threat Only Asynchronous Synchronous	Threat Only Synchronous Asynchronous	Threat Only Asynchronous Synchronous	Threat Only Synchronous Asynchronous
	Group B (22,24,26,28)	Threat Only Asynchronous Synchronous	Threat Only Synchronous Asynchronous	Threat Only Asynchronous Synchronous	Threat Only Synchronous Asynchronous	Threat Only Asynchronous Synchronous

Table 1. Trial Order for experimental paradigm.

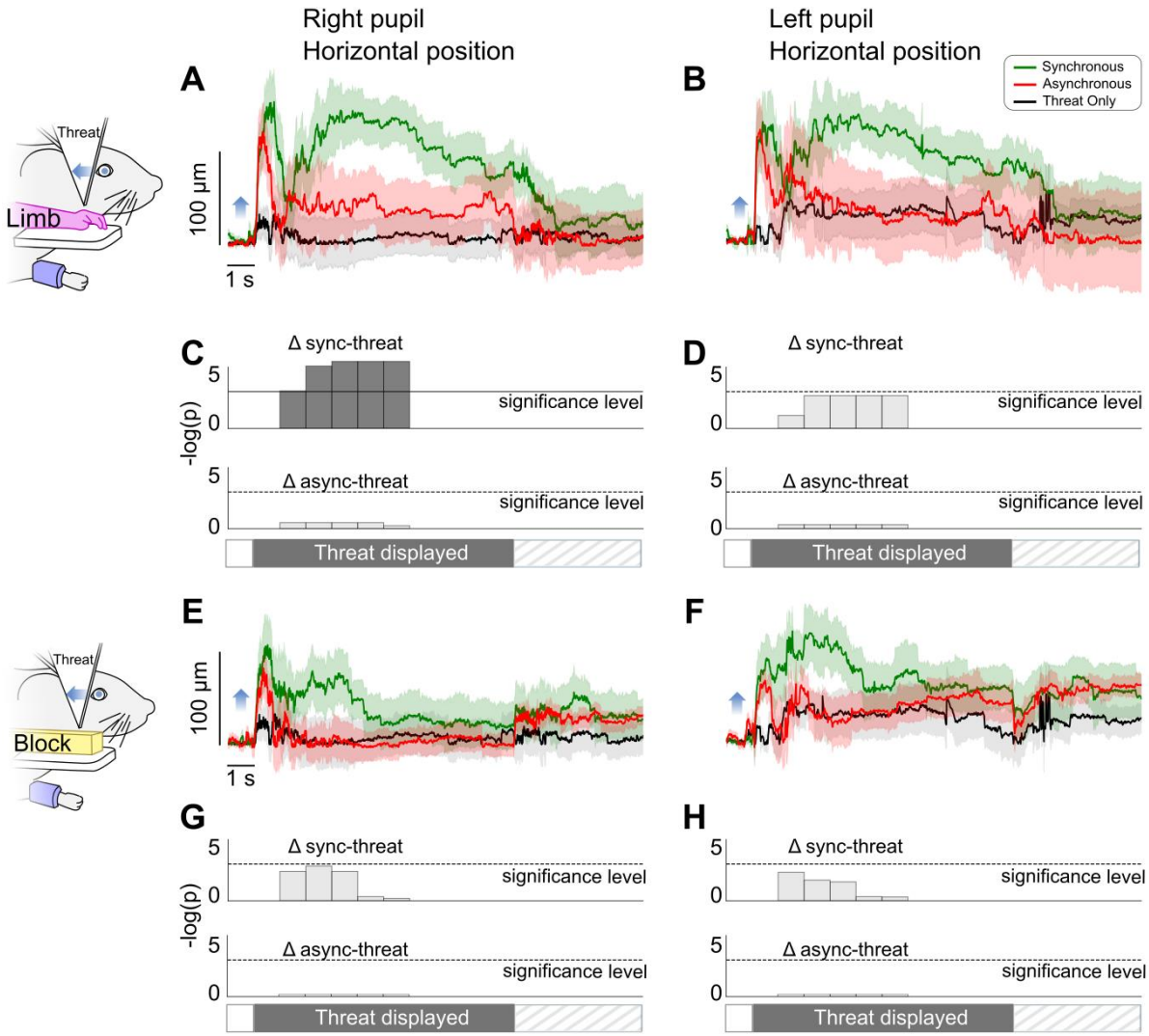

Figure S1. Baseline responses to the threat presented alone without the prosthesis and brush strokes.
(**A**) Average horizontal movements of the right pupil following the threat onset, normalized relative to the average position 1 s before the threat (n=10). Conditions represented are Artificial limb synchronous/asynchronous and Threat Only. Light background: SEM. Blue arrow: direction of pupil movement.
(B) Same as A for the left pupil position.
(**C**) Statistical comparison of right pupil positions averages, 1-6 sec after threat (1 s bins). Bars show -log of the Mann-Whitney p-values with Benjamini-Hochberg correction. Synchronous/Threat: p= 0.047, 0.0062, 0.0043, 0.0043, 0.0043. Asynchronous/Threat: p= 0.61, 0.61, 0.61, 0.61, 0.78.
(D) Same as C for left pupil. Synchronous/Threat: p= 0.35, 0.069, 0.069, 0.069, 0.069. Asynchronous/Threat: p= 0.71, 0.71, 0.71, 0.71, 0.71.
(E) Same as A for the white block condition.
(G) Same as C for the white block condition. Bars show -log of the Wilcoxon p-values with Benjamini-Hochberg correction. Synchronous/Threat: p= 0.091, 0.059, 0.091, 0.71, 0.82. Asynchronous/Threat: p= 1.0, 1.0, 1.0, 1.0, 1,0.
(H) Same as G for the left pupil position Synchronous/Threat: p= 0.098, 0.19, 0.21, 0.71, 0.73. Asynchronous/Threat: p= 0.91, 0.91, 0.91, 0.91, 0.91.

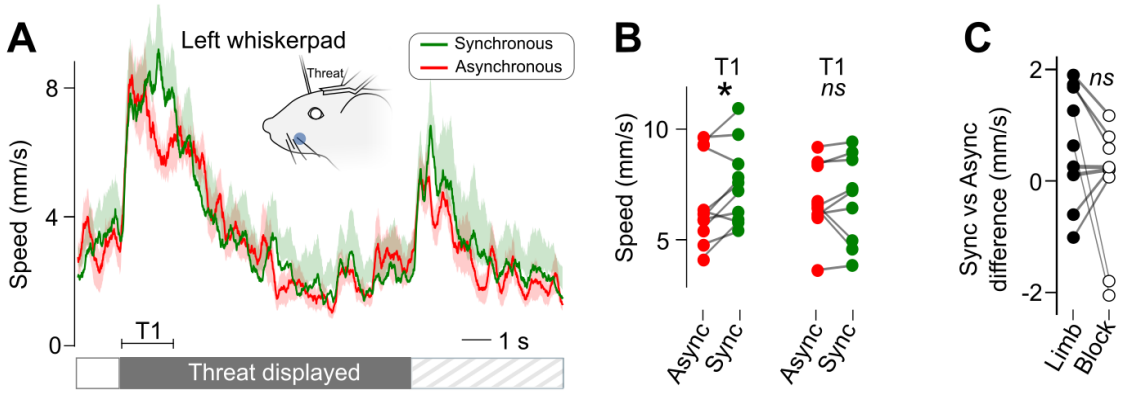

Figure S2. Increased whisking speed after the synchronous stimulation of the artificial limb
(**A**) Averaged speed of the left C1/B1 whisker (n=9) smoothed using 200 ms moving average filter via convolution. Light background: SEM. Blue dot represents area being tracked.
(**B**) Average of whisking speed for each mouse (n = 9) during interval T1 (see **A**) for the artificial limb (test) and white block (control) conditions. *: Wilcoxon p = 0.049, ns: Wilcoxon p = 0.57.
(**C**) Difference between left ear speed in the synchronous and asynchronous conditions for the artificial limb condition (black) versus white block condition (white) during interval T1. There is no significant difference between the block versus artificial limb contribution to the reaction. ns: Wilcoxon p = 0.1

2.3 EMBODIMENT OF AN ARTIFICIAL LIMB THROUGH DIRECT CORTICAL OPTOGENETIC STIMULATIONS

Our first study showed that mice display signs of embodiment towards an artificial limb in a mouse analogue of the rubber hand illusion. Mice displayed a stronger reaction to the threat of an artificial limb after congruent tactile feedback is provided. These findings confirm that the peripheral tactile feedback embodiment paradigm can be effectively studied in mice.

Building on this, and following our aim to develop a mouse platform for neuroprosthetic research, we wanted to adapt this paradigm to investigate whether we can provide effective tactile feedback with direct optogenetic cortical stimulations instead of peripheral brush strokes. Particularly, we wanted to test if replacing the brush strokes applied to the periphery by a direct cortical targeting of the forelimb area in S1, would induce the embodiment of an artificial limb.

As discussed in the introduction of this thesis (Chapter 1.3.1), working with mice provides us with a variety of brain imaging and optogenetic tools that are available first in this model. Optogenetic tools combined with our in-house 1-photon light patterning system (introduced in 1.3.3.1 of the thesis) can provide cortical stimulations that are arbitrarily patterned, both spatially and temporally, allowing us more flexibility in the exploration of the effect of this type of feedback on embodiment.

Our goal here was to probe the use of optogenetic stimulations to generate tactile percepts that would induce embodiment of the prosthesis by the mice. To achieve this, we aimed to implement optogenetic feedback that would closely resemble the physiological cortical activity generated by brush strokes. This involved targeting the representation of the forelimb in the primary somatosensory cortex and ensuring that the photostimulation pattern was consistent with the activity observed during tactile stimulation with a brush. To achieve this, we first explored the cortical activity provoked by brush strokes on the periphery with mesoscale calcium imaging. Secondly, we used this information to mimic the strokes on the paw through an artificial optogenetic feedback during the illusion to induce embodiment.

2.3.1 Mesoscale calcium imaging during tactile stimulations

2.3.1.1 Participation statement

This work was done in collaboration with Anton Dogadov, a postdoctoral researcher in the team. He developed a setup for mesoscale calcium imaging (Celestine), as well as the corresponding algorithms to analyse the cortical waves observed in the brain. The tactile stimulation system used was the one that I developed for our first study of the rubber paw illusion. I made a replica of the pod allowing the restriction of the mouse's forepaw, and installed the motor carrying the brush providing the tactile stimulations on the mouse's limb. The window and head implant surgeries were performed by Anton Dogadov and Clément Picard. The data collection was done by both myself and Dr Dogadov on the Celestine setup.

2.3.1.2 Materials and methods

2.3.1.2.1 Animal preparation

Four Emx-Cre x Ai95 male mice (expressing GCaMP6f, a calcium-sensitive fluorescent protein, in pyramidal neurons) were used for this experiment. Three of these mice were previously trained on a task where they had to generate cortical waves to obtain rewards as part of Dr Dogadov's experiments. Finally, one additional mouse was implanted by PhD student Clément Picard and was naïve to this task (mouse 02).

Surgeries for the optical window implantation followed the same steps as previously described *(See BioRxiv Pre-Print Chapter 2.1)* for the head implant surgery, up to the skull cleaning step. In this protocol, a craniotomy was performed to remove a 6 mm diameter disk of skull over forelimb S1/M1 on the left brain hemisphere (Guo et al., 2020). A glass cover slip of the same diameter was then glued on the sides with cyanoacrylate glue, after that a layer of dental cement was added to cover all exposed skull.

After 5 days of recovery post-surgery, a fluorescence mesoscale imaging session was performed to locate the position of forelimb S1/M1 areas in the cortex. Mice were placed under 1% isoflurane

anaesthesia (Induction Isoflurane 3%). Either the right forelimb (FL) or the right hindlimb (HL) was placed on top of a piezoelectric bender (Physics Instruments) to receive mechanical stimulations (100 Hz trains). At the same time, we imaged at 100 Hz a 6 x 6 mm patch of the somatosensorimotor cortex. Each frame was acquired with both blue (480 nm) and with green (530 nm) illumination. This pair of images was used to minimize the hemodynamic-related component of the signals by dividing one by the other (Figure 2.2A) (Y. Ma et al., 2016). The signal acquired was filtered using a 2D median filter and the areas that showed activation during piezoelectric stimulation were traced using an extended maximum transform. Mouse 02 was not exposed to this paradigm; thus, these areas have not been traced for this animal.

2.3.1.2.2 Imaging calcium activity during brush strokes

The second imaging session was conducted one to two weeks after the completion of the initial experiments involving the animals (2 months after implantation surgery for mouse 02). During this second session, the animals were head-fixed, and their paws were restrained using the same handcuff mechanism employed in our first study that focused on the classical rubber-hand illusion. Like in the initial study, a soft brush was used to administer tactile stimulations to the mouse forelimb. Brush strokes were applied to the mouse's right forelimb at random intervals ranging from 2000 to 2500 msec for a duration of 10 minutes (Figure 2.2A-B). These intervals were longer than those used previously to account for the decay time of calcium transients (Chen et al., 2013). Two type of brush strokes were applied: all animals received brush strokes going from the wrist to the tip of the paw, the same ones applied in the initial rubber paw illusion study. We also added, for mouse 28 and 02, the reverse stimulation from the tip of the paw to the wrist to test if we could detect a correlation between the direction of the brush and the direction of propagation of the activity wave.

The recording frequency was set at 100 Hz. The baseline fluorescence (F0) was calculated by averaging the data 400 msec before the onset of each stimulation. ΔF/F0 was computed on data ranging from 100 msec before to 3 seconds after each stimulus onset (Figure 2.2B). We calculated the mean of ΔF/F0 obtained for each brush stroke during the 10-min period to obtain an averaged signal for each mouse.

In our observations, brain activity propagated from 300 to 1000 msec following the stimulus onset (Figure 2.2C). A Ricker wavelet transform was then applied to smooth the data, in order to extract the static blobs or moving waves of activity. Thanks to this, we could track over time the x/y coordinate of the local maxima of the waves of activity observed at the surface of the GCaMP6f-expressing cortex. This allowed us to locate the wave in forelimb S1 that was associated with the brush stimulations.

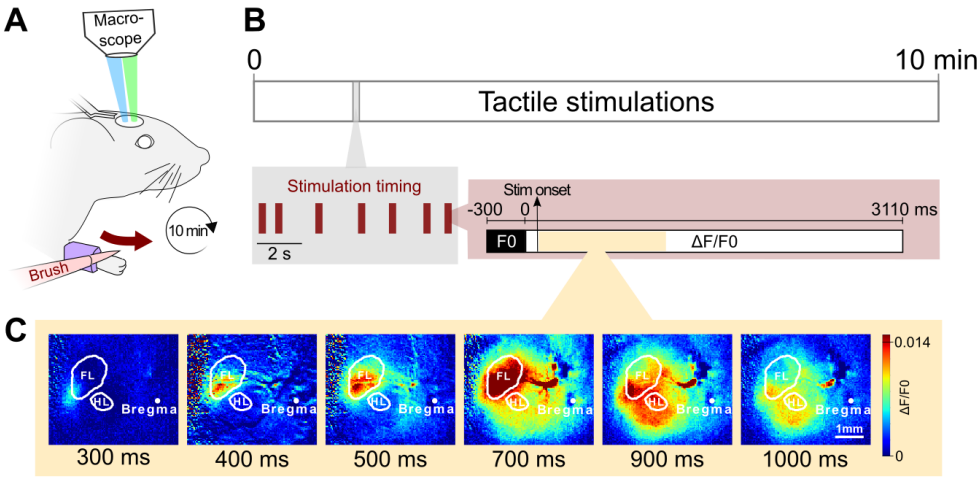

Figure 2.2: Experimental setup for mesoscale imaging of cortical activity generated by brush strokes.
(A) Head fixed mice are placed on the setup with right forelimb restricted. Brush strokes are applied for 10 min at random intervals between 2000 and 2500msec. Alternating blue and green LED flashes allow the recording of images at a 100Hz corrected for hemodynamic artefacts.
(B) Data acquisition timing. The dark red bars represent the TTL signals recorded indicating the activation of the motor applying the brush strokes, these signals last for 300 msec.
(C) Example mouse 28: Cortical activity is observed 300 msec after stimulus onset. Cortical waves propagate in forelimb S1 for up to 700 msec after the tactile stimulation is applied. The FL/HL areas indicated in white have been previously traced during a separate imaging session with a piezoelectric sensor.

2.3.1.3 Results

We observed cortical activation approximately 300 msec after stimulus onset, when the brush made contact with the animal's forelimb (Figure 2.3C). During wrist-to-tip stimulations, all mice exhibited activity starting in the middle/lower regions and progressing along the

anterior axis over 700 to 800 msec (Figure 2.3). In mice 22 and 24, this progression was primarily along the anterior axis (Figure 2.3A), whereas in mice 28 and 02, the wave advanced along both the anterior and lateral axes towards Bregma (Figure 2.3B). This variability can be partially attributed to differences in paw positioning and brush contact, which varied among individual animals due to the limited control we had over the positioning of the limb.

Moreover, when we reversed the direction of stimulation, we observed similar activity, but it moved in the opposite direction, starting from the endpoint of the previous stimulations and ending at the starting point of the wrist-to-tip stimulation (Figure 2.3B).

The positions of the peak of the brush-triggered activity did not always locate into the previously traced forelimb area. To investigate this further, we performed an intrinsic imaging session on mouse 24 *(see 2.2.2 for methods)*. The intrinsic imaging revealed a forelimb area much larger than that found in the initial calcium imaging session, although their positions overlapped, and the larger intrinsic imaging area encompassed the smaller calcium imaging one. This suggests that the size and shape of the forelimb area vary depending on the type of stimulus applied (brush stroke vs. piezoelectric sensor). Nonetheless, the forelimb areas were anatomically accurate, differing mainly in shape and size.

Despite the observed variability, a clear pattern emerged. In all mice, we noted a consistent back-and-forth progression of cortical activity generated by the brush strokes on a distance of 4 to 6 mm, following the direction of the applied stimulus. This provided us with a clearer framework for the type of activity we aimed to mimic using optogenetic photostimulations to create the embodiment of the artificial limb

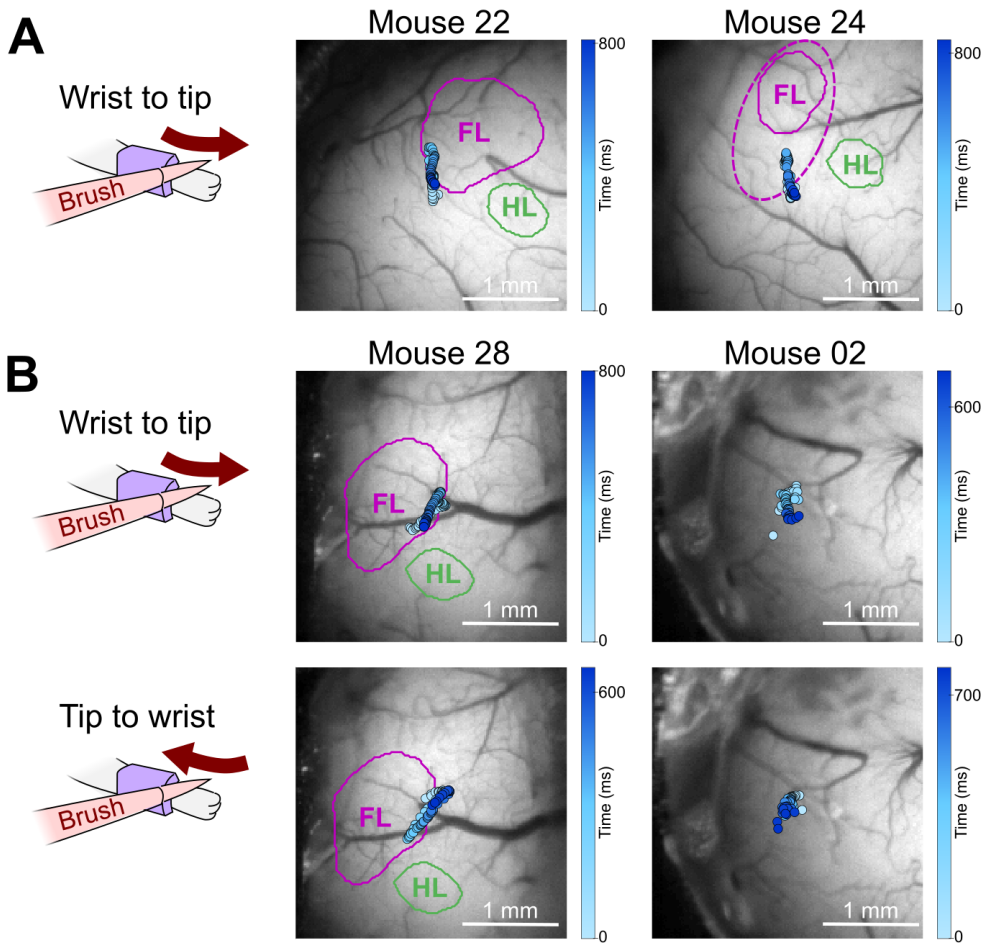

Figure 2.3: Cortical signal progression during brush strokes

(A) Mouse 22 and 24 were exposed to one type of stimulation from wrist to top. The blue gradient represents the waves progression over time, progressing similarly for both mice along the anterior axis. The magenta and green areas represent the forelimb and hindlimb areas traced on the first calcium imaging session. The dashed magenta line represents the FL area traced for mouse 24 during the intrinsic imaging session.

(B) Mouse 28 and 02 were exposed to the same wrist to tip stimulation as well as the reverse direction. The averaged signal of the repeated stimulations shows a propagation that seems to follow the direction of the brush strokes applied.

2.3.2 Optogenetic rubber paw illusion

2.3.2.1 Participation statement

This work was conducted in collaboration with Océane Samarasinghe, an M2 student who interned in our lab during the first half of 2024. I developed the setup for optogenetic stimulations and created the codes for data analysis. Additionally, I performed all the surgeries and carried out some of the experiments. I trained Océane Samarasinghe to perform the tests, and she subsequently carried out the majority of the experiments presented here and analysed a portion of the data. I performed the perfusions necessary to extract the brains and subsequent histology and staining were performed by Guillaume Hucher.

2.3.2.2 Materials and methods

2.3.2.2.1 Installation of optical window and intrinsic imaging

The surgeries for the optical window implantation follow the same steps as previously described for the head implant surgery until the skull cleaning *(See BioRxiv Pre-Print Chapter 2.1)*. After this step, a craniotomy was performed to remove a 4 mm diameter disk of skull over forelimb S1 on the left hemisphere, located on coordinates 0.0 – 0.7 mm posterior and 2.2 – 2.5 mm lateral (Guo et al., 2020). A glass cover slip of the same diameter was then glued on the sides with cyanoacrylate glue, after that a layer of dental cement was added to cover the rest of the skull.

After 10-15 days of recovery post-surgery, a session of intrinsic imaging was performed to locate the position of forelimb S1 in the cortex. Mice were placed under 1% isoflurane anaesthesia. The paw was placed on top of a piezoelectric bender (Physics Instruments) applying stimulations at 100 Hz while 625 nm red light illuminated the window. Images (659 x 494 px) were acquired with a CCD camera at 60 fps. Space-time fluctuations in luminescence were analysed to locate the position of forelimb S1 (Optimage, Thomas Deneux, NeuroPSI)(Lassagne et al., 2022). 20 repetitions of the stimulation were applied to the right forelimb, right hindlimb, and vibrissae E1 using a piezoelectric actuator. The signals obtained 2 seconds before the

stimulation were averaged to establish a baseline signal, by which each signal from the session was divided. The images were normalized by averaging a 2-second post-stimulus interval to obtain an image of the cortical response. The boundaries of the cortical representation of the stimulated area were manually traced as visually selected regions of minimal luminance in the response image (Figure 2.4C-D).

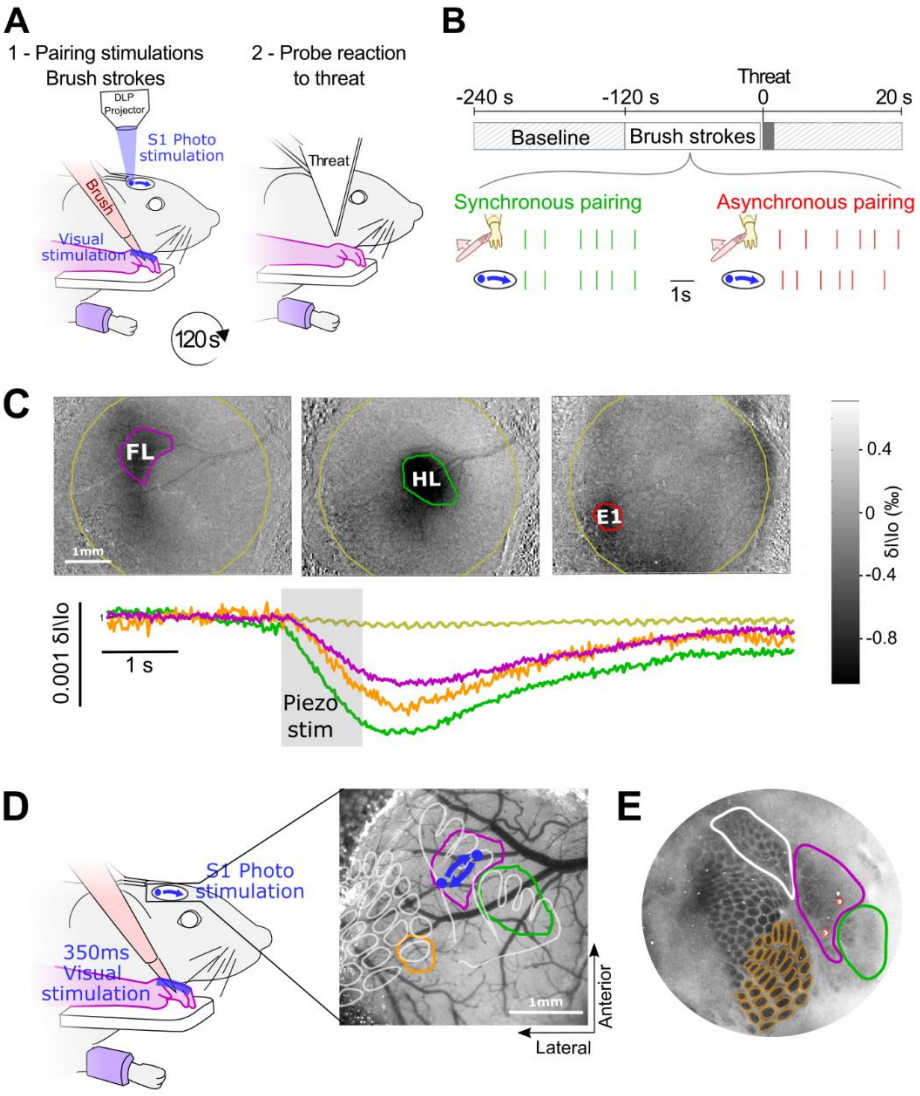

Figure 2.4: Experimental protocol for optogenetic rubber paw illusions
(A) Protocol schematic. During 120 seconds, brush stimulations were applied to the artificial limb (visual stimulation) while photostimulations were applied simultaneously on forelimb S1. Embodiment of the artificial limb was then tested by threatening the artificial limb, and probing the intensity of the reaction of the mouse.

(B) Top: timeline of the pairing and test sessions. 9 mice were exposed to the protocol: 5 sessions were run for each experiment (1 session per day). During each session, the mice were exposed to 2 trials (synchronous and asynchronous pairing). Bottom: example brush stroke and photostimulation times for the synchronous (green) and asynchronous (red) strokes.
(C) Intrinsic imaging to locate the forelimb (FL), hindlimb (HL) and E1 representation in the somatosensory cortex. The areas traced were manually traced based on a visual selection of the region that showed minimal luminance.
(D) Setup for optogenetic stimulation. The zoom-in window shows a picture of the cortical window, overlayed with a reconstruction of the map of somatosensory barrels borders (white). The forelimb intrinsic imaging signal border is in cyan. Hindlimb in green. E1 whisker barrel in yellow. The general spatial extent and dynamics of the brush-mimicking stimulation is shown as blue arrows showing the trajectory of the photostimulation stop performing back and forth movements at the surface of the cortex.
(E) Marking by cytochrome oxidase of a tangential section of flattened cortex corresponding to layer 4, observed under epifluorescence microscopy (X10). The orange points represent the visible fluorescence markings of DiI used to delineate the endpoints of the photostimulation. Shades of grey depict the cytochrome oxidase staining of the barrels in layer 4 of the somatosensory cortex. The manual delineations highlight the cortical regions: orange for the barrel cortex, cyan for the forelimb areas, green for the hindlimb areas, and white for the face/tongue regions.

2.3.2.2.2 Behavioural optogenetic stimulations:

The goal of the third experimental step of protocol was to carry out, in the implanted mice, the rubber paw illusion, while replacing the peripheral touch stimulations applied to the right forelimb of the mouse by a brush stroke-like photostimulation of forelimb S1.

To this aim we used Emx-Cre Ai32 mice expressing Channelrhodopsin2 in pyramidal neurons (Madisen et al., 2012). Stimulations were applied through the optical window with our mesoscale patterning system: A Digital Light Processing module (DLP Vialux V-7001) with a 1024 x 768 Texas Instruments micro mirror ship illuminated by a blue LED at 462 nm. We focused light coming from the DLP with an L shape optical pathway with, light entering a 150 mm SMC Pentax-A 645 Lens and going through a dichroic beam splitter placed at 45° (Semrock 640 nm edge BrightLine). This beam splitter reflected the light to a second lens (45 mm SMC Pentax-A 645) demagnifying the image projected on the mouse cortex by a factor of three (Figure 1.22B). The patterns projected can be updated every millisecond (Abbasi et al., 2018).

Using this mesoscale projection strategy, we conducted tests on 11 animals, and 9 were kept for analysis after histology confirmation of the location of the photostimulation inside the forelimb primary somatosensory cortex *(See histology section below)*.

The mice were first placed in a pod with their heads fixed and their paws restrained by a handcuff for 5 days to habituate them. Following the initial 10-minute session on the first day, the next four sessions lasted 20 minutes each. The mice were given sweetened water to associate the habituation with a positive event and minimize stress.

This experiment follows the same protocol as the rubber paw illusion test based on peripheral touch inputs that we first carried (See 2.1 paper for methods). The only difference consisted in replacing the brush strokes previously applied on the right forepaw by direct cortical photostimulations. Brush movements on the artificial limb covered a distance of 6 mm between the end of the distal limb and the beginning of the fingers, in a back-and-forth motion lasting 350 msec (Figure 2.3A). During the test condition, these brush movements were synchronously coupled with the movement of a light spot sweeping across the representation of the right forelimb on the surface of the somatosensory cortex, mirroring the spatiotemporal dynamics of the brush at the periphery. The photostimulation pattern involved a back-and-forth movement of a 200 µm light spot, traveling a distance of 600 µm on the cortex over a duration of 350 msec (Figure 2.3D).

2.3.2.2.3 Histology

To verify that the photostimulations were correctly located in the forelimb area, we systematically performed histological analysis of the photostimulation sites on the mice's cortex in relation to the sensory map obtained by revealing mitochondrially dense cortical areas using cytochrome oxidase staining.

For this purpose, we marked the start and end points of the photostimulation locations with a DiI dye deposit administered under anaesthesia (Isoflurane, 4% for induction, 1.5 - 2% for maintenance). The procedure was followed by a pentobarbital injection (Dolethal, 150 mg/kg) before exsanguinating and perfusing the animals with 4%

paraformaldehyde (PFA). We then extracted the brains, isolated and flattened the cortices. These were stored overnight in 4% PFA and subsequently transferred to PBS (phosphate-buffered saline). The transverse sections (50 µm) were treated with cytochrome oxidase, and the results were examined using visible light microscopy (cytochrome oxidase) and fluorescence microscopy (DiI) (Figure 2.4E).

In two mice, the histological analysis revealed that the photostimulations were not located within the S1 area associated with the forelimb. They were excluded from the analysis.

2.3.2.2.4 Statistical analysis

The average right pupil positions and whisker positions were averaged for the 9 mice used for this experiment. To correct for baseline shifts, we subtracted the mean position of the tracked position measured during the 120 sec baseline that proceeded the brush stimulations. When looking at the effect after the threat, we subtracted the mean values 1s before the threat so we could normalize to pre-threat positions that may not be the same from trial to trial.

Statistical significance was assessed with a non-parametric Wilcoxon (paired) test. We performed statistical tests on the time window 1-6 sec after the threat's arrival, which comprised of 5 bins of 1 sec each. For the whisker we also repeated this analysis on a 10-15 sec time window, corresponding to the removal of the threat.

2.3.2.3 Results

2.3.2.3.1 Right pupil shifts towards the threatened artificial limb

During the pairing phase, which involved synchronous or non-synchronous photostimulations and brush stimulations of the artificial limb, we observed a significant change in the vertical position of the right pupil. Specifically, the pupil was positioned higher during synchronous stimulation compared to asynchronous stimulation. There were no significant differences observed in the horizontal position of the pupil during this phase (Figure 2.5B).

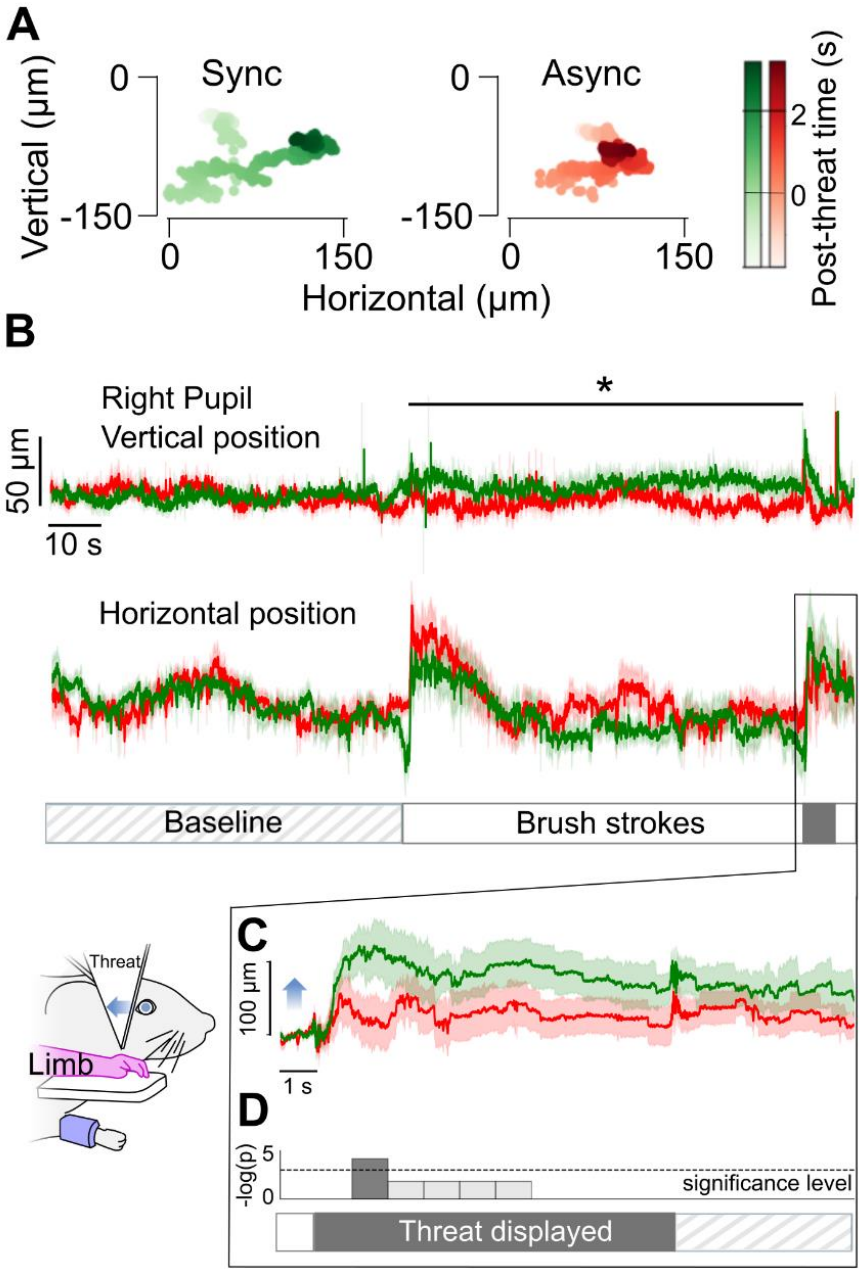

Figure 2.5: Stronger right pupil shifts during synchronous pairing.

(A) Spatial distribution of the right pupil position 0 to 2 sec after the threat onset. Mice shift their pupil in the direction of the threatened area. The average pupil positions in the synchronous condition are the most

(B) Average vertical (top) and horizontal (bottom) movements of the right pupil during synchronous and asynchronous condition trials, normalized to the average position during the 120 sec baseline (n = 9). The sequence includes a Baseline, Brush strokes pairing, and a threat to the artificial forelimb (n = 9). Horizontal axis, Wilcoxon p= 0.50. vertical axis: Wilcoxon p = 0.02.

(C) Average horizontal movements of the right pupil following the threat onset, normalized relative to the average position 1 sec before the threat (n=9). Light background: SEM. Blue arrow: direction of pupil movement
(D) Statistical comparison of right pupil positions averages, 1-6 sec after threat (1 sec bins). Bars show -log of the Wilcoxon p-values with Benjamini-Hochberg correction. p= 0.016, 0.16, 0.16, 0.16, 0.16. Lighter gray: time bins that did not reaching significance, Darker gray: time bins reaching significance. Dark gray: time bins reaching p = 0.05 threshold.

After the introduction of a threatening object to the artificial limb, we noted a significant difference in horizontal pupil shifts between the two conditions (Figure 2.5A,C,D). In the first time bin (1-2 sec after the threat presentation), the right pupil shifted more significantly in the direction of the threatened prosthesis area during synchronous stimulation compared to asynchronous stimulation (Figure 2.5D). This initial difference was notable, indicating a stronger gaze shift towards the threatened object in the test condition.

Note that tracking of the left pupil was not possible, as several mice developed a transient white cast on the left eye, that made it difficult to track the pupil. We are not sure why this white cast was present. We hypothesize that it may be related to stress or illumination.

2.3.2.3.2 Shifts in whisker positions are stronger in synchronous conditions

In addition to monitoring pupil shifts, we assessed whisker movements as indicators of artificial limb embodiment. During the initial response to the threat, whiskers exhibited a pronounced movement forward and downward. We observed significant differences in whisker positions during the final time bin (5-6 seconds post-threat) (Figure 2.6A,B,D,E). Specifically, both horizontal and vertical whisker positions differed significantly between synchronous and asynchronous conditions during this period.

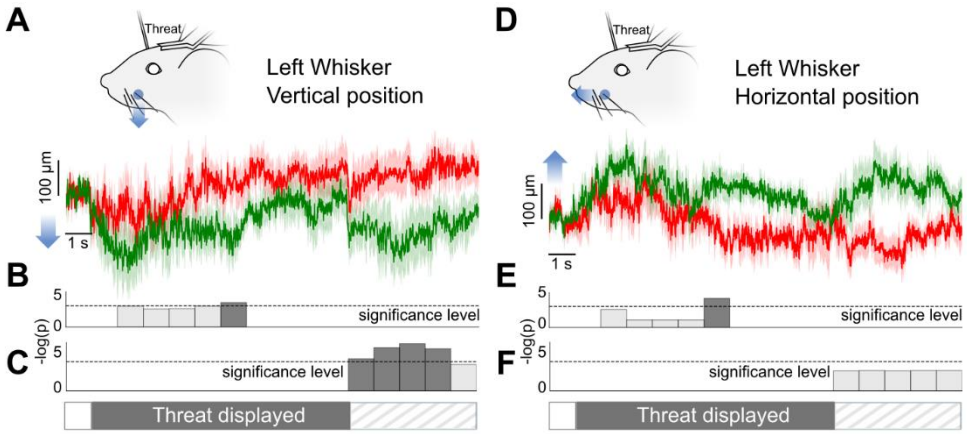

Figure 2.6: Stronger whisker movements after synchronous pairing

(A) Top: Average vertical movements of the left whisker following the threat onset, normalized relative to the average position 1 sec before the threat (n=10). Light background: SEM. Pink arrow: direction of whisker movement

(B) Statistical comparison of left whisker positions averages, 1-6 sec after threat (1 sec bins). Bars show -log of the Wilcoxon p-values with Benjamini-Hochberg correction. p= 0.078, 0.34, 0.34, 0.34, 0.016. Lighter gray: time bins not reaching significance, Darker gray: time bins reaching significance. Dark gray: time bins reaching p = 0.05 threshold.

(C) Same as B for the time window 10-15 seconds after threat (0-5 sec after removal of threat) p=0.037, 0.012, 0.0078, 0.013, 0.066.

(D) Same as A for the horizontal axis.

(E) Same as B for the horizontal axis p= 0.052, 0.078, 0.074, 0.052, 0.031.

(F) Same as C for the horizontal axis p=0.13, 0.12, 0.13, 0.12, 0.12.

Following the retraction of the threatening object, we repeated the analysis of whisker movements (Figure 2.6B,F). In this phase, no significant differences were found in horizontal whisker positions between the synchronous and asynchronous conditions (Figure 2.6F). However, vertical whisker movements showed significant differences in all time bins except the final one. This indicates that the impact of the threat's retraction on vertical whisker positions persisted over time after the removal of the threat (Figure 2.6C).

2.3.2.3.3 Partial conclusion

In our experiments, we observed that cortical photostimulations synchronous to visual brush strokes elicited stronger reactions to the threat, in both pupil and whisker movements, when compared to asynchronous stimulation. These findings mirror the results from our initial study using peripheral stimulations, where we reported enhanced responses in pupil shifts and whisker speed after synchronized stimulation of the artificial limb. This preliminary data suggests that it is feasible to provide artificial somatosensory feedback to mice in a manner that promotes the embodiment of an artificial limb. By aligning the timing of somatosensory inputs with the corresponding artificial limb stimuli, we can enhance the animal's integration of the artificial limb, shown through their heightened reaction to its threat.

It is important to note that these results are still preliminary. Further research is needed to confirm the robustness and reliability of these findings. One area that warrants additional exploration is the investigation of different feedback structures. representation is critical for inducing the embodiment of artificial limbs.

There are numerous parameters that we can tweak to optimize our results. We could experiment with different shapes and sizes of the photostimulus, as well as varying direction, frequency, and intensity of the stimulations. These modifications could potentially strengthen the responses observed, as our optogenetic results are not as prominent as those achieved with peripheral stimulations. Fine-tuning these parameters is essential to determine the most effective conditions for inducing artificial limb embodiment in mice.

In our experiments, extensive histological analysis and intrinsic imaging were essential for accurately localizing the forelimb area in S1. While intrinsic imaging provided a general location for the forelimb area, it did not offer precise topographical details. The regions identified through this technique appeared to encompass the upper arm, paw, and fingers, making it challenging to determine the exact placement for photostimulations to generate illusory touch sensations, such as brush strokes, on the paw. Additionally, the signal in the

forelimb area tended to be more diffuse compared to other regions.

To refine our targeting, we incorporated histological checks, which helped us understand how we might have missed the precise area in question. We adapted our approach by adding two other reference regions to our intrinsic imaging: the hindlimb (HL) and the E1 whisker barrel, both of which show reliable signals. By comparing our histological maps with those found in the literature (Gămănuţ et al., 2018; Hoover et al., 2003; Sigl-Glöckner et al., 2019; Vanni et al., 2017; Yamawaki et al., 2021), we used these reference points to better locate the specific area of interest in our forelimb S1 region. We observed that the paw area is generally somewhat perpendicular to the hindlimb region and more anterior compared to the E1 whisker barrel (Figure 2.3C-E). Notably, we found that our areas of interest in the forelimb S1 tended to be parallel to the E7 and E8 whiskers. However, due to the small size of these whiskers, targeting a larger, easily identifiable whisker like E1 proved more effective.

These adjustments allowed us to better locate the correct region in the forelimb S1 area. By leveraging the anatomical landmarks provided by reliable signal regions, we were able to improve the precision of our optogenetic stimulations and enhance the effectiveness of our experiments.

Another point that be discussed is the use of EMX-Cre-AI32 mice for this experiment which express ChR2 in all pyramidal neurons. Another strategy could be targeting layer 4 of the cortex, which is the thalomorecipient layer of the cortex in which the anatomical and functional segregation of sensory responses is maximal *(See 3. Discussion)*.

Overall, while our findings provide a promising foundation for using optogenetic stimulations to provide somatosensory feedback for embodiment, additional exploration is needed to confirm these results.

2.4 MOTORIZED PROSTHESIS PROTOTYPING

Existing brain-machine interface systems for mice provide valuable insights, but they don't allow us to explore the embodiment of an artificial limb and the role it may play in enhancing integration and motor control. To address this and to allow us to investigate questions related to more naturalistic limb control and the concept of embodiment, a part of my work during my first year as a PhD student in the lab was to contribute to the development of a prosthetic limb that closely resembles the natural forepaw of a mouse.

Our goal was to create a motorized forepaw that would enable upper limb neuroprosthetic research in mice, thus enhancing our understanding of how somatosensory and motor integration can improve the functionality and acceptance of a prosthetic limb. I have presented the general principles of this prosthesis in the thesis introduction as a context to the embodiment work (part 1.3.1). Here, I aim to detail my contribution to this effort.

2.4.1 Participation statement

I was involved in the first stages of the design of the prosthesis, which was then further developed by PhD student Edouard Ferrand. This work was carried in collaboration with Marie Engel, an M2 student in our lab during the first half of 2021. Marie was responsible for designing the 3D models used to print the prosthesis, as well as printing and assembling the robotized limb. Prior to Marie's arrival, I developed the initial prototypes of the prosthetic limb. This involved modifying the 3D components, printing and assembling prototypes of the robotized arm, and experimenting with various textures and materials for the prosthesis envelope.

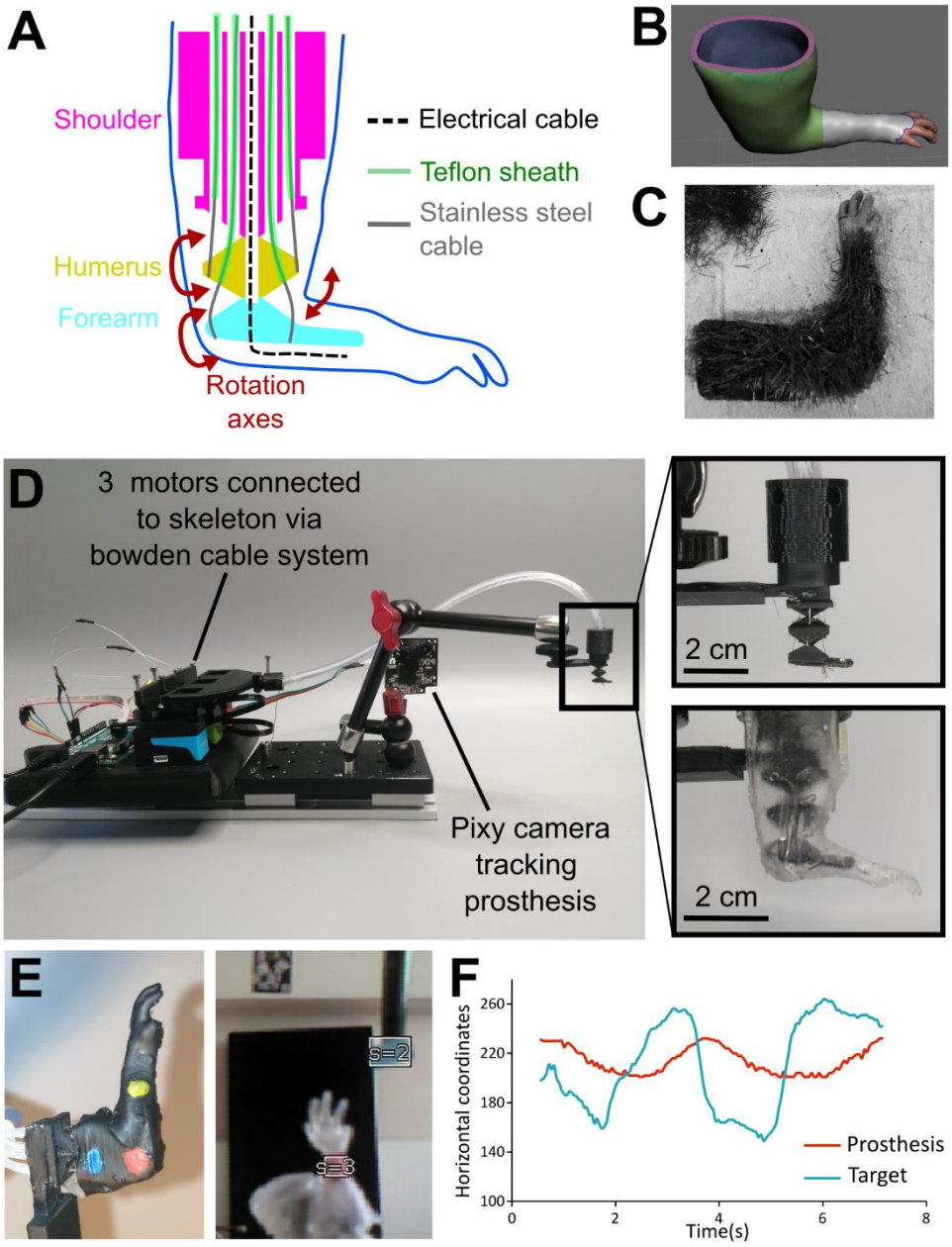

Figure 2.7: Motorized mouse forelimb prototype

(A) Side view of the internal structure of the prosthesis: 3 pairs of sheathed cables are attached to the ends of the humerus and forearm, allowing for 3 degrees of freedom. A capacitive cable connects all parts of the prosthesis through a central channel (Adapted from Marie Engel).

(B) 3D model of prosthesis envelope modified in Meshmixer (Autodesk), and printed in flexible or elastic resin (Formlab Form 3)

(C) Texturizing the artificial limb. The use of static grass (miniature modelling grass) was explored to apply synthetic fibers on the paw to give the subject the illusion of fur.

(D) Motor system controlling the prosthesis: This prototype had 3 degrees of freedom. Each pair of steel cable is attached to one motor, effectively controlling one degree of freedom. Close up pictures show the printed prosthesis skeleton and the skeleton when covered with the elastic envelope.
(E) Left: Envelope painted with acrylic paint and marked with colourful points for tracking. Right: Pixy camera feed: Envelope painted with a red dot that is being tracked in real time by pixy camera. The blue dot is a target that the prosthesis has to follow
(F) Tracking of horizontal coordinates of the prosthesis and of a target that the prosthesis tracked.

2.4.2 Functioning of the prosthesis

This first prosthesis prototype was composed of three parts representing the shoulder, humerus, and forearm. It was designed using SolidWorks software and printed with both filament and resin printers, the latter being used for components requiring a resolution finer than 0.01 mm. These three parts, stacked one on top of the other were physically connected by a shielded electrical cable that runs through a central channel. This cable serves both to hold the parts together and to potentially carry information from sensors (Figure 2.7A).

The prosthesis featured three degrees of freedom: two for the shoulder (one around the anteroposterior axis and another around the transverse axis), and one for the elbow joint (around the transverse axis). To facilitate movement, pairs of stainless-steel cables (0.2 mm diameter) were attached to the ends of the components using cyanoacrylate glue. They operated in opposition, with one being pushed and the other pulled. For this flexible transmission of movement, the stainless-steel cables operated as Bowden cables. In this transmission system, a tension-resisting cable that can flex slides within a pressure-resistant sheath that can also flex laterally. This system allows for the transmission of force over a distance, while allowing for bending (Figure 2.7A,D).

For this first version of the prosthesis, motor control was achieved by three servo-motors Smart-Servo MS-12A (Makeblock). Thanks to the Bowden cables system, the servomotors — which were connected to the prosthesis each by a pair of stainless-steel cables (Figure 2.7D) — could operate at a distance of the prosthesis.

To ensure the components and their assembly functioned correctly, I conducted multiple printing tests to prototype and refined the design. This involved testing and ordering different types of Bowden cables, addressing the specific requirements for successful operation, and making necessary modifications. Through these tests, I was able to identify materials and settings as well as construct a functional first prototype and identify its limitations. This iterative process highlighted challenges such as servo-motor performance issues, or the twisting of the Bowden cables, which reduced the force transmitted by the servomotors to the joints. This informed subsequent adjustments and improvements on the motorized limb.

2.4.3 Prosthesis envelope

This skeleton provided the necessary movements at 3 articulations but didn't resemble the mouse forelimb (Figure 2.7D), an element that we know is important for prosthesis embodiment in humans, where non-limb objects do not give rise to embodiment (Tsakiris & Haggard, 2005). For this reason, the motorized skeleton required an envelope to closely mimic the appearance of an animal's paw.

To address this need, we tested a range of options, including taxidermy techniques, resin casting, and 3D printing. Among these, 3D printing of the envelope emerged as the most flexible and adaptable solution. We initially tested Formlabs Flexible Resin V2. This was the same resin that was used to print the static artificial limb used in the rubber paw illusion protocol. For this, I modified the 3D model of the rubber paw previously printed to fit the skeleton. This involved making this model into a hollow envelope. However, it turned out to be too brittle and prone to breaking under stress (Figure 2.7B). This led us to switch to the more durable Formlabs Elastic 50A resin. Although Elastic 50A posed some challenges in maintaining the necessary thickness at such a small scale, this material allowed us to construct a functional prototype successfully (Figure 2.7D). Moreover, the elastic resin could withstand acrylic paint, which remained firmly adhered and was not displaced by the prosthesis movements. This allowed us to make the prosthetic limb more resembling to a mouse's paw (Figure 2.7E).

To enhance the prosthetic limb's texture, we also tested the possibility of creating a fur-like texture using a static grass system (Figure 2.7C). This method involved applying fine synthetic fibers using electrostatic adhesion, a technique commonly used in model-making to simulate realistic textures. However, preliminary embodiment tests indicated that mice could effectively embody a "smooth" paw *(See Chapter 2.1)*. Consequently, we opted to minimize the use of this system to simplify implementation and avoid issues such as fiber flyways.

Again, this exploration was important in informing the development process. The increased friction introduced by the envelope was a significant factor that restricted the motorized skeleton's movements, highlighting the need for further refinement. This experience underscored the importance of balancing material durability with functional requirements and guided subsequent design improvements to enhance both the prosthesis's appearance and its operational efficiency.

2.4.4 Prosthesis tracking

To track the movements of the prosthesis and potentially correct it, we initially employed a Pixy 2 camera (Charmed Lab, USA). This camera is designed for object tracking through a learning process and provides coordinates with a frequency of 20 Hz. To facilitate this tracking, coloured markers were painted onto the surface of the prosthesis, including one at the tip of the limb, to assess the camera's ability to follow these points (Figure 2.7E,F). Preliminary tests showed the camera's ability to track several coloured points on the image and track the prosthesis position in real time (Figure 2.7F).

However, we encountered significant limitations with this approach. The Pixy 2 camera proved to be highly sensitive to changes in lighting conditions, which affected its tracking accuracy. Variations in ambient light caused inconsistencies in the data, making it challenging to obtain reliable information and imposing stringent requirements for stable lighting conditions to ensure reliable tracking. Moreover, the Pixy interface generated high latencies in our system, proving to be too slow for this application (Figure 2.7F).

This experience highlighted the need for a more robust tracking system. Consequently, we transitioned to using Infrared LED tracking, which has proven to be more effective in maintaining accuracy despite changes in lighting. This method was implemented in the latest iteration of Promice, addressing the limitations encountered with the initial tracking setup and providing a more reliable means of monitoring the prosthesis's movements.

2.4.5 Conclusion

In conclusion, with this work, I contributed in developing a motorized forepaw prosthesis that closely resembles a natural mouse limb. This included prototyping the initial designs and identifying limitations in the early iterations. This process led to crucial redesigns of the 3D models to improve movement and the selection of appropriate materials to better simulate a mouse forelimb. These efforts resulted in a functional first prototype, that guided the development of further iterations of the "Promice" motorized mouse forelimb.

PART 3

GENERAL DISCUSSION

3 GENERAL DISCUSSION

3.1 Embodiment like behaviour in the mouse model

My thesis work focused on developing a protocol to study embodiment-like behaviour in the mouse model and how somatosensory feedback shapes it.

Our main finding is the replication of the rubber hand illusion paradigm in the mouse model. In our main study, we exposed mice to an artificial limb resembling their own and applied simultaneous brush strokes to both limbs either synchronously or asynchronously. When this artificial limb was threatened, we observed that the mice reacted more strongly after congruent anatomical information and tactile feedback were provided. Specifically, we observed pupil shifts in the direction of the threatened area.

Our hypothesis is that two mechanisms occur here: first the animals are focusing their attention on the fake limb after the threat onset. Secondly, mice are trying to turn their heads towards the threatened artificial limb which results in coupled right and left pupil movements. This is a likely interpretation of our findings, as movements tend to be conjugated, in head fixed animals, with attempted head rotations (Meyer et al., 2020). This is also consistent with findings on the rubber tail illusion in mice where non-head fixed mice have been shown to turn their heads towards the fake tail after it is grasped (Buckmaster et al., 2020; Wada et al., 2016).

We also observed changes in pupil diameter and enhanced whisking speed, as well as ear movement speed, although these effects seemed less pronounced than the pupil shifts.

Pupil dilation was observed following the threat to the rubber paw, indicating strong engagement and arousal. Interestingly, in the synchronous condition, the right pupil diameter was significantly smaller within 1-2 seconds after the threat. This delayed pupil constriction, may reflect cognitive processes such as memory recollection or adaptation to novel stimuli (Kafkas & Montaldi, 2015;

Naber et al., 2013).

Additionally, facial expression tracking revealed faster movements of the ear and whisker pad areas in response to the threat of the prosthesis-like object after synchronous stimulations. While these changes were less prominent compared to pupil shifts, they provided valuable insights into the mice's emotional responses. The increased ear and whisker pad movements suggest a heightened grimace response, consistent with the manifestation of fear or anxiety in mice (Dolensek et al., 2020).

To our knowledge, this is the first time that artificial forelimb embodiment is studied in mice, using automated tracking of animal behaviour.

We relied on an affective measurement of embodiment (Table 1.1) to assess the impact of our experimental interventions, considering the challenges inherent to working with non-verbal subjects like mice. Unlike human participants, we could not employ subjective questionnaires or self-reported measures to gauge their experiences or perceptions of embodiment. Instead, we utilized behavioural indicators and physiological responses to infer the level of embodiment experienced by the mice when exposed to the artificial limb.

We argue that our protocol can be directly compared with established rubber hand illusion protocols, where the threat of a limb object results in a more pronounced stress reaction following synchronous stimulations (Armel & Ramachandran, 2003; Ehrsson et al., 2007). In these protocols, the alignment of tactile stimuli between the real and artificial limb fosters a sense of ownership over the artificial limb, leading to heightened stress responses when that limb is threatened. Our study mirrors this phenomenon, as we observed that mice reacted significantly more intensely to threats directed at the artificial limb when they received congruent tactile feedback. We suggest that the observed behavioural responses of the mice — such as pupil dilation, heightened whisking speed, and increased ear movement speed — reflect a threat response linked to the perception of the artificial limb as being integrated into their body schema. This integration likely

occurs through the synchronization of sensory inputs, akin to how humans experience ownership over a rubber hand in similar paradigms.

3.2 Higher cognitive functions in mice

Our findings suggest that mice are capable of self-perception and embodiment, which are often regarded as higher cognitive functions.

Rodents exhibit a range of cognitively complex behaviours, demonstrating their capacity for sophisticated interactions and emotional responses. For example, rats have been shown to engage in complex social play, such as hide-and-seek with humans. In this context, they not only learn the rules of the game, such as seeking hidden humans or hiding themselves, but they also exhibit appropriate responses like vocalizing during seeking and finding, and remaining silent while hiding. This behaviour highlights their ability to understand and participate in social games, driven by the rewarding nature of social interaction (Reinhold et al., 2019).

Furthermore, recent research into the neurological underpinnings of rodent play behaviour reveals that the periaqueductal grey in the brainstem is a key region for play behaviour, and manipulating this area can disrupt playfulness and ticklishness. This suggests that rodents possess intrinsic neural circuits that enable complex interactions, driven by fundamental brain structures (Gloveli et al., 2023).

Similarly, in our experiments, mice not only integrate congruent tactile stimuli, but do so in a specific context that leads to the artificial limb being considered a part of the body, also taking into account the shape of the object being stimulated.

This and previous work in the mouse rubber tail illusion (Wada et al., 2016, 2019) address a question that has historically focused on a narrow set of species, primarily great apes, when exploring complex notions of self. One of the pioneering tests to study this notion was developed in 1970 by Gordon Gallup, who explored the ability of apes to display visual self-recognition. Anesthetized chimpanzees were marked with a red dot on their foreheads. Upon their awakening, they

were placed in front of a mirror and self-directed movements towards the spot was used to assess self-recognition (Gallup, 1970).

Other species have been shown to pass this test such as dolphins or elephants (Plotnik et al., 2006; Reiss & Marino, 2001). However, this test has its limitations, as individuals who fail the test might still exhibit some sense of 'self' in other contexts, and individual differences might play a role.

Birds for instance, fail this test in majority, often reacting to their mirror image as if it were another bird, exhibiting aggressive or social behaviours instead. Such behaviours have been reported in various species of songbirds, gulls, grouse, and parrots... However, some exceptions exist, such as the magpie, which has shown evidence of self-recognition. Additionally, research on petrels and other species indicates that while they may not recognize themselves visually, they use other senses, like olfaction and vocalization, to recognize and differentiate themselves and others in their environment (Derégnaucourt & Bovet, 2016). Thus, the exploration of self-recognition and embodiment extends beyond just visual cues to include a range of sensory modalities and behavioural responses.

Building on this understanding, recent research by Yokose et al. has further expanded our knowledge of self-recognition in rodents. Indeed, the authors developed a variation of the mirror self-recognition test in mice, where they showed that black mice would spend more time grooming themselves in front of a mirror when their head was covered by a white ink dot. They did not exhibit this behaviour with black ink or with smaller dots. Previous habituation to the mirror also helped facilitate this phenomenon (Yokose et al., 2024). This suggests that mice can display visual self-recognition in certain circumstances.

Deafferentation models using rodents can also give an indication of sensory recognition of their body parts. Studies have shown that after deafferentation, where sensory input from a limb is severed, rodents such as rats and mice often display autotomy, or self-amputation, of the affected limb (Coderre et al., 1986; Jergova et al., 2021). This behaviour, linked to phantom pain, suggests that rodents are capable

of a form of body recognition, as they react to the altered sensory input by attempting to remove the non-functional part of their body. This response suggests that they have a concept of "mine-ness" and can detect discrepancies in sensory feedback (Bekoff, 2002).

To probe whether these elements of bodily self-recognition can be transferred to a prosthetic limb, we have developed a mouse forelimb prosthesis and we have found that mice can learn to control this prosthesis by modulating their neuronal activity (Figure 1.16). This expands our previous brain-machine interface systems by incorporating a physical, limb-like object that the mouse can both see and manipulate. This approach provides us with a platform to study the concept of embodiment in relation to motor control. By equipping mice with a visible and controllable artificial limb, we can explore their capacity to adapt to and incorporate this prosthesis into their body schema. Additionally, we aim to enhance this system by integrating somatosensory feedback, using the knowledge acquired from the rubber paw illusion studies. A closed-loop system would be essential for comprehensively studying, motor learning, sensory integration, and embodiment. By combining visual and tactile feedback, we can better understand how sensory inputs are processed and utilized by the brain to achieve coordinated movements with an integrated prosthetic limb.

To fully appreciate the implications of these findings, it's crucial to acknowledge the limitations and unique sensory modalities of our animal model. Although the data we acquired shows results that are consistent with embodiment, our approach remains anthropomorphic. It is important to consider that mice have a different sensory scenery than humans. For instance, mice rely heavily on their olfactory and whisker-mediated tactile senses, whereas humans predominantly use visual and limb touch for equivalent tasks. This difference in sensory reliance means that the way mice perceive and integrate sensory stimuli differs significantly from humans. This could explain some of the variability we see in our data. Indeed, our results reflect an averaged effect across multiple mice, and significant variability exists in individual responses *(See Figure 2A of Part 2 Hayatou et al. manuscript)*. This variability makes it challenging to fully elucidate the specific mechanisms at play for each mouse, especially those showing

minimal or ambiguous effects.

3.3 Advantages and limitations of videography-based approach

In our system, we relied on high-frequency videography to capture the behaviour of mice, which we then analysed using DeepLabCut. This allowed us to quantify the animals' reactivity to the experiment in a non-invasive way.

Indeed, the use of this method facilitated the setup of this paradigm. One of the primary advantages of videography is its flexibility and adaptability. Since it does not necessitate the implantation of additional sensors, it simplifies the setup and allows for easy adjustments to experimental conditions. Videography enables comprehensive behavioural monitoring by capturing a variety of features such as general movements, pupil shifts, whisking, and ear movements. This broad overview of the animals' responses provides valuable data for our analysis.

Additionally, we may use our video data for more sophisticated facial analysis techniques. For instance, Dolensek et al. (2020) employed a machine learning approach to classify different types of facial expressions in mice (Dolensek et al., 2020; Dolensek & Gogolla, 2021). Adopting a similar approach could offer deeper insights into the animals' emotional states and reactions.

However, there are also notable limitations to the videography-based approach we have used. One significant drawback is the difficulty in comparing our results with human studies on the rubber hand illusion, which typically do not use video-based measurements. Human studies often rely on physiological measures such as skin conductance response (SCR) to gauge stress and arousal levels (Armel & Ramachandran, 2003). Incorporating SCR measurements would be particularly interesting, as it is commonly used in human rubber hand illusion studies to provide a direct measure of the autonomic nervous system's response.

Furthermore, while videography allows us to capture visible behavioural features, it lacks the capacity to monitor physiological signals such as electromyographic activity, temperature or heartbeat.

The inclusion of such measures would provide a more comprehensive understanding of the animals' responses.

In conclusion, our setup's adaptability allows it to incorporate various sensors and analytical methods, making it a powerful and flexible platform for studying embodiment and neuroprosthetic integration. This versatility ensures that our research can continue to evolve, incorporating new technologies and methodologies to deepen our understanding of sensory-motor integration and its applications in prosthetic development.

3.4 Optogenetic cortical feedback and topography

In our optogenetics study, we aimed to investigate whether direct cortical stimulation could induce the embodiment of an artificial limb, similar to the effects observed with peripheral tactile stimulations. We replaced the peripheral stimulations with direct cortical stimulations in S1 and observed similar results suggesting that mice exhibit signs of embodiment towards the artificial limb: right pupil shift in the direction of the prosthesis, as well as whisker movements are stronger during the synchronous condition. Our findings suggest that optogenetic stimulations have potential to replace peripheral tactile feedback, and maintain the capacity to induce embodiment.

This study began with the use of calcium imaging to record brain activity during tactile stimulations, specifically brush strokes, on mice's paws. By observing the neuronal activity in response to these peripheral tactile inputs, we gained insights into the patterns of cortical activation within the primary somatosensory cortex. The mesoscale calcium activity displayed back-and-forth movements on the cortex that appeared to correspond to the direction of the brush strokes applied on the paw (Figure 2.3). These recorded patterns served as a reference for our subsequent experiments as we aimed to replicate these physiological responses through photostimulations, intending to create sensory perceptions in mice that were analogous to those generated by natural tactile feedback. This biomimetic approach has been shown to be efficient to produce such percepts (Flesher et al., 2016, 2021).

However, previous studies in our lab, in an optogenetic discrimination task as well as in a closed-loop sensorimotor learning task have also shown that it may not be necessary to mimic natural activity patterns to achieve sensory integration, but that constraints on the structure of the photostimulations still apply. In these specific studies, mice successfully learned an optogenetic sensory or a sensori-motor task that was not inspired by the structure of peripheral inputs. But they could only learn these tasks when the optogenetic stimulation were spatially and temporally contiguous at the surface of the somatosensory cortex (Abbasi et al., 2023; Lassagne et al., 2022).

These previous findings suggest that the spatio-temporal evolution of stimulation patterns within a spatially continuous cortical topography is crucial for effective cortical integration. In particular, these studies showed that learning was significantly impaired when optogenetic stimulations stopped being applied on a topographically continuous area (the whisker barrel cortex) and were instead applied across the borders of multiple adjacent representation of the somatosensory cortex, or even on the non-topographically organized posterior parietal cortex (Figure 1.23) (Lassagne et al., 2022). These results point to a clear challenge regarding the forelimb somatosensory cortex, as this area it is cramped between the jaw representation and the hindlimb representation, and any mistake in the localization of the optogenetic forelimb stimulation would have led to sensory inputs to the mouse that are not matching the visual brush inputs that we provided. And indeed, we found that slightly varying the angle of stimulations within the same general area could impact embodiment behaviour, highlighting the importance of precise spatial and temporal coherence in stimulation. We could investigate further to which degree the angle variations would affect embodiment, in a similar way to human experiments where the rubber hand illusion is maintained when orientation mismatches of the subject's remain small (Costantini & Haggard, 2007) (*See Chapter 1.2.1)*. Extensive imaging and histological analysis were therefore required to identify the exact topography and optimal directions for effective stimulation (Figure 2.4C-E)*, (See Chapter 2.2.2.3.1.3.)*

Many different stimulation parameters can be explored using the setup we built. Beyond representations of inputs that are based on the

topographical maps present at the surface of the primary somatosensory cortex, we may experiment with various shapes, sizes and positioning of photostimulation patterns. And if we remain focused on biomimetic inputs, we could attempt more elaborate input strategies. Indeed, so far, we have used a generic, spotlight photostimulation pattern that moved back and forth on the forelimb somatosensory cortex coherently with the brush. Given our access to GCamP6 imaging of the specific cortical activation patterns that are triggered by actual brush strokes, we may be able to generate patterns of photostimulations that more closely emulate the physiological inputs. Fine-tuning the photostimulation may have a direct impact on the level of embodiment achieved in our experiments, as we have found so far that they remain lower than those that we achieved with peripheral stimulations.

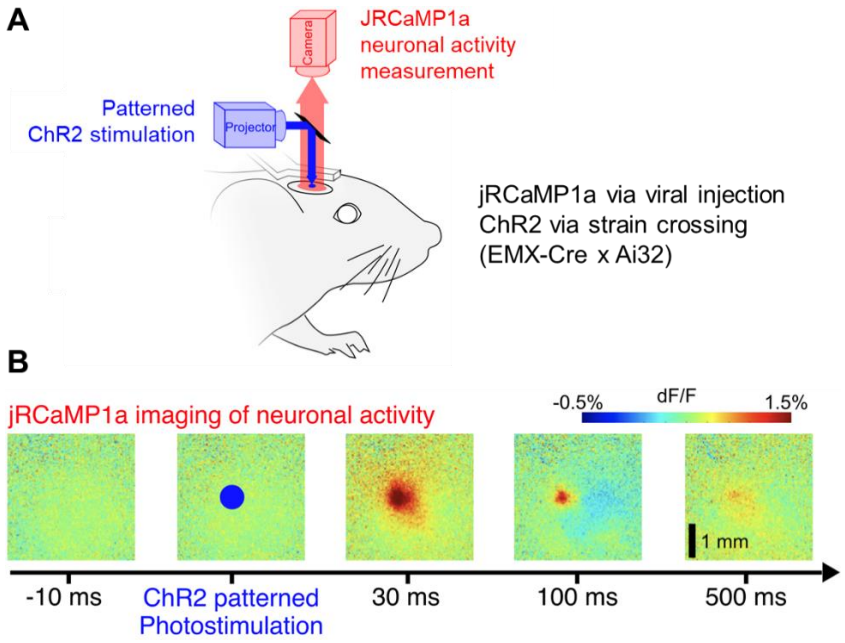

Figure 3.1: Recording of cortical activity during optogenetic stimulations (Adapted from a Cosyne abstract by A. Dogadov 2022).

(A) EMX-Cre x Ai32 mice expressing ChR2 in pyramidal neurons are injected with JRCamp1a, a genetically encoded calcium indicator, to allow simultaneous optogenetic stimulation and calcium imaging.
(B) Example of cortical activity observed after application of a patterned optogenetic photostimulation.

One major limitation to this optimization based on imaging is that we cannot use green GCaMP for simultaneous imaging and channelrhodopsin-2 (CHR2) photostimulation due to overlapping wavelengths, which causes interference. To address this issue, in the team, Post-doctoral researcher Anton Dogadov has combined in the same mice a virally expressed jRCamP1a calcium indicator with the transgenic expression of ChR2 (Emx-Cre x Ai32 mice). Preliminary data (Figure 3.1) suggests that we can indeed both stimulate the cortical tissue and record the impact of this stimulation on neuronal activity, with limited crosstalk between these two optical channels (Scaglione et al., 2024).

Another strategy to optimize feedback would be to target specific cell types within the cortex. In particular, layer 4 excitatory neurons, which play a crucial role as relays from the thalamus to the cortex, could be particularly relevant targets for our stimulation approach. Indeed, it has been shown that photostimulations specific to layer 4 were efficient to produce illusory touch (Figure 1.21)(O'Connor et al., 2013). To carry these experiments, we are now breeding Scnn1a x Ai32 mice crossings, which express ChR2 in layer 4 neurons. Initial tests with one Scnn1a x Ai32 mouse did not yield significant results, but, this is likely due to the need for further adaptation of the photostimulation intensity. Overall, extensive data collection will be required to validate this Layer 4-selective approach and more generally to optimize the parameters of optogenetic stimulation towards inducing embodiment through optogenetic feedback.

3.5 Providing proprioceptive feedback via optogenetic stimulation

To extend our work, we argue that proprioception would be an attractive target. Most investigations concerning sensory feedback in neuroprosthetic research focus on touch percepts. Unlike the tactile inputs that we generated, which provide intermittent feedback, proprioception involves continuous feedback about body position and movement. This constant sensory information, which is seen as crucial for normal motor function *(See Chapter 1.1.2.1),* seems vital for neuroprosthetic development. Indeed, beyond motor control, it could provide to the user, constant embodiment-triggering inputs, thereby significantly enhancing the functional integration of prosthetic limbs.

The preliminary results obtained from our optogenetic study, suggest that optogenetic tactile sensory feedback can induce the embodiment of an artificial limb. We now hypothesize that proprioceptive-like optogenetic feedback could similarly enhance the embodiment effect. This study represents a necessary exploration since we currently have very little understanding of how to selectively activate the cortical areas responsible for proprioception.

Peripheral proprioceptive embodiment will be studied in the laboratory by moving in synchrony the limb and an artificial prosthesis, and then threatening the prosthesis (Figure 3.2A) — a direct analogue to passive movement-induced embodiment in humans (Abdulkarim et al., 2023; Kalckert & Ehrsson, 2014a). Next, optogenetic feedback patterns that mice can associate with prosthesis movement will be designed (Figure 3.2B,C). We hypothesize that replacing peripheral proprioceptive feedback with these optogenetic cues will successfully trigger the embodiment of the prosthetic limb.

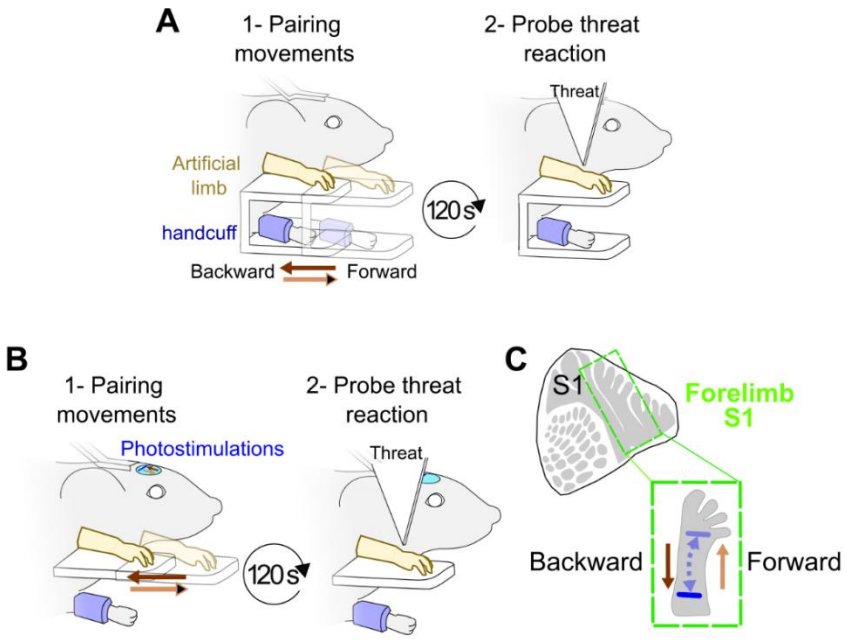

Figure 3.2: Providing proprioceptive feedback via optogenetic stimulations
(A) Embodiment test with proprioceptive feedback: A platform holding both the artificial limb and the mouse's handcuffed right forelimb would make back and forth movements, displacing both limbs. The goal here is to generate a passive movement illusion to give the animal the impression that the artificial limb belongs to them as they receive concurring proprioceptive information.

(B) Embodiment test with optogenetic proprioceptive feedback: In this protocol, the moving platform only carries the artificial limb and the handcuffed paw remains static. Proprioceptive information will be provided via optogenetic stimulation, to test if it is possible for mice to understand this feedback, and compute a change in perceived paw location.
(C) S1 topography map, the stimulation will target the forelimb area and will take the shape of a bar, making back and forth movements on the area of interest.

3.6 Perspectives for human research

Our research platform based on optogenetics provides a unique opportunity to explore various facets of embodiment related to sensory, motor, and proprioceptive feedback, integrating these elements to enhance prosthetic function. This comprehensive approach allows us to investigate how optogenetic stimulation can effectively mimic the natural sensory experiences of owning a body part, potentially improving the overall integration and usability of prosthetic devices.

Our model is particularly relevant for guiding future neuroprosthestic developments towards human applications. Before translating optogenetic technologies to human trials, rigorous evaluations of safety, efficacy, and ethical implications are required. Our research platform offers a valuable tool for addressing these issues. It enables us to refine optogenetic protocols, optimize feedback mechanisms, and assess the impact on embodiment in a controlled environment.

In human trials, optogenetics has already demonstrated its potential, such as in the case of retinitis pigmentosa, where it restored partial vision in a blind patient using optogenetically engineered goggles (Sahel et al., 2021). This success underscores the potential of optogenetic therapies to restore sensory function. However, it is important to note that in this case, it was the retinal ganglion cells that were ontogenetically engineered. This involved retinal viral injections, which are less complex and invasive than cortical injections, and it would take additional time to address the ethical and safety concerns associated with cortical injections before these methods could be accepted for use in humans

Extending these optogenetic techniques to the human cortex presents some challenges. The cortical tissue is much thicker and more

convoluted than the retina, making it difficult to apply optogenetic methods effectively due to limited light penetration and the complexity of cortical layers. A promising strategy to address this, for direct cortical stimulation in humans, is sonogenetics. This technology combines the expression of large-conductance mechanosensitive ion channels with high-frequency ultrasonic stimulation which are able to open these channels. This technology makes for the activation of neurons with millisecond precision deep into the tissue, offering a non-invasive and potentially more practical solution for deep brain stimulation. Recent advancements in this field have shown that sonogenetics can modulate neuronal activity with high spatial and temporal resolution, which could be adapted for visual and sensory restoration (Cadoni et al., 2023). This technique holds promise in overcoming the limitations imposed by cortical thickness and complexity, thereby opening new avenues for the application of optogenetic principles in human brain machine interface research.

Moreover, our research in the mouse somatosensory cortex model provides valuable insights that could inform future human applications. By leveraging the knowledge gained from our studies, researchers can design electrical microstimulation protocols that effectively target specific cortical regions, potentially enhancing somatosensory feedback and prosthetic embodiment.

Beyond classical electrical microstimulations, a promising technology for direct cortical stimulation in humans is functional ultrasound (fUS) neuroimaging. fUS uses high-frequency ultrasonic waves to visualize and modulate brain activity by detecting changes in cerebral blood volume across large brain regions. This technology offers a non-invasive and practical solution allowing the activation of large areas of the cortex with minimal invasiveness. Recent advancements have shown that fUS can modulate neuronal activity with high spatial and temporal resolution, potentially overcoming the limitations imposed by cortical thickness and complexity (Griggs et al., 2024; Rabut et al., 2024).

By exploring the integration of sensory, motor, and proprioceptive feedback through optogenetics, our platform contributes to a broader understanding of how to enhance neuroprosthetic systems and their

seamless integration into the subject body. This research advances fundamental knowledge in the field of somatosensory feedback. It can also pave the way for practical applications that could significantly improve quality of life for individuals relying on prosthetic devices.

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