**Identifying the Neuronal and Molecular Mechanisms Underlying**

**Conditioned Immune Response** (Abstract- one page)

 During evolution, both the immune- and the nervous systems independently developed learning and memory capabilities that enable organisms to flourish and thrive in ever-changing and demanding ecosystems. In the cross talk between the two, it is well established that the immune system affects the brain in health and disease situations. Less is known about if and how our mental state, which is defined by our brain, reciprocally affects the immune system. The benefits for such an interaction are obvious. Internal representations of the outside world can predict and prepare the immune system correctly, to appropriately react against different challenges before they occur. This phenomenon has been modelled in a form of conditioning, which pairs sensory information as the conditioned stimulus (e.g. a newly experienced taste) with a drug that activates or represses the immune system, in an associative learning paradigm called conditioned immune response (CIR). CIR was described many years ago and is temporally similar to another long-delayed association between bodily state and taste, known as conditioned taste aversion. We and others have shown that the internal representation of taste and its valance is encoded in the anterior insular cortex. Recently, it was found that the internal representation of two different types of immune response are represented in the posterior insula. We hypothesize here, that the internal representation of CIR is at least in part dependent on the internal reciprocal circuit between the anterior and posterior insula. Very little is known about the structure and function of this proposed circuit. Our preliminary results, which tested one case of CIR that activates the immune system, strongly support our hypothesis. CIR has two different phenotypic reads: immune response and aversive behavior. We will measure these phenotypes, and with the advantage of our long experience in the field, we will investigate and describe the underlying cellular and molecular mechanisms in the insula. Specifically, we will first identify correlations at the different levels of analysis and later will use different tools to prove or refute causative relationships between the identified mechanisms and immunological and/or behavioral read-outs. Our expected results will reveal for the first time the functional connectivity within the insula, a cortical structure, integrating both interoceptive and sensory information about the world. Moreover, CIR is a fascinating phenomenon that proves that the brain can modify/control immune responses in different directions. Our proposed research will explain how sensory information is associated with immune response and how they are stored as long-term memories with the potential to affect future healthy- or disease states. This will potentially open a window for brain manipulations and treatments that can tune the immune system to better react in future encounters with pathogens. Moreover, it may propose a basic explanation for non-declarative forms of placebo/nocebo effects, which pervades our lives and the biomedical research of every drug one aims to develop.

**Main Research proposal 15 pages including fig.**

**Scientific Background. Conditioned immune response and the interaction between the brain and the immune system.** In recent years, pioneering studies demonstrated how the body, and in particular the immune system, affects brain functions in health and diseases states1,2. For example, the bacteria in our digestive system can determine brain function3–5. However, the interactions between our thoughts and bodily states, which is part of the complex brain to body interaction, are still largely unknown. A practical part of this unsolved philosophical debate is the well documented phenomenon of placebo and nocebo effects. Placebo and nocebo occur when health outcomes are affected by the patient's previous experiences and/or expectations6–8. In the pharmaceutical industry, a placebo is usually used as a sham compound, lacking any active component and is employed in clinical studies to confirm the effectiveness of a specific treatment or drug. Multiple studies show clear effect of placebo on symptomatology in patients with different disorders such as depression9,10, Parkinson’s disease11–14, pain15,16 asthma17 and many others. Nocebo effect relates to negative outcome by a sham compound. In fact, expecting that a treatment will work can induce real responses such as neurotransmitter release, hormone production, and an immune response, easing symptoms of various conditions. Research of placebo/nocebo effect focuses mainly on two neuropsychological underlying mechanisms: 1. The expectations that patients have in terms of the impact of the compound/treatment and/or 2. Classical conditioning 6,18,19. Early experiments have shown that learning processes mediate the placebo/nocebo response and this opinion was further strengthened by various experimental studies17,20–22, indicating that associative learning processes are an essential component in the placebo/nocebo effects. Importantly, many associative learning paradigms are non-declarative (i.e. can be learned unconsciously) and thus can be studied both in human and animal model. One central paradigm to study nocebo effect via the interaction between the brain and the immune system is a phenomenon termed conditioned immune response (CIR). In a classical conditioning paradigm, a sensory information, usually a novel taste, serves as a conditioned stimulus while an aversive drug stimulating or repressing the immune response serves as an unconditioned stimulus (UCS). Following a time frame of days to weeks, re-exposure to the CS will induce a conditioned response, i.e. a measurable immune response similar but not identical to the one induced by the UCS. The time frame of CIR is longer than classical conditioning and similar to conditioned taste aversion (CTA), which is another associative paradigm in which a novel taste is paired with malaise inducing agent. We and others have shown that taste and taste valance are encoded in the anterior insula (aIC)23 and recently showed that specific immune response is encoded in the posterior insula (pIC)24. Assuming CIR is an associative process similar to others, one would predict that the neuronal ensemble encoding for CIR resides, at least in part, within the insula circuit containing and connecting anterior and posterior parts reciprocally. The IC is an elongated structure with unique anatomical and functional divisions in human and rodent. Unfortunately, we know very little about functional connectivity within the insula.

**Here, we will test the hypothesis that CIR is encoded in the cortical circuit connecting between the representation of taste information (i.e. aIC) and state of immune response (i.e. pIC). Later, we will elucidate how this connectivity within the IC enables formation and retrieval of conditioned immune responses in mice.** **Our preliminary experiments indicate that such network underlies one type of CIR.**

**Neuronal mechanisms underlying learning processes.** Learning and memory are processes defined by time. First, an animal acquires the information and creates an internal representation in the central nervous system (CNS). This short-lived representation of the external or internal world (i.e. an engram or neuronal ensemble), can be consolidated to form long term memory that is integrated with previous knowledge25,26. The stored information can then guide the behavior in certain scenarios to increase the likelihood to survive and flourish in a given ecosystem. In associative learning paradigms, the animal learns to associate between two independent stimuli, a conditioned stimulus (CS) and an unconditioned stimulus (UCS) occurring close enough in time, to evoke a conditioned response (CR). Such associative learning paradigms include, the famous experiments carried out by Pavlov in dogs, where the sound of a bell was associated to food delivery27. Other examples include conditioned taste aversion (CTA), where an animal associates a novel taste with malaise (e.g., induced by LiCl injection)28. This aversive associative learning paradigm results in learned aversion and avoidance of the taste in subsequent encounters. Others and we have shown that the anterior IC (aIC) is a crucial forebrain structure for the acquisition and retrieval of CTA. In a similar way, immunological responses can also be learned and memorized by associative learning or conditioning in the form of CIR. We propose here that the IC is a cortical mediator of the interaction between external sensory and interoceptive information in CIR and aim to identify the underlying neuronal mechanisms.

**CIR, an associative form of learning and memory.** CIR, which was demonstrated many years ago, depends on the bidirectional communication between the central nervous system (CNS) and the peripheral immune system. The conditioning of immune responses typically includes the pairing of an immunomodulatory compound (US) with a sensory, neutral (conditioned) stimulus (CS, usually flavor or taste). An association between the two stimuli is established following one or more CS/UCS pairings (i.e., learning), so that a subsequent exposure to the same CS will induce an avoidant behavior (as in CTA) and an immune response resembling the one induced by the UCS. This indicates that peripheral immune responses can be suppressed or stimulated by associative learning processes. CIR can be achieved across experimental approaches, and does not appear to be restricted to the use of a specific immunopharmacological drug or compound (the UCS) 29. In conditioned immunosuppression, the CS (taste/odor) can be paired to different compounds that act as immunosuppressors (i.e. cyclophosphamide (CY), Cyclosporine A). On the other hand, immune activation is achieved by pairing to immunostimulatory agents (i.e. polyinosinic poly-cytidylic acid (Poly I:C), Ovalbumin (OVA), Bovine serum albumin (BSA), Lipo-poly saccharide (LPS)). This conditioning using immune suppressors/stimulators compounds causes an aversion towards the taste as well as immune-response which is elicited when CS is presented alone, days or weeks following association30–34. Both immunosuppressive and immune-stimulating conditioned effects were analyzed in different species including mice, rats, and humans.

The neuroanatomical pathways involved in CTA learning includes the nucleus of the solitary tract, the parabrachial nucleus, medial thalamus, amygdala, and IC 23,35–40. Importantly, lesion experiments proposed that the IC is necessary in learned immune responses. However, our preliminary experiments are the first clear indications to suggest that the circuit **within** the insula is correlative and necessary for learning and retrieving CIR. **The IC is where cortical representations of visceral state, the immune system and taste sensory information representations communicate.** The IC integrates external and internal information, specifically gustatory and visceral stimuli39,41 which are particularly relevant for acquiring and retrieving an association of a bodily state in relation to external cues23,37,42–44. In order to identify the brain regions that are involved in CIR, correlative markers, neuroanatomical specific lesions or local injection of antagonists have been used 45. The IC and the central nucleus of the amygdala play a prominent role in mediating the acquisition phase of the learned increase in antibody response to lysozyme46. Re-exposure of conditioned rats to the conditioned stimulus leads to an increase of c-Fos immunoreactivity in the IC47 and activity within the IC is crucial for the acquisition of conditioned immunosuppression48. These relatively old experiments suggest for IC participation but does not explain **how** the IC is involved in the neural mechanism underlying CIR. Importantly, we have shown recently that immune-related information is stored in the posterior IC (pIC)24, and that neuronal ensembles of the pIC which were active under a specific inflammatory state, retrieved this inflammatory state upon artificial reactivation of the captured neuronal ensemble. Taking together, identifying the internal representation of taste and its valance in the anterior IC (aIC23,37) and that of immune response in pIC, opens the door to study the cellular and molecular mechanisms subserving CIR for the first time. Moreover, our proposed research aimed at dissecting the functional divisions of different areas within the IC using both correlative and causative approaches. **Information transfer from/to the IC and PNS modulating immune response.** The IC receives a vast amount of interoceptive information, ascending through deep brain structures, from multiple visceral organs. The autonomic nervous system (ANS) is responsible for processing and transmitting interoceptive information to the brain from the visceral organs that maintain survival functions, including the gastrointestinal, cardiovascular, respiratory, thermoregulatory, hormonal and immune systems49. However, no conclusive evidence has been suggested regarding mechanisms by which such information transfer occurs.  Within the frame of the proposed research we will not study the way information is transferred from the immune system to the brain and back but will focus on brain mechanisms underlying CIR similarly to any other learning paradigm.

**Research objectives and expected significance.** Here, we aim to reveal, for the first time, the underlying molecular, cellular and circuit mechanisms enabling the formation and retrieval of CIR. We will identify the functional connectivity within the insula allowing at least in part, the formation and retrieval of CIR. Brain to body interaction is one of the fundamental philosophical questions occupying humanity for many centuries. Revealing the biological mechanisms underlying the ways mammalian brain modulates immune systems, could be a crucial step in the effort to tackle the subject. Moreover, revealing mechanisms underlying CIR, which can be regarded as a reduced form of placebo/nocebo effect (i.e. mediated by non-declarative association), will enhance our very basic understanding of how novel treatments can be beneficial for our health in different and innovative perspectives. From a brain function angle, the IC has drawn a lot of attention in the last few years as the site where interoceptive information is encoded, computed and possibly integrated with external information50.

**Detailed description of the proposed research. Working hypothesis:** Assuming the internal state of an immune response is represented at least to a certain extent in the pIC24 and taste and its valance in the aIC41, ***we hypothesize that functional connectivity within the IC is central for CIR and that CIRs obey the rules of associative conditioning, with a long delay between CS and UCS*.**

We will assess these two basic hypotheses through two work packages (WPs) divided into different tasks as listed below. The research proposal has three main dimensions through which the hypotheses will be tested. The first dimension is *the types of data* we will collect: **1.** Correlations between the two measured phenotypes: immunological state or aversive behavior and circuit/cellular/molecular measurements in the IC. **2.** Causality experiments, where inhibition of cellular or molecular function/s within the IC affects behavior and/or immunological read-outs. **3.** Following 1 and 2, that will allow a novel understanding of at least some components of the association, we will assess the necessity and sufficiency of the identified circuit/cells/molecules within the IC in evoking CIRs. The second dimension is *the kinetics of CIR learning* and its representation in the IC. First, acquiring the internal representation, consolidating it and only later retrieving it. In terms of experimental set up, the temporal phase of retrieval is the time of testing the mice, days or weeks following the conditioning and comparing it with mice experiencing the CS, UCS or both but not one after the other in a way that induce an association between the taste and the agent that activates or deactivates the immune system as detailed below. The third dimension is *cellular versus molecular measurements*. Following our preliminary results identifying the aIC-pIC as a major hub underlying CIR of taste and LPS, we will first better define that circuit and the cell types in the IC involved in different forms of CIR (**WP1**) and only later identify the molecular components underlying the cellular/circuit mechanisms. Thus, first effort and WP will be the cellular approach and only later the molecular one, while aiming to identify the molecular processes taking place within the identified cells and circuit (**WP2**). Our laboratory is experienced working with the three dimensions which compliment each other to explain biological mechanisms underlying CIR.

**Research plan.**

**Strategy:** we will start by demonstrating correlations between CIR at the different levels of analysis before conducting causality experiments. Only after correlation and causality experiments we will aim at sufficiency (i.e. artificially activate the relevant circuit and/or molecules).

**General:** The IC is an elongated cortical structure with a unique laminar organization51. It is segregated into granular, dysgranular and agranular regions as defined by the disappearance of layer 4 and differential composition of GABAergic interneurons52. The CS in our experiments will be taste. Specifically, we will use a pleasant taste with low metabolic value (e.g. saccharin), which elicits robust activation of the anterior to medial insula53. In parallel, medial to posterior insula receives more inputs from internal organs and the peritoneum including pain and immune responses via the peripheral nervous system 54. In the last century, Penfield already proposed that internal organs are represented in the human insula55, a notion that was reinvigorated following seminal reports by Craig56. More recently, using new genetic tools, it was shown that the pIC encodes information of two different types of inflammation in mice 24, obeying at least in part the principle of forebrain internal representation of bodily experience57 . This, together with our better understanding of taste valence coding in the aIC allows us to propose the current application to test how CIR is formed and retrieved in the mammalian brain. From a neuroanatomical perspective, we have a better understanding of functional connectivity between the IC and other brain structures both in humans and rodents53,58. However, the microcircuitry within the insula is largely unknown and thus represents a major gap in knowledge. Using retrograde adeno-associated viral vectors (AAVs), expressing fluorescent marker, injected in a minute amount to anterior and/or posterior insula in combination with pERK as neuronal activity marker, we mapped for the first time a functional connectivity within the insula (Fig's 1 and 3).

WP1, Cellular approaches (Tasks 1, 2, and 4, years 1-5): We will use both pERK (i.e. level of ERK phosphorylation which is indicative to its activated state) and c-Fos promoter activity as measured by IHC or TRAP mice, as a proxy, to capture neuronal engrams or neuronal ensembles24,59,60. This will allow us to identify neuronal ensembles correlative with the different phases of CIR with high spatial and low temporal resolution. In parallel, using single unit activity measurements in behaving mice, we will capture the temporal dynamics governing neuronal encoding of CIR. In addition, we will use opterodes together with retrograde AAVs injection to anterior (A) or posterior (P) insula to isolate the neurons projecting anterior (A) to posterior (P) or P to A within the insula, while recording single unit activity in the behaving mice. WP2, Molecular approaches (Tasks 3, 4, and 6, years 3-5): We will define the neurotransmitters, neuromodulators, signal transduction and synaptic proteins underlying CIR in the circuit and cell types identified in WP1. In addition, will study the consolidation phase of CIR focusing on reduced eIF2 phosphorylation in specific cell types as we did in the hippocampus previously 61,62. If we can indeed show bi-directionality in pivotal measurements (i.e. if we inhibit or activate cell type or an enzyme and measure opposite phenotypes), the validity of our interpretation and proposed model will increase dramatically.

**Power calculation and subjects**- we have a rich experience with manipulating the insula and measuring behavior and cellular and/or molecular correlates. Our experience and preliminary results allow us to conclude for immunology measurements. N=8-10 per group should suffice to conclude if we prove or refute our hypothesis. We are obliged to begin with males' subjects as continuation to previous and submitted research. In addition, the proposed variables are too big at this stage to add variables.

**Summary of preliminary cellular results obtained with saccharin (CS) and LPS (UCS) in CIR**

**Fig 1:** (right panel, correlative measurements) A retrieval of association between saccharin and LPS induces both aversive behavior and induction of immune response with a correlative activation of aIC-pIC reciprocal neuronal connectivity. (left panel, causality study) Inactivation of aIC-pIC but not pIC-aIC pathways, during retrieval of CIR is necessary for behavioral retrieval but both reciprocal connections are necessary for retrieving the immune response.

**T1-Correlative cellular measurements in the IC during the different phases and types of CIR.**

T1-1**.** We will first look for correlative measurements at the different level of analysis: we will use transgenic mice, viral vectors, immunohistochemistry (IHC), optogenetics, electrophysiology in behaving mice (i.e. measuring spike activity with tetrodes), and patch-clamp in slices (i.e. measuring x-vivo intrinsic properties and synaptic activity). We will measure the correlations following CS (i.e. taste), following UCS (induction or suppression of the immune response as will be explained later) following the association of the two and following retrieval. T1-2. In parallel, we will identify functional and neuroanatomical connectivity within the hemispheric IC (i.e. anterior-posterior axis and granular-dysgranular-agranular axis) and between left and right IC. Different correlative measurements have pros and cons; we will use different methods as described below in order to have more comprehensive picture as to the IC circuit underlying the acquisition and retrieval of CIR. Following T-1 and -2, we will have a detailed description of the neuronal and non-neuronal circuit activated in the IC during CIR.

**Behavioral conditioning of immune functions (suppression and activation).** Conditioned immune response is based on the intrinsic relation of food and drink ingestion with possible immune consequences that will lead to behavioral adjustment after the experience. In CIR, the association step will involve the pairing of a taste (e.g., saccharin), as a CS with a stimulus that has immune consequences as a UCS (e.g., immunomodulation drug, or antigen), administered intraperitoneal. At recall time, mice will be exposed to the CS alone. One can measure immunosuppression (A), immune stimulation (B) and behavioral aversion (C) to the given taste. Recently, we have published that inflammatory conditions (i.e. DSS-induced colitis and Zymosan-induced peritonitis) and the immune information related to these conditions are stored as neuronal ensembles within the posterior insular cortex (pIC). Moreover, we showed that reactivation of these neuronal ensembles was sufficient to retrieve the inflammatory state24. Therefore, we concluded that the brain could encode and recall at least in part specific immune responses. **We hypothesize that these rules are valid also in the case of conditioned immune response. More specifically, we hypothesize that specific neuronal representations within the IC encode CIR, which is an associative form of learning and memory.** Our preliminary experiment demonstrates CIR using LPS, an immunostimulant, as the UCS (Fig's 1,2). Later, we will use other UCS to analyze the effects of different drugs that stimulate or inhibit the immune system as explained below.

**A. Behaviorally conditioned immunosuppression.** Mice will be behaviorally conditioned by applying a previously published protocol with some modifications63,64. Animals (n=8) will be placed on a water restriction regime, allowing them 30 min of drinking per day. On the day of experiment (day 4), we will start a sequence of 5 conditioning trials with 72h intervals and 3 evocation trials with 24 h intervals. The period between the last association and the first evocation trial for all groups will be 72h. Mice will be divided into 4 experimental groups: a. conditioned (CS+). b, conditioned not re-exposed (CN). c. Untreated (UNT). d. Cyclosporine A (CsA) treated.  For association trials, CS+ and CN treatment groups will receive a 0.2% w/v saccharin solution as CS. Immediately after the drinking session, the animals will receive a CsA injection (20 mg/kg i.p.) as UCS. For evocation periods, conditioned mice will be re-exposed to saccharin only (CS), whereas the CN group will receive water instead. CsA-treated mice will receive water and 20 mg/kg CsA at all association and evocation trials. UNT mice will be water deprived and will received saline instead of CsA. On the last evocation day, 1 h post drinking, animals will be anesthetized and relevant tissues will be collected (i.e. blood, spleen) for further analysis. On the behavioral level, we will test for taste aversion by measuring saccharin consumption in a choice test using two bottles23. Inhibition of calcineurin by cyclosporine prevents the dephosphorylation of NFAT and its subsequent translocation from the cytoplasm to the nucleus, in an IL-2-mediated process. Inhibition at this level thereby prevents the activation of T-cell activation's promoters and overall of the immune response. Accordingly, collected tissues (spleen, blood) will be subjected to a set of ex-vivo experiments. Spleens will be harvested for isolation of splenocytes that will be divided for flow cytometric analysis, ex-vivo culturing, or further purification of CD4+ T cells by magnetic separation. To determine any changes in proliferative capacity of T-cells following learned immunosuppression, we will utilize the CellTrace CFSE Cell Proliferation Kit (Thermo). To examine cytokine secretion, splenocytes will be cultured and challenged with ConA (5μg/ml) in a 96-well plate at a cell density of 1 × 105 cells/well. Cytokine concentration in culture supernatant will be determined with a cytometric bead array (Th1/Th2 and inflammatory/anti-inflammatory cytokines) using flow cytometry according to the manufacturer’s instructions (BD Pharmingen, San Diego, CA). Moreover, cell populations in blood and spleen samples will be further examined in using cell surface marker staining (i.e. CD45, CD4, CD8, CD161, SIRPα) and flow cytometric analysis. Given that CD4+ T cells express receptors for neuromodulators and neurotransmitters such as noradrenaline65, dopamine66, acetylcholine67, and glutamate68 , we will purify splenic CD4+ T cells using magnetic beads and expose them to the aforementioned stimulations (agonists/antagonists). Subsequently, we will determine calcineurin activity, and cytokine production. According to the obtained ex-vivo results, we will manipulate the IC in the CIR paradigm and perform the same analysis. Main pitfalls and alternatives- the literature suggest that CIR works better in rats29. We prefer mice because of better genetics tools. However, if the above experiments result in marginal and/or unreliable immune responses, we will use rats and viral vectors that allow for analogous manipulations. Our preliminary results using single association with LPS as UCS demonstrate reliable clear measurement of CIR (Fig 2).

**Figure 2: Taste immune conditioning results in both immune and behavioral responses.**

**a,** Schematic representation of the CIR; On the conditioning day, mice were presented with saccharin (0.5% dissolved in tap water) and were IP injected with LPS or PBS 40 minutes later. On the retrieval day, mice were presented with a saccharin/water choice test, and aversion index was assessed. **b,** LPS treated mice (CIR group) were significantly more averse to saccharin (97.02±1.627%) compared to the PBS-treated controls (vehicle group; 33.88±7.61%). **c,** Schematic representation of CIR, second exposure to saccharin (vehicle), LPS-water, LPS-LPS and water experimental groups; On the conditioning day CIR and vehicle groups were presented with a novel saccharin and 40 minutes later they received intraperitoneal injection of LPS (0.5mg/Kg) (CIR) or PBS (vehicle). Water group animals were presented with water; LPS-Water and LPS-LPS animals were intraperitoneal injected with LPS (0.5mg/Kg). On the retrieval day (day 9), CIR and vehicle mice were presented with 1 ml of saccharin, water and LPS-Water groups were presented with 1ml of water, whereas LPS-LPS animals were injected again with LPS (0.5mg/Kg). 3 hours following each treatment, animals were sacrificed and their peritoneal lavage fluid was collected. **d,** Representative dot plots of Flow cytometry. The frequency of monocytes/macrophages in peritoneal lavage were determined by immunostaining for F4/80 and Ly6C. **e,** Normalized percentage of monocyte/macrophage populations was similar in CIR (n=13; Monocytes: 97.84±6.517%; macrophages: 91.500±8.858%), vehicle (n=12; Monocytes: 96.079±8.237%; macrophages: 98.372±4.107%), LPS-Water (n=11; Monocytes: 100.909±6.763%; macrophages: 101.952±8.568%) and LPS-LPS (n=15; Monocytes: 112.149±11.449%; macrophages: 82.795±8.749%) experimental groups (2-way ANOVA, F(1,94)=1.785, p=0.1848).Monocytes/macrophages were sub-gated and analyzed for CD80+ or CD86+ frequencies. **f,** Normalized percentage of CD80+ monocytes/macrophages in LPS-LPS (n=7; Monocytes: 209.864±25.15; macrophages: 107.489±1.934) group was significantly higher than vehicle (n=9; Monocytes: 154.870±13.731; macrophages: 101.883±0.455; p=0.0152) and LPS-Water (n=3; Monocytes: 129.956±11.873; macrophages: 9.3435±5.021; p=0.0095) groups, but was not significantly different than the CIR (n= 7;Monocytes: 206.643±21.747; macrophages: 101.851±1.954) experimental group. Normalized percentage of CD80+ monocytes/macrophages in CIR group was significantly higher than the LPS-Water group (p=0.132) and the vehicle group (p=0.0236) (2-way ANOVA; F(1,44)=43.24, p<0.0001). **g,** Normalized percentage of CD86+ monocytes/macrophages expressing in CIR (n=7; Monocytes: 172.105±31.873; macrophages: 105.416±3.692) group was not significantly different than vehicle (n=9; Monocytes: 124.393±15.236; macrophages: 103.080±3.384), LPS-Water (n=3; Monocytes: 104.203±62.562; macrophages: 110.181±0.249) and LPS-LPS (n=7; Monocytes: 276.088±100.660; macrophages: 93.114±7.895) experimental groups (2-way ANOVA, F(1,20)=14.89, p=0.001). Normalized percentage of CD86+ monocytes/macrophages expressing in LPS-LPS group was significantly higher than the vehicle group (p=0.0376). Data are shown as mean ± SEM. n≥4 \*p<0.05, \*\*p< 0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.

 **B. Behaviorally conditioned immune-stimulation.** Behaviorally conditioned immune activation using taste as CS in mice requires calibrations. We aim to test both T-dependent (OVA) and T-independent (Poly I:C) antigens as the UCS. For T-independent antigen conditioning, we will adapt odor-based protocols69,70 with modifications to taste. More specifically, animals will be placed on a water deprivation regime, allowing them 30 min of drinking per a day. On day 1 of the experiment mice will be subjected to CS (0.2% w/v saccharin solution) – UCS (poly I:C, 30μg i.p. injection per animal) conditioning. Control groups will include untreated animals, conditioned but not re-exposed, and UCS treated. On day 4, mice will be re-exposed to saccharin only (CS), whereas the conditioned but not re-exposed group will receive water instead. Following the treatment, animals will be sacrificed at different time intervals (0, 6, and 24h), blood and immune tissues (BM, spleen, and LN) will be extracted and phenotype characterization of the immune system, including activation state of different immune cells, will be carried out using mass spectrometry (CyTOF). CyTOF can multiplex up to 45 cellular markers with limited need for spectral overlap compensation, well-suited for deep phenotyping of cells in complex systems71. Initially we will apply a marker antibody panel to analyze different (~37) immune cell populations (Maxpar Direct Immune Profiling Assay). This assay includes profiling of monocytes, dendritic cells, granulocytes, lymphocytes and their subpopulations, as well as NK cells. According to the obtained results we will get deeper profiling of the immune populations identified by the assay and their activation state by customizing and expanding the marker backbone. This analysis will enable us to determine specific immune phenotypes in different immune tissues in a temporal way. Moreover, we will test humoral immune response in blood serum taken from the same animals. Based on our preliminary results using LPS (Fig's 2,3) and given that published data indicate that CIR is not restricted to a specific immunomodulatory compound, we expect to succeed in establishing a reliable protocol of behaviorally conditioned immune-activation using taste as CS. The established protocol will include the behavioral paradigm as well as the expected immune phenotypes and will be used in subsequent experiments. In T-dependent antigen experiments, we will condition mice with adjuvanted-OVA (10mg i.p. injection per animal) and re-expose mice to CS on day 30, during the declining phase of the primary antibody response. Blood samples will be collected by tail incisions 5, 10, 15, 20, 25, 30, 35, and 40 days after re-exposure to the CS and we will determine anti-OVA IgG titers by ELISA47. Pitfalls and Alternatives-Conditioned immune activation is more a straightforward and robust association protocol, and thus less challenging for the investigation and manipulation of underlying mechanisms. As for conditioned immunosuppression, the current protocols in mice remain more challenging, but feasible. If needed, we can use rats whereby responses tend to be more reliable29 and appropriate viral vectors for the different manipulations72,73.

**T2-Identifying the cellular code of CIR- Correlative activity measurements in the IC during the different phases of CIR acquisition and retrieval.** We will use different methods to propose a model for CIR coding in the IC. We will aim at integrating the different types of measurements in order have a coherent model of CIR internal representation both in time and space.

T2.1 identification of the activated cells during the presentation of the CS (e.g. taste), following the presentation of the CS (i.e. maintaining the information ready for an association), UCS (e.g. agents as described above to induce immunosuppression or immune activation) both (i.e. the association) or retrieval following presentation of the CS days following the association. The time frame for these experiments is during the presentation of the stimulus and up to 5hrs following the presentation of the UCS in order to account for both the acquisition and molecular consolidation phases of learning. Our preliminary data with LPS as UCS, demonstrate clear involvement of both A-P and P-A insula projections as modifiers of CIR (Fig's 3 and 4).

**Figure 3: ERK is activated in both projections from and to aIC and pIC following retrieval of CIR.** (a, b) Representative coronal IC sections immunostained for pERK (light blue) and DAPI (blue) from mice injected with retroAAV at the aIC (red) and pIC (green) following saccharin, 20x. Number of double-labeled (pERK+, rAAV+) neurons was calculated as a percentage of all rAAV+ neurons. (c) Percentage of pERK+ in pIC-projecting neurons of the aIC was significantly higher following immune-conditioned (14.82±1.139%) compared to non-conditioned (5.298± 0.4%) saccharin consumption (unpaired t-test:p=0.0014). (d) Percentage of pERK+ in aIC-projecting neurons of the pIC was not significantly higher following immune-conditioned (14.34±2.232%) compared to non-conditioned (7.019±1.374) saccharin consumption (unpaired t-test:p=0.057).

Capturing, exposing, tracking and manipulating the relevant circuit underlying CIR acquisition: we will use two different set-ups, we used before successfully: the first one is the Target Recombination in active population (TRAP) mice which is based on the c-Fos promoter. The system allows access to activated cell through the monitoring of c-Fos promoter activity and thus can be used to visualize activated neurons or can induce an expression of Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) or optogenetic tools to show causality and/or sufficiency of the captured circuit. In the TRAP mice CreERT2 is active only in the presence of tamoxifen, allowing capturing of cells that were activated 12-24hr following tamoxifen injection. Later, for causality experiments, we will use in tandem local injection of AAV expressing DREADDs or optogenetic tools to manipulate their activity with ligands or light respectively 53. Importantly, we used these tools recently to identify the neurons that were activated during inflammation24. Thus the TRAP system is currently established as a means of monitoring pIC activity and the type of inflammation, but not for the internal representations in relation to the CS and/or CS/USC associations (i.e. following CS alone or UCS alone or CS/UCS association). Therefore, we will initially calibrate the system for these conditions. As for causality experiments, we prefer using DREADDs for inhibition of relevant cell/circuit and optogenetic tools for activation as explained in 74,75. Our preliminary results demonstrate that inhibition of A-P but not P-A pathways during behavioral retrieval of CIR (i.e. measuring aversion index to the conditioned taste) inhibit taste aversion (preliminary results not presented due to space limitations). On the contrary, both A-P and P-A are necessary for retrieval of the increase in CD80 presenting monocytes following one trial of CIR (fig 2 above and fig 4 below).

**Fig. 4: Inhibition of aIC-pIC reciprocal connectivity impairs the immune retrieval of the conditioned immune response.a,** Normalized monocyte/macrophage frequencies in aIC→pIC ,pIC→aIC and CTRL experimental groups. Monocytes/macrophages were sub-gated and analyzed for CD80+/CD86+ frequencies. **b,** Normalized percentage of CD80+ monocytes/macrophages in aIC→pIC and pIC→aIC were significantly higher than in CTRL experimental group. **c,** Normalized percentage of CD86+ monocytes/macrophages in aIC→pIC, pIC→aIC, and CTRL experimental groups were not different. Data are presented as means (n≥6, \*p<0.05, \*\*p<0.01, \*\*\*\*p<0.0001).

 Alternatives: The TRAP system, explained above, allowed us to identify a neuronal circuit within the pIC24, and we will use it similarly to capture the CS and CS/USC associations. However, it is limited since different cell types and neurons are differentially sensitive to c-Fos promoter as an activity marker. In order to overcome this built-in limitation, we will use another system, which allowed us to identify the role of parvalbumin (PV) interneurons of the IC in CTA memory retrieval37. The Robust activity marking (RAM) system is an alternative for genetic identification of neurons responsible for encoding learned experiences *in vivo*. It is composed of a synthetic promoter that is strongly activated by neuronal activity and a downstream reporter gene to allow subsequent investigation and manipulation. A modified doxycycline (Dox)-dependent Tet-Off system that provides temporal control to label neurons that are activated by a specific experience, which occurs in the absence of Dox. It was recently shown that the RAM system selectively labels neuron ensembles activated by contextual learning in various brain regions60. Moreover, Cre-dependent RAM (CRAM) allows for the study of active ensembles of a specific cell type and anatomical connectivity. The CRAM system, in which the effector gene can only be expressed in cells that express Cre, is also effective in labeling GABAergic neurons60,76,77. For the CRAM experiments, cell specific Cre-driver lines (e.g. Gad2-Cre mice for GABAergic interneurons), will be injected with CRAM-tdT virus, which results in the expression of the fluorescent protein tdTomato in activated neurons. In order to manipulate the activated neurons, one of the DREADDs receptor or opsins (e.g. Channelrhodopsin (ChR2) or Archaerhodopsin (ArchT)) can be inserted into the multiple cloning site (MCS) at the effector gene position. This system allowed us recently to capture activity in both excitatory and inhibitory neurons in the aIC during CTA formation and retrieval37. Moreover, we demonstrated that the temporal resolution for capturing the activated cells is similar to the one described above for TRAP mice. We will first aim to calibrate the system for the complementary experiments we have not performed so far (i.e. use the system to capture the circuit underlying the internal representations of CIR). T2.2Electrophysiology- Aims: 1. Analysis of neuronal activity (i.e. both single unit activity in the behaving mice and synaptic or intrinsic properties using patch clamp is a slice preparation) during the acquisition, the maintenance, and the retrieval of a CIR; 2. Define the circuitry underlying conditioned immune response. 3. Compare data from acquisition, maintenance and retrieval. This will allow us to portrait cellular modifications that enables CIR learning and retrieval. T2.2.1 Patch clamp in identified (neuroanatomical and/or functionally) set of neurons: We will prepare slices following CS, UCS and association of CIR and measure synaptic and intrinsic properties from neuroanatomicaly and functionally defined neurons as explained above37,53. **We hypothesize that excitability will decrease in both A-P and P-A pathways, following CIR retrieval similarly to IC-BLA projections following CTA retrieval37. From synaptic perspective, following our preliminary data (not sown due to space limitation), we hypothesize that presynaptic but not post synaptic EPSCs and IPSCs will increase in both pathways following learning when mice retrieve CIR.** Preliminary results indicate for unevenly changes in excitability reduction between the A-P and P-A pathways and a trend to an increase ****in inhibitory and excitatory presynaptic but not post synaptic events following retrieval of CIR (Fig 4 below and unpublished synaptic measurements not shown due to space limitation).

**Fig. 5: Excitability of aIC-pIC projecting neurons differs at their baseline levels and following the retrieval of conditioned immune response**. **a,** A representative image shows the fluorescently labeled neurons and patch pipette positions following the injection of rAAV-GFP construct (green) at the pIC and rAAV-mCherry construct (red) at the aIC. Scale 10um. The recordings were made from the right insular LV projection neurons. **b,** Representative traces show each group's firing frequencies from the 350pA current injection step. Scale bar 20 mV and 50 ms. **c,** The dependence of firing rate on current step magnitude is significantly higher in pIC-to-aIC (red) compared to aIC-to-pIC projecting neurons (green) at the baseline level (two-way repeated measurements ANOVA, n=9–10 cells per group; p=0.0019; F(8, 136)=3.277).**d,** Experimental design for electrophysiological investigations. The recordings were made from the right insular LV projection neurons. **e,** Representative traces shows aIC-to-pIC firing frequencies following the retrieval of CIR (yellow) or vehicle (orange), compared to the basal levels (green)- From 350 pA current injection step. Scale bar 20 mV and 50 ms. **f,** The dependence of firing rate on current step magnitude of aIC-to-pIC projecting neurons is significantly higher in vehicle group compared to CIR and the baseline level (two-way repeated measurements ANOVA, n=10–14 cells per group(N=4-5); P<0.0001; F (16, 264) = 5.805). **g,** Representative traces show LV pIC-to-aIC firing frequencies following the retrieval of CIR (red-purple), vehicle (yellow) and baseline levels (red). From 350 pA current injection step, Scale bar 20 mV and 50 ms. **h,** The dependence of firing rate on current step magnitude is significantly higher in pIC-to-aIC at the baseline compared to CIR and vehicle (two-way repeated measurements ANOVA, n=9–11 cells per group (N=3-5); p=0.0085; F (16, 208) = 2.126). Values are expressed as mean ± SEM. \*p<0.05, \*\*p< 0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.

T2.2.2 In vivo electrophysiology in freely behaving mice. We will implant tetrodes and record single neuron activity in behaving mice. Recordings in awake, freely behaving mice will be performed using an optrode drive consisting of an electrode array of 128/256 contacts (NeuroNexus Technologies, Unites States) attached to an 18-pin electrical connector, concentrically arranged around an optical fiber in a mechanically adjustable drive. Extracellular waveform signals will be collected using a RHD2000 Evaluation system (Intan Technologies, United States) that allows recording of bio-potential signals from up to 256 low-noise amplifier channels. A head-stage amplifier (Intan Technologies) will be used to amplify the signals (RHA2000 system) which convert the analog signal to a digital one, and transfer it to the recording system. The electrode array assembly can be lowered using the microdrive holder to a new recording site at the end of each recording day, leaving at least 20hr before the experiment to ensure stable recordings. Optical stimulation will be applied through a ferrule-terminated optical fiber (ThorLabs) attached to the patch-chord by a zirconia sleeve (ThorLabs). Our preliminary results demonstrate, we record stably single unit in IC in a homemade licking system we built recently and provide rich behavioral data (preliminary data not presented due to space limitations). In order to identify the neurons, we record from, we will implant a tetrode with a fiber optic78. We will use light stimulation to identify correspondent activity and thus to verify connectivity or/and history of activation information about the recorded neuron. In order to identify the neurons, we record from, we will implant a tetrode with a fiber optic78. **We hypothesize that both learning and retrieving of CIR will enhance fire frequency of set of excitatory neurons. The dynamic (i.e. change over time) is an open question. In addition, we hypothesize that A-P and P-A identified neurons will be differently activated from general population neurons following retrieval of CIR.**

Alternatives: we purchased recently fiber photometry set-up and are integrating it with our taste behaving set up. It will be used as an alternative for the in vivo electrophysiology set up described above.

**WP 2 Molecular approach (years 3-5)- Molecular measurements in the IC during the different phases of CIR acquisition and retrieval**. Similarly, and in parallel to the cellular approach, we will use clarity protocols79,80, viral vectors as explained above with fluorescence markers, and IHC to identify known molecular pathways crucial for learning and retrieval of CIR. We will look at the entire IC and will aim at identifying activation of major molecular pathways correlated with CS, UCS and CIR. We published many papers on the subject and thus describe below in short, the main principles behind these approaches, without getting into the methodological details. Additionally, light sheet fluorescent microscopy (LSFM) together with the clarity protocol, should reduce light scattering in the brain81,82 and enable us to produce of high resolution 3D maps of the entire IC (we have good preliminary results using the Technion microscopy facility). This will allow us to increase the resolution, quality, and clarity of our imaging studies. **T3 Correlative Molecular measurements in the IC during the different phases and types of CIR.** Following T1 and T2 we will focus on the cells and circuit identified in order to explain what are the molecular process that drives the cellular/circuit changes.T3.1 CIR induces synaptic protein expression and post translation modification**.** We will test similar mechanisms that were identified in CTA28. Specifically, we will measure GluR and NMDA-R subunit expression (e.g. NR2A versus B) and their post translation modifications (e.g. tyrosine phosphorylation of the NR2B)83–85. In parallel, we will measure similarly the expression of GABA-R and their post translation modifications86. T3.2 CIR induces neuromodulator efflux**.** CTA induces a prolonged release of different neuromodulators in the IC. We will use sensitive microdialysis87,88 in order to measure the temporal release of the different neuromodulators across the rostrocaudal axis of the IC (i.e. aIC to pIC) following CS, UCS and CS/UCS association. This will allow us to identify the neuromodulation involved and the temporal dynamic of the release independently in the rostrocaudal divisions of the IC following the different phases of CIR. T3.3 Identification of signal transduction downstream to neurotransmitter activation. There are few important signal transduction cascades that are known to be necessary for memory formation and consolidation. We will focus on ERK-MAPK cascade and mRNA translation regulation via phosphorylation of the eIF2. ERK/MAPK is necessary for the different stages of learning, including acquisition and extinction learning89,90. ERK/MAPK is unique with excellent Ab’s that can recognize the dual phosphorylated state which is the activated form91. Our preliminary results demonstrate an increase in pERK in both neurons projecting from anterior to posterior insula and vise-versa following retrieving of CIR (see Fig 4). Levels of eIF2 phosphorylation at serine 51 is a marker for its deactivation which reduces initiation phase of mRNA translation dramatically in different cell types including neurons. We have previously benefited from the use of reliable commercial antibodies to detect protein and phosphorylated levels of ERK/MAPK or eIF2 which in conjunction with cell-type specific markers will allow us to dissect these molecular changes across the rostrocaudal IC, and in relation to the different phases of CIRs. **T4 Defining the neurotransmitters and neuromodulators necessary for CIR acquisition and retrieval.** According to the results obtained in T1-4 we will use local application of antagonists to identify the synaptic processes necessary for the formation of CIR association. The elongated structure of the IC will allow us to identify the neurotransmission necessary for the CS, the UCS and the association of CIR across the rostrocaudal axis of IC. These experiments will be followed by more precise experiments using relatively new genetics tools. We will use Cre driver lines in which Cre recombinase is expressed specifically in neurons producing the neuromodulators combined with injection of retrograde AAV to the aIC or pIC. Recently, we used similar approaches to identify the source of dopaminergic neurons releasing dopamine to CA1 region of the hippocampus during fear conditioning97. We will use the same strategy to express iDREADDs in the specific population of neurons producing the relevant neuromodulator (e.g. Acetylcholine or Dopamine) projecting to the aIC or pIC. This will allow us to identify not only the necessary neuromodulators for learning CIR, but also its source. Similarly, we can activate these identified neurons and express either qDREADDs or opsins (see above for optogenetic tools) to activate them at the relevant time (as part of bidirectional approach in the proposed study). **T5.** **Detailed description of functional organization of the IC microcircuit and a model for the molecular and cellular mechanisms underlying CIR.**  In recent years number of studies, including ours, have examined the functional circuit between IC and other brain structures such as the different divisions of the amygdala and/or other cortical areas23,53,92–94. However, surprisingly, almost nothing is known about the functional circuit within the IC itself. This is a major gap in the field. Importantly, even though studies have examined the significance of hemispheric lateralized connectivity95,96, little is known regarding the functional role of discrete neuroanatomical structures within the IC. Following the data collected in T1-T4, we will aim at producing detailed model of intra-insula (i.e. within insula and between hemispheres) structure to function connectivity model in baseline and underlying CIR. This dynamic model (according to the results and literature) will summarize the results of tasks 1-4 but also will direct of causative experiments in the last two years.

**Concluding remarks.** Brain and body reciprocal interactions are a subject for intense research ranging from philosophy, biology, medicine and physics. The placebo/nocebo effect is one such wonder where past experience can affect the function of the immune system at a given situation. For many years, we studied molecular and cellular mechanisms taking place in the IC underlying valence of sensory information. At the same time, lesion studies suggest that a main forebrain structure to be involved in CIR learning is the IC. We entertained the idea of studying the neuronal mechanisms underlying CIR for a decade, but realized, that though we knew a lot about taste valence presentation in the aIC, we knew nothing about the neuronal representation of the immune response (i.e. the UCS, the internal bodily information). Recently, Koren et al., 2021, have shown that two different types of immune responses are represented in the pIC. This, together with new tools in molecular, cellular, circuit and behavioral neuroscience, will enable us to explain mechanistically the long-standing mystery, of whether and how mammalian mental experiences affects its immune system to promote sickness or health.

**Bibliography**

(1) Morimoto, K.; Nakajima, K. Role of the Immune System in the Development of the Central Nervous System. *Front. Neurosci.* **2019**, *0*, 916.

(2) Dantzer, R. Neuroimmune Interactions: From the Brain to the Immune System and Vice Versa. **2017**, *98* (1), 477–504.

(3) Zhu, S.; Jiang, Y.; Xu, K.; Cui, M.; Ye, W.; Zhao, G.; Jin, L.; Chen, X. The Progress of Gut Microbiome Research Related to Brain Disorders. *J. Neuroinflamm 2020 171* **2020**, *17 1-20*.

(4) Willyard, C. How Gut Microbes Could Drive Brain Disorders. *Nature* **2021**, *590* (7844), 22–25.

(5) Mohajeri, M. H.; La Fata, G.; Steinert, R. E.; Weber, P. Relationship between the Gut Microbiome and Brain Function. *Nutr. Rev.* **2018**, *76* (7), 481–496.

(6) Price, D. D.; Finniss, D. G.; Benedetti, F. A Comprehensive Review of the Placebo Effect: Recent Advances and Current Thought. *Annual Review of Psychology*. 2008, pp 565–590.

(7) Kirsch, I. Response Expectancy Theory and Application: A Decennial Review. *Appl. Prev. Psychol.* **1997**, *6* (2), 69–79.

(8) Pacheco-López, G.; Engler, H.; Niemi, M. B.; Schedlowski, M. Expectations and Associations That Heal: Immunomodulatory Placebo Effects and Its Neurobiology. *Brain. Behav. Immun.* **2006**, *20* (5), 430–446.

(9) Dworkin, R. H.; Katz, J.; Gitlin, M. J. Placebo Response in Clinical Trials of Depression and Its Implications for Research on Chronic Neuropathic Pain. *Neurology*. Lippincott Williams and Wilkins 2005.

(10) Kirsch, I. Antidepressants and the Placebo Response. *Epidemiol. Psichiatr. Soc.* **2009**, *18* (4), 318–322.

(11) De la Fuente-Fernández, R.; Ruth, T. J.; Sossi, V.; Schulzer, M.; Calne, D. B.; Stoessl, A. J. Expectation and Dopamine Release: Mechanism of the Placebo Effect in Parkinson’s Disease. *Science (80-. ).* **2001**, *293* (5532), 1164–1166.

(12) Benedetti, F.; Colloca, L.; Torre, E.; Lanotte, M.; Melcarne, A.; Pesare, M.; Bergamasco, B.; Lopiano, L. Placebo-Responsive Parkinson Patients Show Decreased Activity in Single Neurons of Subthalamic Nucleus. *Nat. Neurosci.* **2004**, *7* (6), 587–588.

(13) De La Fuente-Fernández, R.; Lidstone, S.; Stoessl, A. J. Placebo Effect and Dopamine Release. In *Journal of Neural Transmission, Supplement*; Springer Wien, 2006; pp 415–418.

(14) Lidstone, S. C.; Schulzer, M.; Dinelle, K.; Mak, ; Edwin; Sossi, V.; Ruth, T. J.; De La Fuente-Ferná Ndez, R.; Phillips, A. G.; Stoessl, ; A Jon. *Effects of Expectation on Placebo-Induced Dopamine Release in Parkinson Disease*; 2010; Vol. 67.

(15) Colloca, L.; Benedetti, F. Placebos and Painkillers: Is Mind as Real as Matter? *Nature Reviews Neuroscience*. Nature Publishing Group 2005, pp 545–552.

(16) Colloca, L.; Petrovic, P.; Wager, T. D.; Ingvar, M.; Benedetti, F. How the Number of Learning Trials Affects Placebo and Nocebo Responses. *Pain* **2010**, *151* (2), 430–439.

(17) Kemeny, M. E.; Rosenwasser, L. J.; Panettieri, R. A.; Rose, R. M.; Berg-Smith, S. M.; Kline, J. N. Placebo Response in Asthma: A Robust and Objective Phenomenon. *J. Allergy Clin. Immunol.* **2007**, *119* (6), 1375–1381.

(18) Finniss, D. G.; Kaptchuk, T. J.; Miller, F.; Benedetti, F. Biological, Clinical, and Ethical Advances of Placebo Effects. *The Lancet*. Elsevier B.V. 2010, pp 686–695.

(19) Enck, P.; Benedetti, F.; Schedlowski, M. New Insights into the Placebo and Nocebo Responses. *Neuron*. Neuron July 2008, pp 195–206.

(20) Goebel, M. U.; Trebst, A. E.; Steiner, J.; Xie, Y. F.; Exton, M. S.; Frede, S.; Canbay, A. E.; Michel, M. C.; Heemann, U.; Schedlowski, M. Behavioral Conditioning of Immunosuppression Is Possible in Humans. *FASEB J.* **2002**, *16* (14), 1869–1873.

(21) Benedetti, F.; Pollo, A.; Lopiano, L.; Lanotte, M.; Vighetti, S.; Rainero, I. Conscious Expectation and Unconscious Conditioning in Analgesic, Motor, and Hormonal Placebo/Nocebo Responses. *J. Neurosci.* **2003**, *23* (10), 4315–4323.

(22) Benedetti, F.; Mayberg, H. S.; Wager, T. D.; Stohler, C. S.; Zubieta, J. K. Neurobiological Mechanisms of the Placebo Effect. In *Journal of Neuroscience*; Society for Neuroscience, 2005; Vol. 25, pp 10390–10402.

(23) Kayyal, H.; Yiannakas, A.; Kolatt Chandran, S.; Khamaisy, M.; Sharma, V.; Rosenblum, K. Activity of Insula to Basolateral Amygdala Projecting Neurons Is Necessary and Sufficient for Taste Valence Representation. *J. Neurosci.* **2019**.

(24) Koren, T.; Yifa, R.; Amer, M.; Krot, M.; Boshnak, N.; Ben-Shaanan, T. L.; Azulay-Debby, H.; Zalayat, I.; Avishai, E.; Hajjo, H.; Schiller, M.; Haykin, H.; Korin, B.; Farfara, D.; Hakim, F.; Kobiler, O.; Rosenblum, K.; Rolls, A. Insular Cortex Neurons Encode and Retrieve Specific Immune Responses. *Cell* **2021**, *184* (24), 5902-5915.e17.

(25) Attardo A, Lu J, Kawashima T, Okuno H, Fitzgerald J, B. H.; Schnitzer M. Long-Term Consolidation of Ensemble Neural Plasticity Patterns in Hippocampal Area CA1. *Cell Rep.* **2018**, *25* (3), 640-650.

(26) de Sousa, A. F.; Chowdhury, A.; Silva, A. J. Dimensions and Mechanisms of Memory Organization. *Neuron* **2021**.

(27) (1927), P. I. P. Conditioned Reflexes: An Investigation of the Physiological Activity of the Cerebral Cortex. *Ann. Neurosci.* **2010**, *17* (3), 136.

(28) Gal-Ben-Ari, S.; Rosenblum, K. Molecular Mechanisms Underlying Memory Consolidation of Taste Information in the Cortex. *Front. Behav. Neurosci.* **2012**, *5* (January), 1–15.

(29) Hadamitzky, M.; Lückemann, L.; Pacheco-López, G.; Schedlowski, M. Pavlovian Conditioning of Immunological and Neuroendocrine Functions. *Physiol. Rev.* **2020**, *100* (1), 357–405.

(30) Husband, A. J.; Lin, W.; Madsen, G.; King, M. G. A Conditioning Model for Immunostimulation: Enhancement of The Antibody Response To Ovalbumin By Behavioral Conditioning In Rats. In *Psychoimmunology CNS-Immune Interactions*; CRC Press, 2019; pp 139–148.

(31) Russell, M.; Dark, K. A.; Cummins, R. W.; Ellman, G.; Callaway, E.; Peeke, H. V. S. Learned Histamine Release. *Science (80-. ).* **1984**, *225* (4663), 733–734.

(32) PEEKE, H. V. S.; ELLMAN, G.; DARK, K.; SALFI, M.; REUS, V. I. Cortisol and Behaviorally Conditioned Histamine Release. *Ann. N. Y. Acad. Sci.* **1987**, *496* (1), 583–587.

(33) Ader, R.; Cohen, N. Behaviorally Conditioned Immunosuppression. *Psychosom. Med.* **1975**, *37* (4), 333–340.

(34) Ader, R.; Cohen, N. Conditioning of the Immune Response. *Neth. J. Med.* **1991**, *39* (3–4), 263–273.

(35) Bermúdez-Rattoni, F. Molecular Mechanisms of Taste-Recognition Memory. *Nature Reviews Neuroscience*. Nature Publishing Group 2004, pp 209–217.

(36) Bermúdez-Rattoni, F.; Introini-Collison, I.; Coleman-Mesches, K.; McGaugh, J. L. Insular Cortex and Amygdala Lesions Induced after Aversive Training Impair Retention: Effects of Degree of Training. *Neurobiol. Learn. Mem.* **1997**, *67* (1), 57–63.

(37) Yiannakas, A.; Kolatt Chandran, S.; Kayyal, H.; Gould, N.; Khamaisy, M.; Rosenblum, K. Parvalbumin Interneuron Inhibition onto Anterior Insula Neurons Projecting to the Basolateral Amygdala Drives Aversive Taste Memory Retrieval. *Curr. Biol.* **2021**, 1–15.

(38) Nerad, L.; Ramírez-Amaya, V.; Ormsby, C. E.; Bermúdez-Rattoni, F. Differential Effects of Anterior and Posterior Insular Cortex Lesions on the Acquisition of Conditioned Taste Aversion and Spatial Learning. *Neurobiol. Learn. Mem.* **1996**, *66* (1), 44–50.

(39) Sewards, T. V.; Sewards, M. A. Cortical Association Areas in the Gustatory System. *Neurosci. Biobehav. Rev.* **2001**, *25* (5), 395–407.

(40) Yamamoto, T.; Shimura, T.; Sako, N.; Yasoshima, Y.; Sakai, N. Neural Substrates for Conditioned Taste Aversion in the Rat. *Behavioural Brain Research*. Behav Brain Res December 1994, pp 123–137.

(41) Yiannakas, A.; Rosenblum, K. The Insula and Taste Learning. *Frontiers in Molecular Neuroscience*. Frontiers Media S.A. 2017.

(42) Bermudez-Rattoni, F.; McGaugh, J. L. Insular Cortex and Amygdala Lesions Differentially Affect Acquisition on Inhibitory Avoidance and Conditioned Taste Aversion. *Brain Res.* **1991**, *549* (1), 165–170.

(43) Cubero, I.; Thiele, T. E.; Bernstein, I. L. Insular Cortex Lesions and Taste Aversion Learning: Effects of Conditioning Method and Timing of Lesion. *Brain Res.* **1999**, *839* (2), 323–330.

(44) Fontanini, A.; Katz, D. B. Behavioral Modulation of Gustatory Cortical Activity. In *Annals of the New York Academy of Sciences*; 2009; Vol. 1170.

(45) Riether, C.; Doenlen, R.; Pacheco-López, G.; Niemi, M. B.; Engler, A.; Engler, H.; Schedlowski, M. Behavioural Conditioning of Immune Functions: How the Central Nervous System Controls Peripheral Immune Responses by Evoking Associative Learning Processes. *Reviews in the Neurosciences*. Freund Publishing House Ltd 2008, pp 1–17.

(46) Ramírez-Amaya, V.; Bermudez-Rattoni, F. Conditioned Enhancement of Antibody Production Is Disrupted by Insular Cortex and Amygdala but Not Hippocampal Lesions. *Brain. Behav. Immun.* **1999**, *13* (1), 46–60.

(47) Chen, J.; Lin, W.; Wang, W.; Shao, F.; Yang, J.; Wang, B.; Kuang, F.; Duan, X.; Ju, G. Enhancement of Antibody Production and Expression of C-Fos in the Insular Cortex in Response to a Conditioned Stimulus after a Single-Trial Learning Paradigm. *Behav. Brain Res.* **2004**, *154* (2), 557–565.

(48) Ramírez-Amaya, V.; Alvarez-Borda, B.; Ormsby, C. E.; Martínez, R. D.; Pérez-Montfort, R.; Bermúdez-Rattoni, F. Insular Cortex Lesions Impair the Acquisition of Conditioned Immunosuppression. *Brain. Behav. Immun.* **1996**, *10* (2), 103–114.

(49) Fermin, A. S. R.; Friston, K.; Yamawaki, S. An Insula Hierarchical Network Architecture for Active Interoceptive Inference. *R. Soc. Open Sci.* **2022**, *9* (6).

(50) Namkung, H.; Kim, S.-H.; Sawa, A. The Insula: An Underestimated Brain Area in Clinical Neuroscience, Psychiatry, and Neurology. *Trends Neurosci.* **2017**, *40* (4), 200.

(51) Gogolla, N. The Insular Cortex. *Curr. Biol.* **2017**, *27*, R580–R586.

(52) Maffei, A.; Haley, M.; Fontanini, A. Neural Processing of Gustatory Information in Insular Circuits. *Current Opinion in Neurobiology*. 2012.

(53) Kayyal, H.; Chandran, S. K.; Yiannakas, A.; Gould, N.; Khamaisy, M.; Rosenblum, K. Insula to MPFC Reciprocal Connectivity Differentially Underlies Novel Taste Neophobic Response and Learning in Mice. *Elife* **2021**, *10*.

(54) Craig, A. Pain Mechanisms: Labeled Lines versus Convergence in Central Processing. *Annu. Rev. Neurosci.* **2003**, *26*, 1–30.

(55) PENFIELD, W.; FAULK, M. E. The insulafurther observations on its function. *Brain* **1955**, *78* (4), 445–470.

(56) Craig, A. D. Interoception: The Sense of the Physiological Condition of the Body. *Curr. Opin. Neurobiol.* **2003**, *13* (4), 500–505.

(57) Berntson, G. G.; Khalsa, S. S. Neural Circuits of Interoception. *Trends Neurosci.* **2021**, *44* (1), 17–28.

(58) Ghaziri, J.; Nguyen, D. K. Structural Connectivity of the Insula. *Isl. Reil Hum. Brain Anat. Funct. Clin. Surg. Asp.* **2018**, 77–83.

(59) Rao-Ruiz, P.; Couey, J. J.; Marcelo, I. M.; Bouwkamp, C. G.; Slump, D. E.; Matos, M. R.; van der Loo, R. J.; Martins, G. J.; van den Hout, M.; van IJcken, W. F.; Costa, R. M.; van den Oever, M. C.; Kushner, S. A. Engram-Specific Transcriptome Profiling of Contextual Memory Consolidation. *Nat. Commun. 2019 101* **2019**, *10* (1), 1–14.

(60) Sørensen, A. T.; Cooper, Y. A.; Baratta, M. V.; Weng, F. J.; Zhang, Y.; Ramamoorthi, K.; Fropf, R.; Laverriere, E.; Xue, J.; Young, A.; Schneider, C.; Gøtzsche, C. R.; Hemberg, M.; Yin, J. C. P.; Maier, S. F.; Lin, Y. A Robust Activity Marking System for Exploring Active Neuronal Ensembles. *Elife* **2016**.

(61) Sharma, V.; Sood, R.; Khlaifia, A.; Eslamizade, M. J.; Hung, T. Y.; Lou, D.; Asgarihafshejani, A.; Lalzar, M.; Kiniry, S. J.; Stokes, M. P.; Cohen, N.; Nelson, A. J.; Abell, K.; Possemato, A. P.; Gal-Ben-Ari, S.; Truong, V. T.; Wang, P.; Yiannakas, A.; Saffarzadeh, F.; Cuello, A. C.; Nader, K.; Kaufman, R. J.; Costa-Mattioli, M.; Baranov, P. V.; Quintana, A.; Sanz, E.; Khoutorsky, A.; Lacaille, J. C.; Rosenblum, K.; Sonenberg, N. EIF2α Controls Memory Consolidation via Excitatory and Somatostatin Neurons. *Nature* **2020**, *586* (7829), 412–416.

(62) Shrestha, P.; Shan, Z.; Mamcarz, M.; Ruiz, K. S. A.; Zerihoun, A. T.; Juan, C. Y.; Herrero-Vidal, P. M.; Pelletier, J.; Heintz, N.; Klann, E. Amygdala Inhibitory Neurons as Loci for Translation in Emotional Memories. *Nature* **2020**.

(63) Pacheco-Lopez, G. Neural Substrates for Behaviorally Conditioned Immunosuppression in the Rat. *J. Neurosci.* **2005**, *25* (9), 2330–2337.

(64) Bauer D, Busch M, Pacheco-López G, Kasper M, Wildschütz L, Walscheid K, Bähler H, Schröder M, Thanos S, Schedlowski M, H. A. Behavioral Conditioning of Immune Responses with Cyclosporine A in a Murine Model of Experimental Autoimmune Uveitis. *Neuroimmunomodulation* **2017**, *24* (2), 87–99.

(65) Sanders, V. M.; Straub, R. H. Norepinephrine, the β-Adrenergic Receptor, and Immunity. *Brain. Behav. Immun.* **2002**, *16* (4), 290–332.

(66) Kustrimovic N, Rasini E, Legnaro M, Bombelli R, Aleksic I, Blandini F, Comi C, Mauri M, Minafra B, Riboldazzi G, Sanchez-Guajardo V, Marino F, C. M. Dopaminergic Receptors on CD4+ T Naive and Memory Lymphocytes Correlate with Motor Impairment in Patients with Parkinson’s Disease. *Sci. Rep.* **2016**, *6*.

(67) Mashimo, M.; Iwasaki, Y.; Inoue, S.; Saito, S.; Kawashima, K.; Fujii, T. Acetylcholine Released from T Cells Regulates Intracellular Ca2 +, IL-2 Secretion and T Cell Proliferation through Nicotinic Acetylcholine Receptor. *Life Sci.* **2017**, *172*, 13–18. h

(68) Pacheco, R.; Gallart, T.; Lluis, C.; Franco, R. Role of Glutamate on T-Cell Mediated Immunity. *J. Neuroimmunol.* **2007**, *185* (1–2), 9–19.

(69) Hsueh, C. M.; Chen, S. F.; Lin, R. J.; Chao, H. J. Cholinergic and Serotonergic Activities Are Required in Triggering Conditioned NK Cell Response. *J. Neuroimmunol.* **2002**, *123* (1–2).

(70) Chao, H. J.; Hsu, Y. C.; Yuan, H. P.; Jiang, H. S.; Hsueh, C. M. The Conditioned Enhancement of Neutrophil Activity Is Catecholamine Dependent. *J. Neuroimmunol.* **2005**, *158* (1–2).

(71) Zhang, T.; Warden, A. R.; Li, Y.; Ding, X. Progress and Applications of Mass Cytometry in Sketching Immune Landscapes. *Clin. Transl. Med.* **2020**, *10* (6).

(72) S, von H.; MS, E.; M, S.; E, N.; M, S.; G, S.; J, V.; A, del R.; M, S.; J, W. Behaviorally Conditioned Effects of Cyclosporine A on the Immune System of Rats: Specific Alterations of Blood Leukocyte Numbers and Decrease of Granulocyte Function. *J. Neuroimmunol.* **1998**, *85* (2), 193–201.

(73) Exton, M. S.; Hörsten, S. Von; Vöge, J.; Westermann, J.; Schult, M.; Nagel, E.; Schedlowski, M. Conditioned Taste Aversion Produced by Cyclosporine A: Concomitant Reduction in Lymphoid Organ Weight and Splenocyte Proliferation. *Physiol. Behav.* **1998**, *63* (2), 241–247.

(74) Vlasov, K.; Dort, C. J. Van; Solt, K. Optogenetics and Chemogenetics. *Meth Enzymol.* **2018**, 181.

(75) Whissell, P. D.; Tohyama, S.; Martin, L. J. The Use of DREADDs to Deconstruct Behavior. *Front. Genet.* **2016**, *7* (MAY).

(76) DeNardo, L.; Luo, L. Genetic Strategies to Access Activated Neurons. *Curr. Opin. Neurobiol.* **2017**, *45*, 121–129.

(77) Weng, F. J.; Garcia, R. I.; Lutzu, S.; Alviña, K.; Zhang, Y.; Dushko, M.; Ku, T.; Zemoura, K.; Rich, D.; Garcia-Dominguez, D.; Hung, M.; Yelhekar, T. D.; Sørensen, A. T.; Xu, W.; Chung, K.; Castillo, P. E.; Lin, Y. Npas4 Is a Critical Regulator of Learning-Induced Plasticity at Mossy Fiber-CA3 Synapses during Contextual Memory Formation. *Neuron* **2018**, *97* (5), 1137-1152.

(78) Klavir, O.; Genud-Gabai, R.; Paz Dr., R. Low-Frequency Stimulation Depresses the Primate Anterior-Cingulate-Cortex and Prevents Spontaneous Recovery of Aversive Memories. *J. Neurosci.* **2012**, *32* (25), 8589.

(79) Tomer, R.; Ye, L.; Hsueh, B.; Deisseroth, K. Advanced CLARITY for Rapid and High-Resolution Imaging of Intact Tissues. *Nat. Protoc. 2014 97* **2014**, *9* (7), 1682–1697.

(80) Du, H.; Hou, P.; Wang, L.; Wang, Z.; Li, Q. Modified CLARITY Achieving Faster and Better Intact Mouse Brain Clearing and Immunostaining. *Sci. Reports 2019 91* **2019**, *9* (1), 1–11.

(81) Stefaniuk, M.; Gualda, E. J.; Pawlowska, M.; Legutko, D.; Matryba, P.; Koza, P.; Konopka, W.; Owczarek, D.; Wawrzyniak, M.; Loza-Alvarez, P.; Kaczmarek, L. Light-Sheet Microscopy Imaging of a Whole Cleared Rat Brain with Thy1-GFP Transgene. *Sci. Reports 2016 61* **2016**, *6* (1), 1–9.

(82) Mano, T.; Albanese, A.; Dodt, H.-U.; Erturk, A.; Gradinaru, V.; Treweek, J. B.; Miyawaki, A.; Chung, K.; Ueda, H. R. Whole-Brain Analysis of Cells and Circuits by Tissue Clearing and Light-Sheet Microscopy. *J. Neurosci.* **2018**, *38* (44), 9330–9337.

(83) David, O.; Barrera, I.; Chinnakkaruppan, A.; Kaphzan, H.; Nakazawa, T.; Yamamoto, T.; Rosenblum, K. Dopamine-Induced Tyrosine Phosphorylation of NR2B (Tyr1472) Is Essential for ERK1/2 Activation and Processing of Novel Taste Information. *Front. Mol. Neurosci.* **2014**, *7*.

(84) Rosenblum, K.; Berman, D. E.; Hazvi, S.; Lamprecht, R.; Dudai, Y. NMDA Receptor and the Tyrosine Phosphorylation of Its 2B Subunit in Taste Learning in the Rat Insular Cortex. *J. Neurosci.* **1997**, *17* (13), 5129–5135.

(85) Lussier, M. P.; Sanz-Clemente, A.; Roche, K. W. Dynamic Regulation of N-Methyl-d-Aspartate (NMDA) and α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid (AMPA) Receptors by Posttranslational Modifications. *J. Biol. Chem.* **2015**, *290* (48), 28596.

(86) Vithlani, M.; Terunuma, M.; Moss, S. J. The Dynamic Modulation of GABAa Receptor Trafficking and Its Role in Regulating the Plasticity of Inhibitory Synapses. *Physiol. Rev.* **2011**, *91*, 1009–22.

(87) Rodríguez-García, G.; Miranda, M. I. Opposing Roles of Cholinergic and GABAergic Activity in the Insular Cortex and Nucleus Basalis Magnocellularis during Novel Recognition and Familiar Taste Memory Retrieval. *J. Neurosci.* **2016**, *36* (6), 1879–1889.

(88) P, M.-C.; R, P.-O.; V, V.-S.; I, B.; F, B.-R. Hippocampal Release of Dopamine and Norepinephrine Encodes Novel Contextual Information. *Hippocampus* **2017**, *27* (5), 547–557.

(89) Medina, J. H.; Viola, H. ERK1/2: A Key Cellular Component for the Formation, Retrieval, Reconsolidation and Persistence of Memory. *Front. Mol. Neurosci.* **2018**, *11*.

(90) Mazzucchelli, C.; Brambilla\*, R. Ras-Related and MAPK Signalling in Neuronal Plasticity and Memory Formation. *Cell. Mol. Life Sci. C. 2000 574* **2000**, *57* (4), 604–611.

(91) Canagarajah, B. J.; Khokhlatchev, A.; Cobb, M. H.; Goldsmith, E. J. Activation Mechanism of the MAP Kinase ERK2 by Dual Phosphorylation. *Cell* **1997**, *90* (5), 859–869.

(92) Gil-Lievana, E.; Balderas, I.; Moreno-Castilla, P.; Luis-Islas, J.; McDevitt, R. A.; Tecuapetla, F.; Gutierrez, R.; Bonci, A.; Bermúdez-Rattoni, F. Glutamatergic Basolateral Amygdala to Anterior Insular Cortex Circuitry Maintains Rewarding Contextual Memory. *Commu. Biol.* **2020**, *3*, 1–11.

(93) Gehrlach, D. A.; Weiand, C.; Gaitanos, T. N.; Cho, E.; Klein, A. S.; Hennrich, A. A.; Conzelmann, K. K.; Gogolla, N. A Whole-Brain Connectivity Map of Mouse Insular Cortex. *Elife* **2020**, *9*, 1–78.

(94) Lavi, K.; Jacobson, G. A.; Rosenblum, K.; Lüthi, A. Encoding of Conditioned Taste Aversion in Cortico-Amygdala Circuits. *Cell Rep.* **2018**, *24* (2), 278–283.

(95) Inberg, S.; Elkobi, A.; Edri, E.; Rosenblum, K. Taste Familiarity Is Inversely Correlated with Arc/Arg3.1 Hemispheric Lateralization. *J. Neurosci.* **2013**, *33* (28), 11734–11743.

(96) Karolis, V. R.; Corbetta, M.; Thiebaut de Schotten, M. The Architecture of Functional Lateralisation and Its Relationship to Callosal Connectivity in the Human Brain. *Nat. Commun. 2019 101* **2019**, *10* (1), 1–9.

(97) Gould, N. L.; Sharma, V.; Hleihil, M.; Chandran, S. K.; David, O.; Edry, E.; Rosenblum, K. Dopamine-Dependent QR2 Pathway Activation in Ca1 Interneurons Enhances Novel Memory Formation. *J. Neurosci.* **2020**, *40* (45), 8698–8714.

(98) Hernandez, P. J.; Abel, T. The Role of Protein Synthesis in Memory Consolidation: Progress amid Decades of Debate. *Neurobiol. Learn. Mem.* **2008**, *89* (3), 293..

(99) Gal-Ben-Ari, S.; Kenney, J. W.; Ounalla-Saad, H.; Taha, E.; David, O.; Levitan, D.; Gildish, I.; Panja, D.; Pai, B.; Wibrand, K.; Simpson, T. I.; Proud, C. G.; Bramham, C. R.; Armstrong, J. D.; Rosenblum, K. Consolidation and Translation Regulation. **2012**, 410–422.

(100) Sossin, W. S.; Costa-Mattioli, M. Translational Control in the Brain in Health and Disease. *Cold Spring Harb. Perspect. Biol.* **2019**, *11* (8).

(101) Rosenblum, K.; Meiri, N.; Dudai, Y. Taste Memory: The Role of Protein Synthesis in Gustatory Cortex. *Behav. Neural Biol.* **1993**, *59* (1), 49–56.

(102) Taha E, Patil S, Barrera I, Panov J, Khamaisy M, Proud CG, Bramham CR, R. K. EEF2/EEF2K Pathway in the Mature Dentate Gyrus Determines Neurogenesis Level and Cognition. *Curr. Biol.* **2020**, *30* (18), 3507-3521.

(103) Hrestha, P.; Shan, Z.; Mamcarz, M.; Ruiz, K. S. A.; Zerihoun, A. T.; Juan, C. Y.; Herrero-Vidal, P. M.; Pelletier, J.; Heintz, N.; Klann, E. Amygdala Inhibitory Neurons as Loci for Translation in Emotional Memories. *Nature* **2020**, *586* (7829), 407–411.

(104) Stern, S. A.; Azevedo, E. P.; Pomeranz, L. E.; Doerig, K. R.; Ivan, V. J.; Friedman, J. M. Top-down Control of Conditioned Overconsumption Is Mediated by Insular Cortex Nos1 Neurons. *Cell Metab.* **2021**, *33*, 1418-1432.

**Please reply to the following questions, point by point (up to a total of 2 pages):**

1. What are you trying to do? Articulate your objectives using absolutely no jargon.

We aim to identify for the first time the underlying brain mechanisms of conditioned immune response. Conditioned immune response is a form of classical psychological conditioning that involves a conditioned stimulus - the pairing of sensory information like a taste, with an immune activating/deactivating drug - and an immune response as an unconditioned stimulus. Days, weeks or months following the conditioning, presentation of the sensory stimulus (taste) by itself will activate/deactivate the immune response similarly to the drug used during the paired association. One may argue that conditioned immune response is a non-declarative form of placebo/nocebo effect. We know almost nothing about underlying mechanisms of placebo/nocebo effect.

1. How is it done today, and what are the limits of current practice?

While conditioned immune response was introduced many years ago as a paradigm, we know very little about the underlying mechanisms. The data that was collected over the years was based mainly on brain lesion/s studies which are limited by their nature. There are very few studies on the subject in general.

The main limitation was the identification of an internal representation of an immune response in the cortex. Our laboratory took part in this research and publication, where we identified the posterior insular cortex as a cortical area where immune responses is neuronal encoded. This finding revolutionized the way we think about how the central nervous system, and specifically the cortex, interacts with the immune system. For the past decade, the laboratory has been contemplating how to approach the subject. However, the way brain/cortical circuits encode internal (i.e. interoceptive) information and states is still largely unknown.

1. What’s new in your approach and why do you think it will be successful?

Others and we identified the anterior part of the insular cortex as the brain area that encodes taste sensory information and its valance. We recently identified the posterior insula as the cortical area that encodes an immune response. In the preliminary data of the proposal, we described for the first time the functional connectivity **within** the insula that convey the information between anterior and posterior insula regions, and can explain the associative learning between taste and immune outcome.

1. Who cares? If you are successful, what difference will it make?

Understanding how the brain modulates/controls immune response is a prime target for understanding basic behavior (e.g. how an animal prepares the immune system for near future experiences) and the way the brain integrates sensory information about the outside world with interoceptive information of the immune system. In addition, from health and clinical perspective, mal-function of the process contributes to what we refer as pshcosomatic diseases. The idea that manipulating the brain has the potential to modulates the immune response is pivotal for different kind of novel treatments.

1. What are the risks and the payoffs?

The proposal integrates different fields of molecular, cellular and behavioral neuroscience, AND immunology. One should design the experiments correctly, master different and complementary methods and have experience with the different aspects involved in order to prove or refuse the main hypothesis. Following our preliminary experiments and data, the possibility that we will have clear results on the cellular/circuit levels, are reasonably high, but we have not yet tested at the molecular level. I assume that if we have clear results on the cellular/circuit levels to explain different forms of conditioned immune responses in insula functional connectivity, that, in itself will be a lot, and we can focus on it. However, our previous experience and publication suggest that we will also be able to give clear answers on the molecular level, and integrate both molecular and cellular levels of analysis into a more coherent description of cortical mechanisms underlying conditioned immune response.

1. Are you internationally competitive as a researcher at your career stage and in my discipline? Explain

We are world experts in revealing underlying mechanisms of learning and memory. In addition, we study both cellular/circuits AND molecular mechanisms, as well as the integration between the two, to explain behavior. In addition, the insula cortex, a main subject in the current proposal, is different in many respects from other cortical/forebrain areas, and our laboratory is a world leader for the neuroscience studies in the insula.

1. Why does your project need such a substantial budget?

The project integrates molecular and cellular techniques, and each one of them is expensive by itself, and each one of them also demands highly qualified post doc/PhD students to perform the research. The budget is not high for the requested research.

1. Why are you the best person to carry out the project?

Our laboratory is the best laboratory to perform the propose project since: 1. We are world experts in the insula function and structure in rodents. 2. We have worked for a long time trying to understand the interaction between bodily states and taste sensory information. 3. We are one of the few laboratories that integrate molecular and cellular mechanisms underlying learning and memory. 3. We published in the last 3 years the most important papers related to the subject of the research proposal. 4. We are also capable of integrating the immune read outs, since we studied the subject recently (published and submitted manuscripts) and Dr Efrat Edri, a research scientist in the lab, is an immunologist by training.

Schedule

|  |  |  |
| --- | --- | --- |
| task | Start date | end |
| T1: Correlative cellular measurements in the IC during the different phases and types of CIR. | 01/10/2024 | 01/10/2027 |
| T2: Identifying the cellular code of CIR and its retrieval. | 1/10/2025 | 1/04/2028 |
| T3: Molecular mechanisms within the IC underlying CIR and its retrieval. | 01/10/2027 | 01/04/2029 |
| T4: Detailed description of functional organization of the IC microcircuit and the model to describe the formation of an internal representation of CIR in the IC. | 01/10/2027 | 01/04/2029 |
| T5: Defining the neurotransmitters, neuromodulators and signal transduction in specific cell population as defined by T1-4' necessary for CIR acquisition and retrieval | 01/10/2028 | 1/10/2029 |

Explanatory notes:

T1 in this task we will look for correlative measurements at multiple levels of analysis: transgenic mice, viral vectors, immunohistochemistry (IHC), optogenetics, electrophysiology in behaving mice (i.e. measuring spike activity with tetrodes), and patch-clamp in slices (i.e. measuring intrinsic and synaptic activity). We will measure the correlations following CS (i.e. taste), following UCS (induction or suppression of the immune response) and following the association of the two. We will do this analysis in both immune stimulation and immune suppression, in different time points following CIR. In parallel, we will identify functional and neuroanatomical connectivity within the IC.

T2- following the calibration of CIR in T1, we will start T2 in which we will identify the activated cells during the presentation of the CS (e.g. taste), following the presentation of the CS (i.e. maintaining the information ready for an association), UCS (e.g. immunosuppression or activation) and both. The time frame for these experiments is during both the acquisition and molecular consolidation phases of learning. In addition, we will perform electrophysiology in slices and in freely behaving animals.

T3 – In the fourth year, we will start to elucidate molecular mechanism underlying CIR. More specifically, we will test similar mechanisms that were identified in CTA such as synaptic protein expression, neurotransmitters activation and their downstream signaling.

T4- following T1 and 2 and in parallel to T3, we will start to integrate the different results from the different methods in order to propose a model for the formation of an internal representation of CIR in the IC.

T5- in parallel to T3 and according to the results in T3, we will use genetic tools to determine the neurotransmitters and neuromodulators necessary for CIR acquisition.

Answering BRG committee comments point by point (see below):

The proposal entitled “Circuit within the insular cortex subserves conditioned immune

response“ (2118/23) has been evaluated by the BRG Review committee. Unfortunately, the

committee decided, after an in-depth discussion, that the proposal does not meet the threshold

level that would justify full external review mainly due to the level of competition in the BRG

program.

Please see below the committee’s comments:

The project aims to dissect the circuit and molecular mechanisms in the insular cortex that

mediate the conditioned immune response (CIR), which represents a link between the brain and

the immune system. The anterior insula (aIC) represents taste and smell information while the

posterior insula (pIC) has recently been shown to represent immune processes. The aim here is

to define the neural circuits in the insular cortex (IC) that could provide a link between the two

insula parts to mediate CIR. The aim is first to show the role of IC in mediating the CIR by pairing

odor/taste with immune suppressants or activations. Then, the cellular and synaptic properties

of IC neurons will be recorded in slices and in vivo. Lastly, molecular quantification of different

neurotransmitters and signaling pathways such as NMDA receptors, ERK/MAPK etc will be done,

to show the changes in IC associated with CIR.

Strengths

• Prof. Rosenblum is a world expert in the study of neural circuitries involved in memory

and learning, and in establishing the connection between taste acquisition and the

insula.

• This is an innovative proposal that aims at defining a novel circuitry involving the

anterior and posterior insula and their role in establishing the Conditioned Immune

Response (CIM), which could potentially explain (among others) the placebo effect. The

project has the potential to establish a new paradigm.

• The experimental plan includes several state-of-the-art approaches for studying neural

circuitries (which is clearly the main expertise of the PI) and establishing the CIR.

I Thank the reviewers for the above.

Weaknesses:

• The preliminary studies are insufficient to provide compelling evidence of the

hypothesis presented in the proposal. Additional convincing results are required to

strengthen feasibility.

Our preliminary results on the subject are under revisions in two different best journals.

In high risk high gain grant research proposal, I need to explain in details some of the methods (behavior, electrophysiology, neuroanatomy, circuit and genetic tools, immunology, molecular at the different levels) and give real alternatives for anticipated problematic measurements. Due to space limitations we provided just a small fraction of our preliminary results. Following reviewers comments I added preliminary results, in text, in figures and added also a summary figure.

The main preliminary results demonstrate:

1. Clear CIR protocol with one of the agents to induce UCS (LPS)
2. Clear correlation of activation of the reciprocal connections between aIC and pIC (correlation).
3. Clear effect of deactivation of the reciprocal pathways on behavior (aversive) AND immune response following retrieval of CIR (causality).
4. Clear effect of electrophysiological measurements (intrinsic properties and synaptic strength) within both pathways following retrieval of CIR (beginning to elucidate mechanisms on the cellular/circuit level).

Due to space limitation again, I can give only few of the preliminary results we have but I hope that following the changes, I better explained the solid data we have so far.

• Some specific questions could be better formulated. For example, regarding the

electrophysiological experiments, the predictions are not sufficiently clear in terms of

the changes expected in the circuits. Is it assumed that there is a synaptic change in

interactions between aIC and pIC?

Following reviewers comments I better formulated predications in general and specifically for the electrophysiological measurements. Our preliminary results identify changes both in intrinsic properties and synaptic strength following retrieval of CIR in the studied pathways. We will have to replicate these experiments and expand the time frame to propose a model for possible underlying mechanisms.