

## Norman Cousins Lecture

## The learned immune response: Pavlov and beyond

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## ABSTRACT

The ability to associate physiological changes with a specific flavor was most likely acquired during evolution as an adaptive strategy aimed at protecting the organism while preparing it for danger. The behaviorally conditioned or learned immune response is an exquisite example of the bidirectional communication between the central nervous system (CNS) and the peripheral immune system. How is it possible that specific immuno-modulating properties of a drug or substance (unconditioned stimulus) can be re-enlisted just by the mere re-exposure to a particular taste, odor or environment (conditioned stimulus)? To answer this key question, we review the neurobiological mechanism mediating this type of associative learning, as well as the pathways and mechanisms employed by the brain to harness the immune system during the execution of the conditioned immune response. Finally, we focus on the potential therapeutic relevance of such learned immune responses, and their re-conceptualization within the framework of “learned placebo effects”.

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## 1. Introduction

The behavioral conditioning of immune functions is a fascinating example illustrating the bidirectional interactions between the central nervous system (CNS) and the peripheral immune system (Ader and Cohen, 2001). While expressions such as learning and memory are fixed components in the immunological terminology, when referring to the processes to improve recognition of antigens by B or T lymphocytes, for a long time it remained enigmatic why peripheral immune responses should be affected by classical, or Pavlovian, conditioning. In recent years, it is becoming increasingly acknowledged and accepted that this “learned immune response” indeed developed during evolution as an adaptive strategy.

Ambulatory organisms evolved to face rapidly changing internal and external environments by acquiring the ability to learn and to modify instinctive behaviors. Classical conditioning can thus be understood as the process of learning about the temporal and/or causal relationships between external and internal stimuli. This process enables the organism to use the appropriate preparatory set of responses before biologically significant events occur (Rescorla, 1988, 2003). From this perspective, the capacity to associate a

specific environmental context or a particular flavor (conditioned stimuli: CS) with specific immune challenges e.g. allergens, toxins or antigens (unconditioned stimuli: US) is certainly of highly adaptive value. Thus, it can be hypothesized that this capacity was acquired during evolution as an adaptive strategy in order to protect the organism and prepare it for danger. For example, the exposure to a specific antigen and its categorization as an allergen might be centrally associated (i.e., a *learning process*) with a specific environment or food. An adaptive response is then elicited (i.e., a *memory process*), consisting first of behavioral modifications to avoid the place or food associated with the antigen (Costa-Pinto et al., 2005; Markovic et al., 1992). If avoidance is not possible, the organism will try to reduce the contact with the allergen, for instance by coughing or sneezing (Pinto et al., 1995). At the same time, the immune system may prepare the body for interaction with the antigen, e.g. by mast cell degranulation (Irie et al., 2001; MacQueen et al., 1989; Palermo-Neto and Guimarães, 2000; Russell et al., 1984) or antibody production (Ader et al., 1993; Alvarez-Borda et al., 1995; Chen et al., 2004; Husband et al., 1993). Although under experimental conditions such associative learning can be extinguished, it is likely that it will last for a long time, since the optimal adaptive strategy in a natural setting would be for the organisms to consistently avoid contact with the environmental cues that signal the CS. However, it should be emphasized that under artificial conditions, i.e. employing potent immunosuppressive drugs, the organism “learns” to mimic the pharmacologic effects,

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desirable under clinical settings, but to a certain extent contra-adaptive, if this would occur under natural settings.

### 1.1. Conditioned taste aversion

Two basic steps compose any classical conditioning protocol: an *acquisition* phase in which one or more CS–US contingent pairings occur in order to induce an associative learning process, and an *evocation* phase where the memory of the newly acquired association is retrieved after exposing the subject to the CS (Pavlov, 1927). The association of food or drink ingestion with its possible post-prandial toxic/immune consequences has been experimentally studied in rodents and humans employing the conditioned taste aversion model (Garcia et al., 1955). In this experimental paradigm, the individual learns to associate a particular taste with a delayed visceral malaise (Bermúdez-Rattoni, 2004). This learning capacity has been conserved across the animal kingdom (Kawai et al., 2004; Marella et al., 2006; Paradis and Cabanac, 2004; Schedlowski, 2006), including humans (Garb and Stunkard, 1974), reflecting its highly adaptive value, currently just attributed to food selection strategies but indeed involved in a more complex and diverse repertoire of physiological responses that the individual evokes in order to avoid, reject and/or initiate defense strategies against harmful unconditioned effects (Niemi et al., 2006). The majority of studies in rodents employ a sweet tasting solution (e.g. saccharin) as a CS and an injection of an immuno-modulating drug or agent as a US. After one or several pairings of CS and US during the acquisition (*learning*) phase, animals are re-exposed to the CS during the evocation phase (*memory*). The re-exposure to the CS is now inducing conditioned behavioral and immunological responses. At the behavioral level this response is characterized by avoidance and/or aversion to the CS reflected by a reduced consumption of the sweet tasting solution. More importantly, the re-exposure to the CS elicits an immune response, which is similar to the response induced by the drug or agent employed as the US (Ader, 2003; Ader and Cohen, 1991, 2001; Brittain and Wiener,

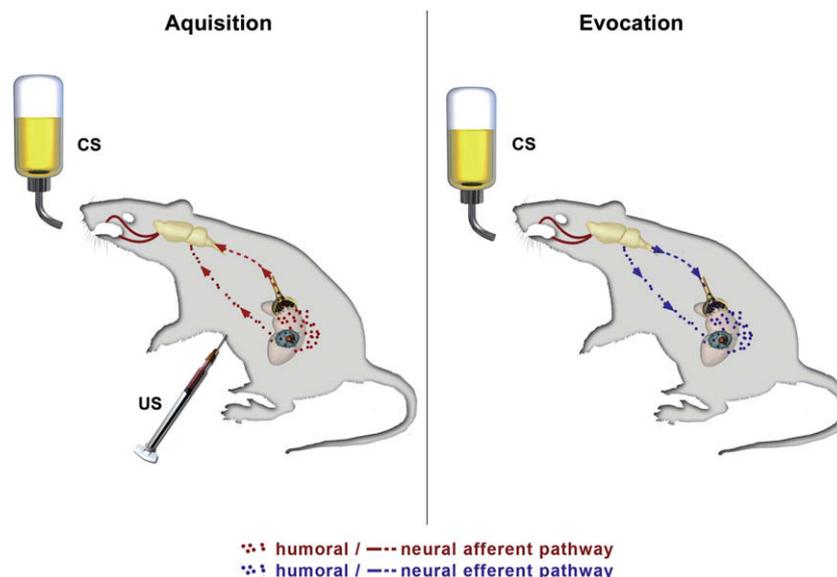
1985; Hucklebridge, 2002; Markovic et al., 1993; Pacheco-López et al., 2006; Riether et al., 2008).

### 1.2. One single experimental model for the bidirectional CNS–immune interaction

The uniqueness of the paradigm of behaviorally conditioned immune responses in analyzing CNS–immune system interaction is manifold (Fig. 1). From a systematic point of view, one advantage is certainly the chance to analyze the *afferent* and *efferent* communication pathways between the brain and the peripheral immune system in one model together with the stimuli processing by the CNS. In addition, by implementing diverse clinical readout systems, the potential therapeutic relevance of the behaviorally conditioned immune response can be investigated. In this review we summarize the neurobiological mechanisms mediating this kind of associative learning and subsequently address the pathways and mechanisms employed by the brain to harness the immune system. Finally, we discuss the therapeutic relevance of such learned immune responses, and their re-conceptualization within the framework of “*learned placebo effects*”.

### 1.3. Cyclosporin A as an unconditioned stimulus

To elucidate the peripheral and central mechanisms mediating conditioned immunosuppressive responses together with the potential clinical meaning of this phenomenon, our laboratory has established a paradigm in which the selective immunosuppressive drug cyclosporine A (CsA) as the US is contingently paired with a distinctive taste (saccharin) as the CS. CsA is a calcineurin (CaN) inhibitor reducing the synthesis of mainly Th1-cytokines, in particular IL-2 and is therefore relatively specifically suppressing the activity of T lymphocytes (Batiuk and Halloran, 1997; McCaffrey et al., 1993).



**Fig. 1.** As any classical conditioning protocol, the conditioned taste aversion paradigm consists of two basic steps. During the *acquisition* phase, the gustatory stimulus (e.g. saccharin solution), which serves as the conditioned stimulus (CS), is paired with an injection of a drug or substance with immunological properties (the unconditioned stimulus/US). The taste is subsequently received by the CNS via neural afferences. In parallel, the drug or substance (US) itself and/or the neural or immunological changes induced by the US is sensed via neural or humoral afferent pathways by the CNS. The CS and the US are subsequently associated in the brain and this association might be stored as any other learned event. During *evocation*, when the organisms is re-exposed just to the CS, the gustatory stimulus is now able to re-enlist the stored information via efferent neural and/or humoral pathways, and to induce the immunological changes formally induced by the drug or substance employed as the US. *Abbreviations:* CNS, central nervous system; CS, conditioned stimulus; US, unconditioned stimulus.

## 2. How the CNS receives the signals: Afferent pathways

In order to associate a CS (such as a taste or an odor) with a US (for example an immuno-modulating agent), the CNS must sense the signals induced by the CS and US. The neural mechanisms of olfactory or gustatory perception are reasonably well understood, including the transduction of taste signals by specific receptors on the taste buds, propagation of the gustatory signals via several peripheral nerves as well as brain stem and thalamic relays, and processing of gustatory perception by a complex neural pathway integrating taste, olfactory and somatosensory inputs (Simon et al., 2006). The neural network involved in taste-visceral associative learning mainly includes sensory and hedonic pathways (Sewards, 2004). The brain structure involved includes the nucleus of the solitary tract, parabrachial nucleus; medial thalamus; amygdala and insular cortex (Yamamoto et al., 1994). The insular cortex is important for the association, retrieval, retention, and extinction of taste-visceral memories (Bermúdez-Rattoni, 2004; Bermúdez-Rattoni et al., 1997; Nerad et al., 1996), and may integrate gustatory and visceral stimuli (Sewards and Sewards, 2001).

In contrast, much less is known about the mechanism(s) which allow the CNS to receive information regarding specific drug-induced immune changes (or other pharmacological effects) from a drug that was employed as a US. The most commonly used US in studies of behaviorally conditioned inhibition of immune functions are immunosuppressive drugs, such as cyclophosphamide or CsA (Ader and Cohen, 2001; Riether et al., 2008). Antigens or immune-enhancing substances such the polyinosinic:polycytidylic acid (poly I:C) have been employed in experiments of conditioned enhanced immune responses (Hiramoto et al., 1993). The most common US to develop an acute phase reaction include the Gram (–) cell wall constituent lipopolysaccharide (LPS) (Exton et al., 1995c,d; Janz et al., 1996), superantigens such as staphylococcal enterotoxin A and B (SEA, SEB) (Kawashima and Kusnecov, 2002; Pacheco-López et al., 2004), or cytokines such as IL-1 (Dyck et al., 1990).

It has been proposed in previous years that the immune system operates as a diffuse sensory system, which detects the presence of specific chemical constituents associated with dangerous microorganisms and then signals the brain to react appropriately (Blalock, 2005; Goehler et al., 2000). Immune-sensory mechanisms have been classified into two general types of pathways: a *systemic* and a *neural* pathway. In the *systemic* or humoral pathway, the putative messengers such as cytokines, neurotransmitters or prostaglandins can cross the blood–brain barrier (Banks, 2005) or they can reach the brain via the circumventricular organs (Goehler et al., 2006), however, the latter may require high plasma messenger levels. The *neural* afferent pathway does not require changes in systemic concentrations but rather the translation of immune-borne messengers into neuronal signals in the periphery. Based on anatomical and functional considerations, the vagus nerve is well-suited for this immune-sensory function (Goehler et al., 2005; Tracey, 2002).

Which pathway(s) are activated during the acquisition process is still unclear since direct experimental evidence is rare. Hiramoto and co-workers employed a model pairing camphor odor (CS) and the viral mimicking drug: poly I:C (US), resulting in behaviorally conditioned enhancement of natural killer cell activity. Interferon- $\beta$  (IFN- $\beta$ ) release by poly I:C injection appeared to be the main cytokine signal in this model, which in this case directly interacted with the CNS via a systemic afferent pathway (Hiramoto et al., 1993; Solvason et al., 1993). There is also evidence to support the notion that the afferent pathway responsible for the conditioned increased NK cell activity differed from the pathway mediating the conditioned fever response in the same associative learning conditioning protocol (i.e. two conditioned responses) (Rogers et al., 1992).

More indirect evidence for the activation of a neural afferent pathway during acquisition comes from studies employing natural substances such as Keyhole limpet hemocyanin (KLH), a T cell-dependent antigen (Ader et al., 1993; Espinosa et al., 2004), hen egg-white lysozyme (Alvarez-Borda et al., 1995; Madden et al., 2001), SEB (Pacheco-López et al., 2004) or LPS (Exton et al., 1995a–d; Oberbeck et al., 2003). To what extent immune-induced neural activation is dependent on neural or systemic afferent pathways is not fully understood. However, the vagus nerve is anatomically well-positioned to detect and inform the brain about changes in visceral immune status (Dantzer et al., 2000; Goehler et al., 2000, 2007; Maier et al., 1998). Consequently, many immune-induced CNS effects are significantly diminished or totally blocked by prior vagotomy (Gaykema et al., 1995, 2000; Goehler et al., 1995; Koonsman et al., 2000; Luheshi et al., 2000). However, this does not exclude the possible existence of redundant pathways, as has been demonstrated under strong immune challenges inducing systemic responses (Hansen et al., 2000; Romanovsky, 2000; Romanovsky et al., 2000, 1997).

Regarding experiments that have employed immunosuppressants such as cyclophosphamide or CsA, it is unknown so far how these signals, either the induced immunosuppression or the direct effects of the drug itself, are sensed by the CNS. Analyses of brain activity after cyclophosphamide injection demonstrated that this drug exerted its main effects at the level of the brain stem as well as on sub-cortical telencephalic structures, and that cyclophosphamide uses both vagal and spinal afferent pathways to reach the brain (Bon et al., 1996, 1997a,b, 1998).

In contrast to cyclophosphamide's general cytotoxicity, the main and specific immunological CsA effect is to inhibit Th1-cytokines such as IL-2 and IFN- $\gamma$  via intracellular blockade of CaN activity (Calne, 2004; Schumacher and Nordheim, 1992). Distribution of CsA depends not only on physicochemical characteristics, but also on biological carriers such as lipoproteins and erythrocytes in blood. Cyclophilin, a binding protein for CsA, influences the distribution and activity of CsA in the body. Initially, (Fahr, 1993) reported no CsA presence in the brain after an acute administration. However, more recently CsA has been detected in brain tissue (Kung et al., 2001; Sato et al., 2007). Furthermore, CsA effects on neural CaN and other neurotransmitter system have been reported (Herink et al., 2003; Mazzanti et al., 2007). Radio-telemetry recordings and c-Fos expression analyses confirmed increased neural activity in the insular cortex as well as in the amygdala 2–4 h after CsA administration (Pacheco-López, unpublished observations). Therefore, a direct action of CsA on the brain within the conditioning paradigm cannot be discarded, being under current investigation.

Overall, at present it is not completely understood how the CNS detects the changes induced by different substances and drugs employed in the immuno-conditioning, and it remains to be elucidated which molecules are the messengers that activates the brain during the acquisition phase of a conditioning protocol.

## 3. Conditioning takes place: Relevant brain structures and neurotransmitters

During the acquisition, both CS and US are associated in the CNS, and in the whole field of neurobiology, learning and memory will be essential to understand the mechanisms of association and long-term information storage (Berman and Dudai, 2001; Bermúdez-Rattoni, 2004; McGaugh et al., 2002). Studies involved in taste aversion learning demonstrated, that the insular cortex is particularly relevant for the acquisition and retention of the associative learning process (Bermúdez-Rattoni and McGaugh, 1991; Cubero et al., 1999), and is able to integrate gustatory and visceral stimuli

(Sewards and Swards, 2001). Definitive evidence regarding the participation of specific brain structures can only be obtained through direct electrophysiological recordings (by electrodes or functional imagery) during the conditioning process. However, such data is scarce. Specific lesions, functional obstruction (neurotoxins) or metabolic mapping (c-Fos) have been so far the methods of choice for determining the effect of a particular brain structure on the acquisition or evocation of a learned immune response. Using an excitotoxic lesioning approach it has been determined that both, the insular cortex and central nucleus of the amygdala, excluding the hippocampus, are involved in the acquisition of the enhancement of the antibody response to lysozyme (Ramírez-Amaya and Bermúdez-Rattoni, 1999). To scope the involvement of the insular cortex and the amygdala is very appealing, seeing as they represent limbic structures widely known to be necessary for conditioned taste aversion (Yamamoto, 2007) as well as fear conditioning (LeDoux, 2003). The acquisition of conditioned immunosuppression using cyclophosphamide as US has also been found to require the specific participation of the insular cortex (Ramírez-Amaya et al., 1996). More recently, using the neuronal activity marker c-Fos, it was possible to confirm the pivotal role of the insular cortex in conditioned antibody production (Chen et al., 2004) in agreement with a previous report (Ramírez-Amaya and Bermúdez-Rattoni, 1999). Furthermore, the insular cortex is needed during the acquisition and evocation process while the amygdala is necessary only for the acquisition phase (Ramírez-Amaya et al., 1998).

In line with these observations, we have been able to identify the neural substrates involved in behaviorally conditioned immunosuppression with CsA as a US in rats (Pacheco-López et al., 2005). The conditioned effect in the immune response, such as lymphocyte proliferation and cytokine production (IL-2 and IFN- $\gamma$ ) was differentially affected by brain excitotoxic lesions. This data demonstrates that the insular cortex is essential for both the acquisition and evocation of this conditioned response. In contrast, the amygdala only appears to mediate the input of visceral information necessary for acquisition, whereas the ventro-medial hypothalamic nucleus appears to participate only in the output pathway to the immune system, which evokes the behaviorally conditioned immune response. Taken together, this data shows that across different conditioning models and substances used as a US, the insular cortex and the amygdala are essential brain areas for learning to take place (Pacheco-López et al., 2005).

In a study employing the model of conditioned augmentation of NK-activity with poly I:C as a US and camphor odor as a CS, chemical destruction of the hypothalamic arcuate nucleus prior to the association phase subsequently blocked the conditioned increase in NK cell activity (Ghanta et al., 1994). This conditioning model was also employed to analyze central neurotransmission responsible for this conditioned effect, demonstrating that central catecholamines were essential for the association process, and glutamate—but not GABA—was also required to evoke the conditioned increase in NK-activity (Hsueh et al., 1999; Kuo et al., 2001).

#### 4. The conditioned immune response: Efferent pathways

Based on the main findings on neural innervation of secondary lymphoid organs such as the spleen (Felten and Olschowka, 1987; Nance and Sanders, 2007) and the expression of receptors for neurotransmitters on lymphocytes (Sanders and Kohm, 2002; Sanders and Straub, 2002), the splenic nerve was identified as one major efferent pathway linking the brain and the immune system. Employing CsA as the US in a taste aversion paradigm, surgical denervation of the spleen completely blocked the conditioned suppression of splenocyte proliferation and IL-2 and IFN- $\gamma$  production

(Exton et al., 1999, 1998b). In parallel, noradrenaline was identified as the predominant neurotransmitter mediating such conditioned effects since chemical sympathectomy (by 6-hydroxydopamine) eliminated the conditioned response (Exton et al., 2002). Moreover, an *in vivo* application of the  $\beta$ -adrenoceptor antagonist propranolol and continuous blockade of  $\beta$ -adrenoceptors with isoproterenol in an *in vitro* model demonstrated that saccharin–CsA conditioned immunosuppression was  $\beta$ -adrenoceptor-dependent (Exton et al., 2002; Xie et al., 2002). In contrast, splenic denervation did not affect conditioned suppression of contact hypersensitivity (CHS) reaction using CsA in the same paradigm, indicating that the splenic nerve seems to be just one of several efferent neural pathways activated during evocation (Exton et al., 2000b). Recently, we were able to demonstrate in the same conditioning model that the particular pharmacological properties of CsA, the inhibition of CaN activity in leukocytes, can be transferred to a neutral taste stimulus through Pavlovian conditioning. The CaN activity was inhibited in splenocytes from conditioned rats after re-exposure to the gustatory signal (Pacheco-Lopez et al., 2009). The relevant sympathetic–lymphocyte interaction was revealed by modeling the conditioned response *in vitro*. This demonstrated that CaN activity in CD4<sup>+</sup> T lymphocytes was reduced by  $\beta$ -adrenoceptor stimulation with terbutaline, and these effects were antagonized by the  $\beta$ -adrenoceptor antagonist nadolol. This identifies CaN as the intracellular target for inducing conditioned immunosuppression by CsA (Pacheco-Lopez et al., 2009).

So far, this experiment indicates that the sympathetic nervous system plays a major role in the efferent pathway of the conditioned immune response (Exton et al., 2001). Hence, the fundamental peripheral mechanisms of behavioral conditioning are still poorly understood and ongoing studies will have to focus particularly on the cellular and intracellular pathways of the conditioned modulation of the immune response. Also, there seems to be no general mechanism sub-serving all conditioned effects on immunity, and extrapolating findings from one experimental model to another is not feasible, since even particularities such as the individual immune history might play a significant role.

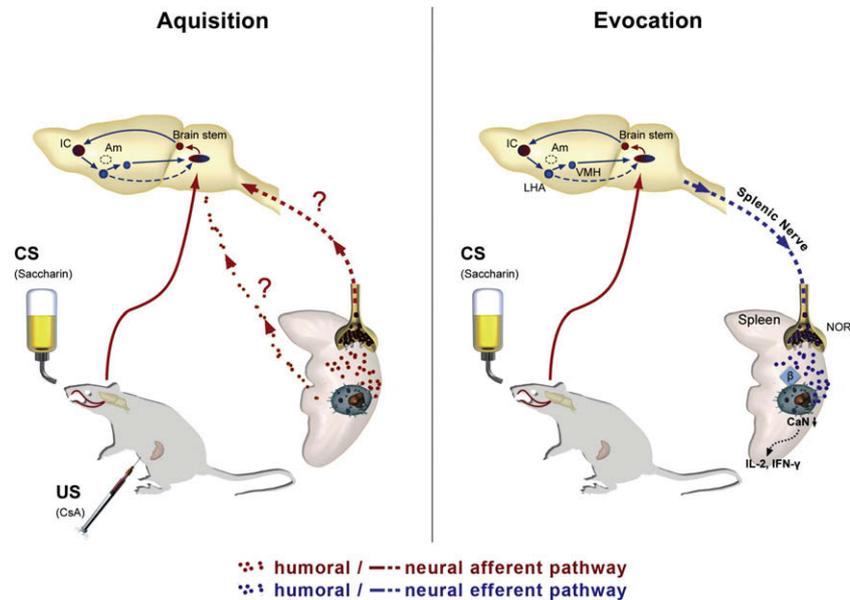
Finally, it has been argued that the conditioned suppression of immune responses might be a generalized conditioned stress effect with an activation of the HPA-axis resulting in increased glucocorticoid release responsible for the immunosuppressive effects (Kelley et al., 1985). Meanwhile, numerous studies indicate that glucocorticoid levels do not differ between the conditioned and control groups, rendering it unlikely that increased corticosterone concentrations are responsible for the conditioned effects (Ader, 1976; Ader and Cohen, 1981; Exton et al., 1998a; Niemi et al., 2007; Roudebush and Bryant, 1991).

Fig. 2 summarizes what we have learned so far about the afferent and efferent communication pathways together with the brain mechanisms involved in the “learned” immune response within the saccharin–CsA model, which our laboratory employed in trying to understand the mechanisms of taste–immune associative learning.

#### 5. The clinical relevance of the learned immune response

##### 5.1. Animal models

A number of studies have addressed the possibility that conditioned changes in immune functions are able to affect disease outcome. Using experimental models of autoimmune or allergic diseases, tumor progression and organ transplantation, the vision to employ Pavlovian conditioning regimens as a complementary therapy supporting pharmacological treatment has been put to the test.



**Fig. 2.** Employing saccharin as a CS and the immunosuppressive drug CsA as an US, the gustatory stimulus (saccharin) is perceived by the CNS via neural afferences during the acquisition phase (see text for details). There are two main possible afferent routes for the CsA to reach the brain: via an indirect afferent route, the CsA may disrupt the peripheral cytokine homeostasis, which could be detected by the CNS immune-sensing capacities or via a direct afferent route, the CsA might reach the CNS through those brain areas with a weak blood–brain barrier, like circumventricular organs, subsequently signaling to forebrain structures. Which of these routes are activated after intraperitoneal CsA application is still unclear. However, the insular cortex and the amygdala are essential structures mediating the input of visceral information which is required at the acquisition time confirmed by excitotoxic lesions of these brain areas as well as enhanced neural activity 2–4 h after CsA administration proved by field potentials recordings (via telemetry) and congruent increased *c-Fos* expression. At the evocation time, the insular cortex together with the neocortical-immune axis, with hypothalamic relays (VMH/LH) and sympathetic peripheral mechanism, seems to be at least one major efferent pathway through which the brain is able to modify in particular T lymphocyte functions in the saccharin–CsA model. The behavioral conditioned suppression of T lymphocyte functions and Th1-cytokine (IL-2, IFN- $\gamma$ ) synthesis and release is mediated via the splenic nerve, noradrenalin (NOR) and  $\beta$ -adrenoceptors with the conditioned inhibition of CaN activity being responsible for the T lymphocyte suppression, similar to the pharmacological effect of CsA. Abbreviations: CaN, calcineurin; CNS, central nervous system; CS, conditioned stimulus; CsA, cyclosporin A; IFN- $\gamma$ , interferon gamma; IL-2, interleukin-2; IC, insular cortex; LH, lateral hypothalamic nucleus; NOR, noradrenalin; US, unconditioned stimulus; VMH, ventro-medial hypothalamic nucleus.

In New Zealand hybrid mice, which are prone to develop an autoimmune disease resembling human lupus erythematosus, a conditioned retardation in proteinuria and mortality was accomplished by evoking a taste–cyclophosphamide engram (Ader and Cohen, 1982). Most interestingly, in a follow-up study the lupus-prone MRL-lpr/lpr mice displayed a weaker conditioned aversion against the saccharin compared to congenic controls. Based on these results, the authors suggested that the lupus-prone animals sought to regain homeostasis by consuming the tasting solution in order to achieve the therapeutic immunosuppressive status elicited by evoking the taste–cyclophosphamide engram (Grotta et al., 1987). In an experimental model of adjuvant arthritis in rats, the joint inflammation was significantly diminished by employing a taste–immune association with either cyclophosphamide (Klosterhalfen and Klosterhalfen, 1983) or cyclosporine A as US (Klosterhalfen and Klosterhalfen, 1990). More recently, a conditioned reduction in disease severity in mice with experimental autoimmune encephalomyelitis was reported following conditioning with saccharin taste (CS) and alpha lipoic acid (US) (Jones et al., 2008).

A series of studies addressed behavioral conditioning of asthma-like symptoms, anaphylactic shock (Djuric et al., 1988; Noelpp and Noelpp-Eschenhagen, 1951a–c; Palermo-Neto and Guimarães, 2000) or histamine release (Irie et al., 2001, 2002, 2004; Peeke et al., 1987). These experiments indicate that especially mast cell function (Marone et al., 2003) is responsive to conditioning protocols.

The delayed-type hypersensitivity response refers to an overreaction produced by the immune system given a pre-sensitized immune state of the host. A behaviorally conditioned decrease (Roudebush and Bryant, 1991) or increase (Bovbjerg et al., 1987)

in this T lymphocyte driven immune response has been observed by pairing saccharin (CS) with cyclophosphamide (US). Similarly, Exton et al. reported a conditioned suppression of the contact hypersensitivity reaction by pairing saccharin with CsA (Exton et al., 2000a).

Behavioral conditioning as supportive therapy has been also studied in the context of cancer treatment (Bovbjerg, 2003; Hiramoto et al., 1991; Spector, 1996). For instance, tumor growth could be enhanced or delayed by applying various types of US (cyclophosphamide and cimetidine, a histamine type II-receptor antagonist) (Gorczyński et al., 1985). In a series of experiments employing camphor odor as a CS and poly I:C as US, tumor growth was reduced and the survival time was prolonged in conditioned tumor-bearing mice (Ghanta et al., 1985, 1987, 1988, 1995). Another example of the potential clinical relevance of the behaviorally conditioned immune response is illustrated by grafting experiments. In an elaborated approach, skin grafting was used as a CS in combination with i.p. injected lymphoid cells of another mouse strain as US (Gorczyński et al., 1982). Re-exposing the conditioned animals to the sham grafting procedure induced an increase in cytotoxic T lymphocyte precursor cells specific for alloantigens on the grafted tissue.

Behaviorally conditioned CsA-immunosuppressive effects prolonged the survival time of heterotopically transplanted heart allografts in rats (Grochowicz et al., 1991). In follow-up experiments, this conditioned prolongation of heart allograft survival could be confirmed employing a similar conditioning paradigm (Exton et al., 1998a). Moreover, a combination of the conditioning procedure with a treatment of sub-therapeutic doses of the immunosuppressive drug CsA and daily re-exposure to the CS lead to long-term survival (>100 days) of transplants in 20–30% of the

animals in two independent experiments, and these effects were completely antagonized by prior surgical denervation of the spleen (Exton et al., 1999). This data indicates that a combination of behavioral conditioned immunosuppression and sub-therapeutic doses of an immunosuppressive drug induces synergistic effects. However, in a recent study we observed that the more a saccharin–CsA engram is activated (either during the association or the extinction phase), the more pronounced the conditioned immunosuppression is (Niemi et al., 2007). These results lead to the striking hypothesis that the prevention of allograft rejection in the above mentioned experiment was not due to the conditioning–drug combination but rather mainly mediated by the learned immunosuppression induced by daily re-exposure to the CS.

## 5.2. Human studies

Up to now, few attempts have been undertaken to specifically investigate conditioned effects which directly modulate peripheral immune functions in human subjects. Early observations on the occurrence of allergic symptoms in the absence of allergens in affected patients together with data in experimental animals resulted in the early hypothesis that asthma could be conceived of as a learned response (Turnbull, 1962). This view has been further supported in patients with allergic rhinitis (Gauci et al., 1994). After the association phase, elevated mast cell tryptase in mucosa was observed, when an intranasal saline application was given simultaneously with the CS. More recently, it has been demonstrated that the anti-histaminergic properties of the H<sub>1</sub>-receptor antagonist desloratadine can be behaviorally conditioned in patients suffering from allergic house-dust-mite rhinitis, as analyzed by subjective symptom score, skin prick test and decreased basophil activation (Goebel et al., 2008).

Another type of allergic reaction, the delayed-type hypersensitivity response, was tested in healthy volunteers who received monthly tuberculin skin tests (Smith and McDaniel, 1983). As a result of the conditioning process, the severity of symptoms was significantly blunted in all the subjects tested. However, a similar protocol using various allergens (e.g. mite dust, fur) did not result in conditioned modulation of skin reactions (Booth et al., 1995).

Associative learning has been consistently reported in the context of cancer treatment, particularly within chemotherapy (Bovbjerg, 2003). Cytotoxic chemotherapy agents generally have also immunosuppressive side effects. These agents are typically administered in cycles, with each outpatient treatment infusion is followed by a period of recovery prior to the next infusion. From a conditioning perspective, clinic treatment visits can be viewed as “acquisition trials” in which the distinctive salient features of the clinic environment (CS) are contingently paired with the infusion of chemotherapeutic agents (US) that have effects on the immune system. For instance, immune function was assessed in 20 cancer patients in the hospital prior to chemotherapy and compared with assessments conducted at home (i.e. neutral environment). Proliferative responses to T-cell mitogens were lower for cells isolated from blood samples taken in the hospital (i.e. after evocation) than for home samples (Bovbjerg et al., 1990). These results were replicated in ovarian patients (Lekander et al., 1995) and pediatric patients receiving chemotherapy (Stockhorst et al., 2000). However, chemotherapy patients often develop conditioned nausea (Andrykowski, 1988; Bovbjerg et al., 1990; Matteson et al., 2002; Morrow et al., 1991), anxiety (DiLorenzo et al., 1995; Jacobsen et al., 1993) and fatigue (Bovbjerg et al., 2005) in response to reminders of chemotherapy. These conditioned nausea and anxiety responses can also be elicited by thoughts and images of chemotherapy (Dadds et al., 1997; Redd et al., 1993), raising the possibility that conditioned effects may affect patients during the course of

normal life for years even after cessation of pharmacological treatment.

Only a few human studies have so far tried to affect immune parameters on the cellular level by employing behavioral conditioning procedures. Acute adrenaline administration increases NK cell numbers and activity (Benschop et al., 1996; Kemeny and Schedlowski, 2007; Schedlowski et al., 1996). Although increased NK cell numbers, as a conditioned response, were reported after evoking a taste–adrenaline association (Buske-Kirschbaum et al., 1994, 1992), these effects could not be replicated in another study (Kirschbaum et al., 1992).

The efficiency of a conditioned immune response was also tested in multiple sclerosis patients. When cyclophosphamide infusions were continuously paired with the taste of anise-flavored syrup, patients showed a conditioned reduction in peripheral leukocyte numbers (Giang et al., 1996). In addition, by pairing s.c. interferon- $\gamma$  injections with a distinctively flavored drink, it was possible to induce an elevation of neopterin and quinolinic acid serum levels after evoking such an association in healthy volunteers (Longo et al., 1999). However, it has been hypothesized that more than a single associative learning trial is required for pairing a distinctive taste (CS) with interferon- $\beta$  injections (US), in order to produce immune conditioned effects (Goebel et al., 2005). This view is supported by experimental data from healthy male volunteers, where the immunosuppressive drug CsA was paired four times with a distinctively flavored/colored solution (Goebel et al., 2002), inducing taste/immune associative learning. After association, the mere re-exposure to the drink resulted in conditioned inhibition of *ex vivo* cytokine (IL-2 and IFN- $\gamma$ ) mRNA expression and cytokine release, as well as in a decreased proliferative responsiveness of peripheral blood lymphocytes. Together, these experimental data from human studies can be taken as a “proof of principle” that behaviorally conditioned immune responses can also be induced in humans. Further, these findings form the basis to cause serious consideration for such protocols as an adjuvant treatment option to pharmacological therapies (Barshes et al., 2004; Cronin et al., 2000).

## 6. The learned immune response: Summary, open questions and future perspectives

The brain's capability to modulate peripheral immune reactivity has been demonstrated by paradigms of behavioral conditioning in animal experiments and human studies. Pavlovian conditioning can be considered adaptive mechanism by which an organism learns to anticipate the onset of a biologically important event, and initiates preparatory responses, including lymphoid- and myeloid cells based responses. Due to the physiological basis of the conditioned effects, the magnitude of the conditioned immune response should not be expected to override the homeostatic balance of the organism. However, this does not mean that conditioned effects on immune functions are not of biological/clinical significance, as has been reviewed here and in previous work (Ader, 2003). A very small increase in the potential of the immune system may be of great value in the fight against pathogens when the system is under allostatic load (McEwen, 1998; McEwen and Lasley, 2003), but it may also increase the occurrence and severity of immune related disorders in other conditions.

### 6.1. Open questions

Current evidence convincingly demonstrates that both innate as well as adaptive immune responses are affected by Pavlovian conditioning. The effects of taste–immune associative learning on immune functions are soon to be clarified and the possible clinical

applications appear to be enormous. However, first we need to understand the reciprocal brain-to-immune communication, together with the brain mechanisms at both the physiological and molecular levels. Furthermore, it is currently unknown how long conditioned immune responses last and how immune-specific they are. Since it may be necessary to apply reinforcement at appropriate intervals, the question also arises as to whether re-conditioning is possible? Since at some time point a pharmacologic therapy will be discontinued, it will be crucial to elucidate the “forgetting pattern” of conditioned immune responses. Another open question concern is the predictability of the conditioned immune response in a human population with different immune and psychological histories. Our understanding of the impact of age and gender on immuno-conditioning is still scarce, as are questions regarding the extent of possible conditioned side effects of immuno-modulating drugs. Finally, why do behavioral conditioned immune responses exist at all? This represents a major scientific challenge, and meeting that goal will give us insights into the possible evolution of endogenous healthcare systems (Benedetti, 2009; Enck et al., 2008).

To date, experimental evidence indicates that behavioral conditioning may have practical implications in the clinical setting as part of a systematically applied placebo response, and to be of use as supportive therapy, with the aim of reducing undesired drug side effects and maximizing the effects of pharmacological therapies (Ader, 1997; Enck et al., 2008; Pacheco-Lopez et al., 2006).

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