Measuring Pavlovian appetitive conditioning in humans with the startle eyeblink and postauricular reflexes

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Abstract

Emotional learning is an essential adaptive function that mainly occurs through aversive and appetitive conditioning. Despite a comparable evolutionary and clinical significance, appetitive conditioning has rarely been studied in humans, in contrast to aversive conditioning. This divergence might be explained by the difficulty in finding effective appetitive stimuli that elicit strong physiological reactions, and/or by a potential lack of sensitivity of the psychophysiological measures typically used to detect appetitive conditioning. However, promising findings suggest that the postauricular reflex (PAR) and the startle eyeblink reflex may be sensitive to appetitive stimuli. The present study therefore aimed to determine whether these two reflexes represent suitable psychophysiological indicators of human appetitive conditioning. To this end, we adopted a differential appetitive conditioning procedure, in which one neutral figure (CS+) was contingently paired with a pleasant odor (US), whereas another neutral figure (CS-) was never paired with any odor. Taken together, our results revealed that the PAR was specifically [...]
Measuring Pavlovian appetitive conditioning in humans with the startle eyeblink and postauricular reflexes

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ABSTRACT

Emotional learning is an essential adaptive function that mainly occurs through aversive and appetitive conditioning. Despite a comparable evolutionary and clinical significance, appetitive conditioning has rarely been studied in humans, in contrast to aversive conditioning. This divergence might be explained by the difficulty in finding effective appetitive stimuli that elicit strong physiological reactions, and/or by a potential lack of sensitivity of the psychophysiological measures typically used to detect appetitive conditioning. However, promising findings suggest that the postauricular reflex (PAR) and the startle eyeblink reflex may be sensitive to appetitive stimuli. The present study therefore aimed to determine whether these two reflexes represent suitable psychophysiological indicators of human appetitive conditioning. To this end, we adopted a differential appetitive conditioning procedure, in which one neutral figure (CS+) was contingently paired with a pleasant odor (US), whereas another neutral figure (CS-) was never paired with any odor. Taken together, our results revealed that the PAR was specifically potentiated in response to the CS+ only during acquisition, demonstrating its sensitivity to the appetitive contingencies. Likewise, CS-US contingency and CS liking ratings reflected successful appetitive conditioning. In contrast, we found no startle eyeblink reflex modulation in response to the CS+, and no effect of appetitive conditioning on SCR. Our findings hence indicate that the postauricular reflex is a sensitive measure of human appetitive conditioning, therefore representing a valuable tool for further investigating the basic mechanisms underlying emotional learning in humans, as well as their dysfunctions in related disorders.
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LIST OF ABBREVIATIONS

ACC: anterior cingulate cortex
Acq: acquisition phase
appCS+: appetitive CS+
avCS+: aversive CS+
cAMP: cyclic adenosine mono-phosphate
CR: conditioned response
CREB: cAMP response element-binding protein
CS: conditioned stimulus
CS+: conditioned stimulus positively predictive of the unconditioned stimulus
CS-: conditioned stimulus negatively predictive of the unconditioned stimulus
CV: conditioned valence
EMG: electromyography
Ext: extinction phase
Hab: habituation phase
ITI: Intertrial interval
Nacc: Nucleus Accumbens
NS: neutral stimulus
NT: neurotransmitter
OFC: orbitofrontal cortex
PAR: postauricular reflex
PIT: Pavlovial to Instrumental Transfer
PKA: cAMP-dependent protein kinase
PKC: protein kinase C
PnC: caudal pontine nucleus of the reticular formation
rmANOVA: repeated measures analysis of variance
SCR: skin conductance response
UR: unconditioned response
US: unconditioned stimulus
UV: unconditioned valence
VAS: visual analog scale
The ability to predict noxious and rewarding events is of critical importance for any organism’s survival (Andreatta & Pauli, 2015). For instance, we can imagine a situation in which an animal is drinking from a source of water in the savannah. Being able to identify the circumstances that predict a predator’s attack, such as the appearance of little movements of the water’s surface created by the presence of a hidden crocodile, could be critical to escape from a life-threatening situation. In a similar vein, recognizing signals that predict a partner’s mating receptivity, as by identifying a signaling part or movement of its body or the sound of its cry, has a selective advantage. In fact, it offers the animal more chances to copulate hence to perpetuate its genes by having eventually a greater number of offspring. Likewise, recognizing signals that predict the availability of food such as the odor or the color of a ripe fruit, or that predict the noxious properties of food that has to be avoided, such as the leaves’ shape of a poisonous berry, gives the animal more chances of persistence.

To enable and foster this ability, emotional learning is undeniably essential. Emotional learning is the process through which a stimulus acquires an emotional significance (Stussi, Brosch, & Sander, 2015), which can be positive or negative. Appetitive and aversive stimuli are positive, respectively negative, emotional stimuli that are linked to the organisms’ ongoing needs. Responses to those stimuli are tempered by motivational salience, the process that motivates and drives an organism towards or away from a particular stimulus, causing approach or avoidance behaviors, respectively (Puglisi-Allegra & Ventura, 2012).

Emotional learning therefore represents a fundamental adaptive functions that allows the organism to shape appropriate behaviors in response to environmental stimuli, by learning to detect their aversive or appetitive features (Stussi et al., 2015). Emotional learning mainly occurs through Pavlovian conditioning, a type of associative learning that produces implicit/non-declarative memory, in which neutral stimuli become associated with motivationally salient aversive or appetitive events. The former type of conditioning is termed aversive conditioning whereas the latter one is termed appetitive conditioning.

Whereas aversive conditioning has been extensively investigated, appetitive conditioning has only been rarely studied in humans. Such a discrepancy might notably stem from the difficulty in finding appetitive stimuli that elicit similarly intense physiological reactions than aversive stimuli. However, this discrepancy might also arise from a potential lack of sensitivity of the psychophysiological measures commonly used to index appetitive conditioning. Here, we therefore aimed to test new physiological indicators of appetitive conditioning, thereby helping to eventually
remedy the scarcity of knowledge about key mechanisms in human emotional learning.

More specifically, we investigated whether the postauricular and startle eyeblink reflexes, two psychophysiological measures that have been shown to be sensitive to appetitive stimuli, may be valid psychophysiological indices of appetitive conditioning in humans.

The following review of literature will start with the description of appetitive conditioning, including the principal types of conditioning. Then, it will address the neuronal bases that underlie appetitive conditioning, and the importance of studying this process by elucidating its applications and some examples of psychological disorders that are caused by its malfunction. Afterward, it will tackle how appetitive conditioning has been commonly investigated so far. Following this line, the postauricular and the startle eyeblink reflexes will be introduced and their connection with appetitive conditioning will be clarified. This latter subject represents the main motive of this work, and will lead to the purpose of our study and to our main hypotheses.

**REVIEW OF LITERATURE**

**Appetitive Conditioning**

**Definition**

In a few words, appetitive conditioning is described as a form of associative learning in which a previously neutral stimulus acquires a new motivational significance through its association with a reward (Martin-Soelch, Linthincum, & Ernst, 2007). A reward is defined as any stimulus, event or situation that has the potential to induce appetitive/approach and consummatory behaviors (Schultz, 2015). Accordingly, new rewards are learned through the process of appetitive conditioning (Martin-Soelch et al., 2007).

There are principally three types of conditioning: classical conditioning (also named Pavlovian conditioning), operant conditioning and evaluative conditioning (Martin-Soelch et al., 2007). Since the present study is based on the first type, the focus will mostly be put on classical conditioning while the others will be only briefly addressed.
Classical conditioning was first intensively studied and described by the Russian physiologist I. P. Pavlov, by reformulating observations made on the salivation of dogs in response to different stimuli. In line with his observations, classical conditioning was defined as the learning process through which an initially neutral stimulus (NS, in his experiment a metronome) is repeatedly paired with an unconditioned stimulus (US, in his experiment food) resulting, after some associations, in the elicitation of the same reaction as the one elicited by the US, called unconditioned response (UR, in his experiment salivation) to the presentation of the NS alone (Martin-Soelch et al., 2007). Thenceforth, the NS is called conditioned stimulus (CS) and the reaction to the CS is called conditioned response (CR) (Martin-Soelch et al., 2007; see Figure 1A).

To deepen a little more the description of the above-mentioned terms, it should be stated that an US, which can be food in appetitive conditioning or an electric shock in aversive conditioning for example, is a biologically significant stimulus which naturally elicits a strong consistent response, the UR, without the occurrence of prior associative learning (Kandel, Schwartz, Jessel, Siegelbaum, & Hudspeth, 2013; Martin-Soelch et al., 2007). In contrast, a CS, such as a light or a tone for example, is a biologically neutral stimulus, which initially elicits no or only very weak response (Kandel et al., 2013). The pairing of the NS and the US is also named contiguity. The UR is a spontaneous reaction that is produced without learning (Kandel et al., 2013). According to Pavlov (Pavlov, 1927), the UR occurs at the physiological level and is consistent with the US, such as salivation or freezing for example. The CR is a response elicited by a CS. Its nature is typically defensive or consummatory depending on whether the type of US is aversive or appetitive (Andreatta & Pauli, 2015).

Nevertheless, the Pavlovian definition of classical conditioning no longer corresponds to the one conceived at present. We will discuss this issue later on.

After successful conditioning, the CS can in turn be paired with a new neutral stimulus, which will then also be able to elicit a CR. This process is called second-order conditioning and is critical for processes such as reward learning and motivation (Martin-Soelch et al., 2007; see Figure 1B). Further, the probability of a CR decreases when the CS is repeatedly presented without the US following. This process is called extinction and it represents an essential adaptive function as it would be maladaptive for an organism to continue responding to environmental stimuli that are no longer significant (Kandel et al., 2013). Correspondingly, the overall human conditioning procedure reproduced in the laboratory is usually formed up by three distinct phases: habituation, acquisition and extinction. The initial habituation phase consists of unreinforced presentations of the NS; in other words, no US is presented upon presentation of the NS. During the acquisition phase, the NS (that
will become the CS) is reinforced by being associated with the US, and the probability of a CR increases. During the final extinction phase the CS is repeatedly presented without being followed by the US, leading to a decline in the CR.

A further distinction must be done when several neutral stimuli are presented in a conditioning procedure and only one of them is predictive of the US. In this case, we talk about discriminative (or differential) learning, the process in which the presence of a specific stimulus (CS+) predicts the occurrence of an US, while another stimulus (CS-) predicts the non-occurrence of the US. After successful conditioning, only the CS+ will be able to elicit the conditioned response (Martin-Soelch et al., 2007; see Figure 1C).

As already mentioned, Pavlovian conditioning is described as a form of learning in which a neutral stimulus becomes associated with an US and, as a result, is able to elicit a conditioned response (Domjan, 2005). However, this description is considered as too simplistic from a modern and functional point of view. Indeed, a more modern view of classical conditioning that diverges from Pavlov’s original view does not see conditioning as a simple process whereby a response is merely transferred from a given stimulus to another, but, on the opposite, as a complex mechanism through which an organism creates his own representation of the world by seeking for information via the use of logical and perceptual relations among events, along with its own preconceptions (Rescorla, 1988).

For instance, with regard to the circumstances that produce learning, it states that the contiguity between US and CS alone is neither sufficient nor necessary and considers instead the predictive relationship of the CS to the US (i.e., a logical relation among these two events, referring to the term “contingency”, which describes the relation of probability between the CS and the US) to be the critical factor that produces learning (Rescorla, 1988). Indeed, as Rescorla demonstrated, conditioning can be obstructed by enough CS and US alone trials (Martin-Soelch et al., 2007), by the so-known blocking effect (which consists in unsuccessful conditioning of a second CS in the presence of a first one when the subject was already conditioned to the first CS, because the second CS holds no predictive value of the US, thus being redundant) or by a negative predictive relationship between CS and US (Rescorla, 1988). In addition, from a functional perspective, an exclusively contiguity-dependent learning would be maladaptive since, in this case, animals would learn to associate whichever events in the environment solely because of their randomly association, even though they have no utility or advantage (Kandel et al., 2013). Moreover, under this modern view, conditioning depends on the properties of the events themselves (e.g., generally more intense CSs generate faster learning and greater CR (Domjan, 2005) and some stimuli are more easily associable than others, as for example interior malaise which is better associable with taste than with audio-visual stimuli.
and on the perceptual relation among these events (such as their similarity and the part-whole relation; see Rescorla, 1988). In addition, a functional perspective of classical conditioning emphasizes the use of conditioned stimuli that are natural precursors of a US, that is that have a pre-existing ecological relation, rather than being neutral and arbitrary (Domjan, 2005). Learning with naturalistic CSs is faster, more resistant to the increase in CS-US interval and more resistant to the blocking effect, as proven by studies of poison avoidance, sexual behaviors, as well as fear and defensive behaviors for instance (Domjan, 2005).

Regarding the content of learning, in contrast to the original Pavlovian view, learning consists not simply in the relation between a neutral event and a significant event, but in many different associations between and within events. For instance, the simultaneous association with the environment in which conditioning takes place (i.e., context learning) (Rescorla, 1985).

With respect to the effects of learning on behavior, opposite to the original Pavlovian interpretation, conditioning is not limited to the transmission of the response primarily evoked by the US to the CS but involves the formation of new responses (e.g., whereas the UR to the shock is increased activity, the CR to a tone signaling that shock is reduced activity; Rescorla, 1988) that depend on the nature of the CS (e.g., a visual signal of food evokes pecking in pigeons, while a diffuse auditory signal does not; Rescorla, 1988). In addition, whereas the Pavlovian view focuses on the CR to the CS, a functional perspective rather underlines the measurement of behavioral consequences of conditioning to the US that are of adaptive significance. As the study of eyeblink conditioning, fear conditioning, drug conditioning, feeding, digestion, sexual, aggressive, and maternal behavior showed, these conditioned modifications of responding to the US increase the efficiency of the organism’s interactions with the US (Domjan, 2005).

In summary, from the original Pavlovian representation of the last century to the contemporary view, classical conditioning has undoubtedly captured a large scientific interest. In experimental conditions, Pavlovian conditioning is often used to produce goal-directed instrumental behavior or to study the effect of different conditioned cues with the Pavlovian to Instrumental Transfer paradigm (PIT, which is described later on), and more generally represents a good model for the study of mechanisms underlying associative learning. Pavlovian conditioning also strongly contributes to the study of motivation and reward processing, as well as their related dysfunctions.

In conclusion, as we have seen before, one learns in classical conditioning to respond to a CS predicting a US. Following this rationale, classical conditioning is considered as an important process that enables the organism to represent the world through multiple associations, thereby being an essential means by which the organism learns to predict events in the environment. This learning allows it to cope with aversive and appetitive situations relevant to the survival and the maintenance...
of its species. In other words, classical conditioning is a key emotional learning process.

![Diagram](image)

**Figure 1.** A) Classical conditioning. B) Second-order conditioning. C) Discriminative learning. Figures adapted from Martin-Soelch et al., 2007.

**Evaluative Conditioning and Operant Conditioning**

Similar to classical conditioning, evaluative conditioning is defined as the process through which the valence of an unconditioned stimulus (US), named unconditioned valence (UV), is transferred to a neutral stimulus (NS) after their repeated association (Martin-Soelch et al., 2007). The NS then becomes a conditioned stimulus (CS) characterized with a conditioned valence (CV) (Martin-Soelch et al., 2007).

Systematically studied by B. F. Skinner, operant conditioning (or instrumental conditioning) represents the process by which a reinforcing stimulus (that can be positive such as a reward or negative such as an electric shock) becomes contingent upon a response (e.g., the pressing of a lever) (Martin-Soelch et al., 2007). The probability of the response is increased when positive reinforcers are presented or negative reinforcers are omitted, and decreased when punishment is presented or positive reinforcers are omitted (Martin-Soelch et al., 2007). While in classical conditioning one learns that a stimulus predicts an event, in operant conditioning one learns to predict the outcome of an action (Kandel et al., 2013).
To understand how appetitive conditioning works, it is important to make a short digression on the structural and cellular bases that are implicated in this process.

At a structural level, the brain areas that have been identified to be involved in appetitive conditioning are principally the amygdala, the orbitofrontal cortex (OFC), the anterior cingulate cortex (ACC), the striatum and the mesolimbic dopaminergic system (Cox, Andrade, Johnsrude, 2005; Gottfried, O’Doherty, & Dolan, 2002; Kirsch et al., 2006; Kirsch et al. 2003; Klucken, Kruse, Wehrum-Osinsky, Schweckendiek, & Stark, 2015; Klucken et al., 2009; Klucken et al., 2013; McClure, Berns, & Montague, 2003; O’Doherty, Buchanan, Seymour, & Dolan, 2005; O’Doherty, Dayan, Friston, Critchley, & Dolan, 2003; O’Doherty, Dayan, Schultz, Deichmann, Friston, Dolan, 2004; Seymour et al, 2005).

Although the central role of the amygdala in fear conditioning (LaBar, Gatenby, Gore, LeDoux, & Phelps, 1998), its role in appetitive conditioning is less clear (Klucken, Wehrum-Osinsky, Schweckendiek, Kruse, & Stark, 2016). The amygdala is essentially thought to be involved in the attribution of emotional significance to events and in attentional processes (see Martin-Soelch et al., 2007). Indeed, animal and human studies have recently brought evidence for its activation in the processing of appetitive stimuli and in appetitive conditioning (Cole, Hobin, Petrovich, 2015; Gottfried et al., 2002; McLaughlin & Floresco, 2007, Politis, et al., 2013; Seymour et al., 2005; Sergerie, Chochol, & Armony, 2008; Setlow, Gallagher, & Holland, 2002a; Setlow, Gallagher, & Holland, 2002b; Voon et al., 2014). For example, Gottfried et al., 2002 found amygdala activation to the CS+ relative to the CS- during human appetitive conditioning using pleasant odors as US. The OFC may encode outcome expectancies and facilitate associative learning (e.g., Cox et al., 2005; Gottfried et al., 2002; Kirsch et al., 2003; O’Doherty et al., 2003) and the ACC may play a role in discriminative learning (Cox et al., 2005; Kirsch et al., 2003) in both animals and humans. The striatum, and particularly the Nucleus Accumbens (Nacc), a key region of the reward system, may participate as a behavioral output region and may be involved in second-order conditioning in animals (see Martin-Soelch et al., 2007). In humans, the dorsal (including dorsal putamen and caudate nucleus; e.g. McClure et al., 2003) and the ventral (mostly Nacc; e.g., Cox et al., 2005; Gottfried et al., 2002; Kirsch et al., 2003; O’Doherty et al., 2005; O’Doherty et al., 2003; O’Doherty et al., 2004) striatum may be activated in response to primary and secondary CS+ and, particularly the ventral striatum, may be involved in the encoding of the stimulus’ salience. In addition, the ventral striatum may play an important role in anticipation, reward processing, and learning (Klucken et al., 2009). Finally, the mesolimbic dopaminergic system (Nacc) may act as potential neurochemical messenger...
of appetitive conditioning (among other neurotransmitter systems) in both animals and humans, as dopamine (DA) in the Nacc has been suggested to amplify the responding to conditioned reinforcement, without being however necessary to its acquisition (for more details, see Martin-Soelch et al., 2007).

Secondly, at a cellular level, storage of implicit memory that results from classical conditioning is coded by molecular changes at the synaptic surface that change the effectiveness of the involved synaptic transmission, through a presynaptic facilitation dependent on pre- and postsynaptic activity (Kandel et al., 2013). These cellular mechanisms were observed in the simple nervous system of the marine sea slug Aplysia californica. More specifically, two neuronal pathways, one mediating the response to the conditioned stimulus and the other the response to the unconditioned stimulus, converge onto the same sensory neuron in a time dependent manner during classical conditioning. The conditioned stimulus must precede the unconditioned stimulus within a very short temporal window in order to produce a greater presynaptic facilitation, a process that is called activity dependence (Kandel et al., 2013). Hence, this mechanism is initiated by the conditioned stimulus sending an action potential to the sensory neuron. The action potential then triggers an influx of Ca\(^{2+}\) ions into the sensory neurons’ pre-synaptic terminal (Kandel et al., 2013). The Ca\(^{2+}\) binds to the Ca\(^{2+}\)-binding protein calmodulin, that in turn binds to adenylyl cyclase, which acts as a coincidence detector, potentiating its subsequent response to the unconditioned stimulus (Kandel et al., 2013). Indeed, immediately after, as the unconditioned stimulus synapses onto the same sensory neuron, serotonin and other released neurotransmitters (NT) bind to specific sensory membrane receptors and activate a stimulatory G-protein which in turn activates an adenylyl cyclase to produce the second messenger cyclic adenosine mono-phosphate (cAMP) (Kandel et al., 2013). cAMP activates the cAMP-dependent protein kinase (PKA) (Kandel et al., 2013). Serotonin also activates a second type of G-coupled receptor that leads to the activation of protein kinase C (PKC) (Kandel et al., 2013). PKA and PKC enhance the release of transmitters from the sensory neurons' terminals through the phosphorylation of several substrate proteins (Kandel et al., 2013). In addition, a postsynaptic component of classical conditioning consists in a retrograde messenger signal triggered by the Ca\(^{2+}\) influx into the motor neuron and which is taken up by the presynaptic terminals of the sensory cell, enhancing further the NT release (Kandel et al., 2013). Finally, the consolidation of long-term implicit memory from short-term memory requires gene expression, new protein synthesis and growth of new synaptic connections which are mediated by the cAMP-PKA-CREB pathway (Kandel et al., 2013).
As already mentioned, one important role of appetitive conditioning is as a model for the investigation of mechanisms underlying implicit memory that derives from associative learning. Moreover, the study of appetitive conditioning contributes to the study of motivation and reward processing, as well as their related dysfunctions. In fact, a stimulus that has acquired an emotional or motivational significance through the process of reward learning mediated by appetitive conditioning will trigger a motivation to approach the appetitive conditioned stimulus and prepare to consummatory behaviors. Thus in a broad sense, appetitive conditioning is strictly linked to motivation and reward processing.

More specifically, appetitive conditioning is essential for the Pavlovian to Instrumental Transfer (PIT) paradigm, in which cues that become associated with rewards through Pavlovian conditioning modulate motivation and choice of instrumental actions (Cartoni, Balleine, & Baldassarre, 2016). This paradigm indeed allows for studying the effects of conditioned cues on behaviors and to measure one’s motivation to achieve a rewarding stimulus by the exposure to such conditioned cues (Cartoni et al., 2016). Moreover, thanks to the PIT, it is possible to dissociate under particular circumstances the different components of the reward system described by the incentive salience theory, namely “wanting” (i.e., the motivation to obtain a reward) and “liking” (i.e., the hedonic pleasure) (Berridge & Robinson, 2003; Pool, Sennwald, Delplanque, Brosch, & Sander, 2016). In particular and in line with the principles of the incentive salience hypothesis, wanting (referring to implicit incentive salience ‘wanting’, which is distinct from explicit wanting that relates to cognitive desires) is measured during or after the presentation of a reward-associated cue, according to the idea that wanting is generated by the interaction between the current physiological state (including the relevance for the organism’s current concerns) and the encounter of a cue associated with the reward (Pool et al., 2016). Conversely, liking (referring to implicit ‘liking’, which differs from conscious pleasure) is measured during or right after the consumption of the reward (Pool et al., 2016). Importantly, a distinction between the different components and the expected pleasantness of a reward is needed, because the latter rather relies on past reward-related experiences underlying cognitive desires, thus representing a potential confound (Pool et al., 2016). The disentanglement of these components allows to investigate pathological systems in which wanting and liking do not go hand in hand, such as pathological gambling, addiction or overeating (Pool et al., 2016).

In this respect, as regards the role of appetitive conditioning in pathological conditions, various psychopathologies, including for example substance abuse disorders, eating disorders,
depression and schizophrenia, are thought to be related to appetitive conditioning and are depicted by motivation deficits (Martin-Soelch et al., 2007).

For instance, substance abuse represents a disorder characterized by deficits in associative learning. It has been demonstrated that the conditioning of stimuli or events (NS) that are present in the environment of the drug (US) administration and which will become associated to the rewarding properties of the drug, is responsible for the subsequent drug abuse, the craving sensation and the relapse following treatment (Martin-Soelch et al., 2007). Additional experimental support for the involvement of appetitive conditioning, in addition to operant conditioning, arises from the association of a neural network that underlies appetitive conditioning (Martin-Soelch et al., 2007).

Similarly, eating disorders are influenced by conditioned cues (CS) - sensory stimuli such as the sight, smell or taste of food (US) - that will trigger the desire for food (craving) and the subsequent maintenance of the food-intake (CR) (Martin-Soelch et al., 2007). For example, bulimia nervosa, which is characterized by binge eating, may develop by a strong craving sensation due to the formation of many food cues that are present in the context of food consumption (Martin-Soelch et al., 2007). The treatment that has been proposed in eating disorders or in the case of smoking-habits cessation was the correct reconditioning of the contextual conditioned cues (Martin-Soelch et al., 2007). That means, the reconditioning of the environmental conditioned cues that trigger the desire for the concerned substance (food or cigarette), in order to extinguish their potential as CS, by repeatedly present them without the reward following.

Concerning depression, one cause of anhedonia - a core symptom of depression - may be related to a deficit in appetitive conditioning as for the normal formation and maintenance of positive association between appetitive US and NS (Martin-Soelch et al., 2007).

Schizophrenia instead, which is characterized by cognitive disturbance linked to attention and information processing deficits, may be caused by impaired latent inhibition (i.e., the delay in learning the association between CS and US when one of the two has been previously presented without being associated with the other) leading to an incapacity to ignore irrelevant stimuli (Martin-Soelch et al., 2007). In other words, the impairment of latent inhibition results in the maintenance of the CR to the CSs even when the CSs are no longer associated with the US (Martin-Soelch et al., 2007). An alternative mechanism underlying the development of schizophrenia may consist in the chronic hyperarousal states of patients, which does not correspond to the contingency of the CSs, forcing the external stimuli to become irrelevant (Martin-Soelch et al., 2007).

These examples of pathologies underline the important role of appetitive conditioning in the clinical picture.
Whilst aversive conditioning represents an intensively studied area of research in both animal and humans (e.g., Delgado, Olsson, & Phelps, 2006; LaBar & Cabeza, 2006; Phelps & LeDoux, 2005), only a few studies have investigated appetitive conditioning in animals (Bouton & Peck, 1989; Koch, Schmid, & Schnitzler 1996; McDannald, Lucantonio, Burke, Niv, & Schoenbaum, 2011; Schneider & Spanagel, 2008), as well as in humans (Andreatta & Pauli, 2015; Delgado, Jou, & Phelps, 2011; Gottfried et al., 2002; Hermann et al., 2000; Klucken et al., 2009, 2013; Kumar, Waiter, Ahearn, Milders, Reid, & Steele, 2008; Prévost, Liljeholm, Tyszka, & O’Doherty, 2012; for a review, see Martin-Soelch et al., 2007). Since appetitive conditioning is known to be a key process in reward learning and motivated behavior (Berridge & Robinson, 2003; Pool et al., 2016) and more generally in emotional learning, and because its malfunction is strictly involved in the development and progress of different pathologies such as depression, schizophrenia, addiction, and eating disorders (Martin-Soelch et al., 2007), appetitive conditioning has at least a similar evolutionary survival and clinical significance as aversive conditioning. Hence, there is an urge to remedy to the scarcity of human appetitive conditioning studies.

The divergence between appetitive and aversive conditioning literature is thought to originate from the difficulty in finding effective appetitive stimuli that elicit strong physiological reactions like the one elicited by the painful or fear stimuli (e.g., electric shock) used in aversive conditioning (Hermann et al., 2000; Martin-Soelch et al., 2007). In addition, appetitive conditioning might entail a higher complexity compared to aversive conditioning, for instance by its sensitivity to satiation when using primary rewards such as food (Andreatta & Pauli, 2015). Another further explanation might be the potential lack of sensitivity of the psychophysiological measures typically used in order to detect changes induced by appetitive conditioning in humans.

Indeed, efforts to point out a reliable index of human appetitive conditioning have been truly challenging (Sandt, Sloan, & Johnson, 2009) and the extant research has been inconclusive so far. Human appetitive conditioning has been investigated using different methods such as subjective, behavioral, brain imaging, and psychophysiological measures. Even though it has usually been successfully evidenced using subjective measures (e.g., Andreatta & Pauli, 2015; Van Gucht, Baeyens, Vansteenwegen, Hermans, & Beckers, 2010; Van Gucht, Vansteenwegen, Van den Bergh, & Beckers, 2008), behavioral measures (e.g., Pool, Brosch, Delplanque, & Sander, 2014; Pool, Delplanque, et al., 2014; Van Gucht et al., 2008), or brain activity, such as hemodynamic response (e.g., Delgado, 2007; Gottfried et al., 2002, 2003; Klucken et al., 2009; Prévost, McNamee, Jessup,
Bossaerts, & O’Doherty, 2013) and electrophysiology (e.g., Franken, Huijding, Nijs, & van Strien, 2011), these measures involve some disadvantages.

Subjective measures refer to the use of self-reported questionnaires that allow for collecting information about the valence and arousal ratings of the appetitive conditioned stimulus and of the subjective CS-US expectancy ratings after or during the appetitive conditioning procedure. However, it remains an indirect and subjective measure of appetitive conditioning, which can be influenced by intentional control and which is susceptible to demand and interpretation biases (Grillon & Baas, 2003; Johnson, Valle-Inclán, Geary, & Hackley, 2012). The same disadvantages hold for behavioral measures such as reaction times (Grillon & Baas, 2003). Brain activity measures are expensive and incompatible for some populations (Johnson et al., 2012). Finally, the use of psychophysiological measures, as for example skin conductance response (SCR), salivation, heart rate and facial EMG activity, has yielded incongruent results.

SCR is a non-specific measure of physiological arousal that indicates orienting toward a stimulus regardless of its valence, reflecting the sympathetic activation and being thus increased to both appetitive and aversive stimuli (Andreatta & Pauli, 2015; Grillon & Baas, 2003; Lang, Bradley, & Cuthbert, 1990). Another psychophysiological measure proposed as a potential measure of appetitive responding is salivation (Bradley, Codispoti, Cuthbert, & Lang, 2001; Lang et al., 1990; Sandt et al., 2009). Nevertheless, some limitations, as the difficulty of collection methods, the delay in the response time and the fact that it does not generalize to the different categories of appetitive stimuli still persist (Johnson et al., 2012; Sandt et al., 2009). The use of heart rate, which is principally influenced by the metabolic activity and attentional orientation (Lacey, 1967), and EMG activity of the corrugator supercilii and zygomaticus muscles, which reflect emotional expressions (Dimberg, 1990) but cannot distinguish between felt smiles and grimaces (Johnson et al., 2012), have both brought little congruent results (e.g., Hermann, Ziegler, Birbaumer, & Flor, 2000).

The lack of an objective measure of appetitive conditioning precludes its use as a diagnostic and therapeutic tool (Grillon & Baas, 2003). Finding an appropriate psychophysiological index of appetitive responding would hence represent an essential contribution to the study of appetitive conditioning and therefore allow to better understand key mechanisms underlying emotional learning in human subjects and its related pathologies.

However, two psychophysiological measures, the startle eyeblink and postauricular reflexes, have been shown to be sensitive to appetitive stimuli and may therefore constitute potential psychophysiological indices of appetitive conditioning in humans.

Indeed, the startle eyeblink reflex may be a promising psychophysiological measure as startle eyeblink reflex inhibition has been found in response to an appetitive CS (Andreatta & Pauli, 2015).
Besides, advantages of the startle methodology are the low cost, the ease and applicability (Grillon & Baas, 2003). In addition, as a reflex, it is not primarily susceptible to intentional control and it is resistant to demand and interpretation biases (Grillon & Baas, 2003). Finally, it represents a valuable link between preclinical animal and clinical human research (Grillon & Baas, 2003). Comparably, the postauricular reflex has been found to be potentiated by pleasant/appetitive stimuli relative to unpleasant/aversive and neutral stimuli (Sandt et al. 2009). The next section will therefore address these two promising measures and outline their potential value in indexing appetitive conditioning.

Postauricular and startle eyeblink reflexes

The startle eyeblink reflex is one component of the startle response, an automatic defensive response to an abrupt strong and unexpected stimulus (Koch, 1999). The startle eyeblink reflex constitutes a rapid contraction of the orbicularis oculi muscle, which is innervated by the facial nerve (Grillon & Baas, 2003), resulting in an involuntary blinking of the eyelids. In experimental conditions, it is normally elicited by an acoustic startle probe, a very brief loud white noise (normally lasting for 50ms with an intensity of 95-105 dB SPL). Despite the high intensity, the acoustic startle probe is not dangerous for the auditory system because of its very short duration (Grillon & Baas, 2003). The startle eyeblink reflex seems to be based on a relative simple neuronal circuitry involving the cochlear root neurons, the caudal pontine nucleus of the reticular formation (PnC) and the spinal motorneurons (Andreatta & Pauli, 2015; Fendt & Franselow, 1999; Koch, 1999; Lee, Lopez, Meloni, Davis, 1996), among which the facial motor nucleus is most likely responsible for the eyeblink reflex (Grillon & Baas, 2003).

The postauricular reflex (PAR) is a vestigial muscle microreflex in humans that pulls the ear backwards and upwards (Benning, 2011; Benning, Patrick, & Lang, 2004; Bérzin & Fortinguerra, 1993; Gray, 1901/1995) by exposure to the acoustic startle probe. The PAR is measured by recording the EMG activity of the postauricular muscle located behind the ear (O’Beirne & Patuzzi, 1999), which is innervated by the postauricular branch of the facial nerve (Benning, 2011).

The acoustic startle probe is therefore used to evoke both the PAR and the startle eyeblink reflex, allowing a concurrent assessment of these two reflexes (Hackley, 1993). Moreover, the PAR latency (9-11 ms) to the acoustic startle probe is faster than the one of the startle eyeblink reflex (45-50 ms) (Hackley, 1993; Hackley, Woldorff, & Hillyard, 1987), indicating a simpler underlying neural circuitry (Benning et al., 2004; Hackley, 1993; Hackley, 2015). Indeed, the PAR is thought to rely on
a similar neural circuitry as the startle eyeblink reflex, including the same input (cochlear nucleus) and output (facial-motor nucleus) components, but bypassing the PnC (Casella & Davis, 1986; Hackley, 2015) – a structure known for receiving inputs from the amygdala (Hackley, 1993), which is involved in the animal fear-potentiated startle (Davis, Gendelman, Tischler, & Gendelman, 1982). Animal studies on the pinna reflex (Li & Frost, 1996), the analog of the human postauricular reflex, showed that the retrorubral nucleus, a midbrain dopaminergic nucleus which has been associated with sensitivity to reward stimuli (Waraczynski & Perkins, 2000), sends additional synapses to the motoneurons of the facialis nerve that innervate the pinna (Benning et al., 2004).

The PAR is thought to be a vestigial remnant of the ancestral pinna-orienting system, which has been lost during primate evolution (Hackley, 2015). Pinna movements, which originally served to express emotion, to defensively retract ears when startled and to orient toward novel, salient, relevant stimuli, are no longer possible in humans (Hackley, 2015). All that remains from the pinna-flexion is a brief, ineffectual excitation of the postauricular muscle (Johnson et al., 2012). This is due to the weakness of the ear muscles, to the way the muscles’ tendons attach the ears, and to the rigidity of the ears themselves (Hackley, 2015).

Connections between postauricular and startle eyeblink reflexes and appetitive conditioning

Startle Eyeblink Reflex

A large body of literature has shown that the magnitude of the startle eyeblink reflex to an acoustic startle probe in humans is modulated by their ongoing motivational state (Amodio, Harmon-Jones, & Devine, 2003; Grillon & Baas, 2003; Hawk & Kowmas, 2003; Lang, 1995; Lang et al., 1990; Vrana, Spence, & Lang, 1988), indicating that the use of this reflex may be effective for assessing emotions (Schneider & Spanagel, 2008).

According to Lang’s motivational priming hypothesis (Lang, 1995; Lang et al., 1990), when someone is experiencing a negative emotion (such as fear) in response to an unpleasant stimulus, the underlying aversive system circuitry is activated (Hess, Sabourin, & Kleck, 2007). As a consequence, defensive reflexes are primed (Lang et al., 1990). If a sudden, intense stimulus (such as the acoustic startle probe) is then presented, the startle eyeblink reflex will be enhanced (Hackley, 2015). The opposite would be true if someone is experiencing a positive emotion by the exposure to a pleasant stimulus; that is, appetitive reflexes would be primed and the startle eyeblink reflex would be
attenuated because the current approach disposition inhibits the defensive startle reaction (Benning et al., 2004). A reverse pattern would be expected for an appetitive reflex: a potentiation during presentation of pleasant/appetitive stimuli and an inhibition during presentation of unpleasant/aversive stimuli (Benning et al., 2004). Accordingly, Lang (1995) proposed that emotions modulate the way the organism acts in response to environmental stimuli by enhancing appetitive or defensive motivational states, which in turn promote approach and avoidance behavior (see also Schneider & Spanagel, 2008). Bradley et al. (2001) proposed that contexts that support sustenance, nurturance and procreation activate the appetitive motivational system and mobilize an organism toward ingestion, caregiving and copulation, whereas contexts involving threat and death activate the defensive motivational system and support withdrawal, escape and attack (Sandt et al., 2009).

Modulation of the startle eyeblink reflex has been documented with different types of stimuli including emotion eliciting pictures (Lang et al., 1990; Vrana et al., 1988), sounds (Bradley & Lang, 2000), and odors (Miltner, Matjak, Braun, Diekmann, & Brody, 1994) (see Hackley, Munoz, Hebert, Valle-Inclán, & Vila, 2009).

In this respect, it is by now clear that the startle eyeblink reflex is sensitive to affective modulation and, more importantly, that it does reflect defensive responding, as human startle researches repeatedly confirmed that the startle eyeblink reflex is specifically potentiated in response to unpleasant stimuli and attenuated in response to pleasant stimuli compared to neutral stimuli (Lang et al., 1990; Vrana et al., 1988), thereby perfectly fitting the expected pattern for a defensive reflex.

In addition, intense emotional stimuli that activate most strongly the appetitive or defensive motivational systems should result in extreme modulatory effects on the eyeblink reflex (Benning et al., 2004). Accordingly, Cuthbert, Bradley, and Lang (1996) found higher startle eyeblink potentiation to high- versus low- arousal aversive images and higher startle eyeblink attenuation for high- versus low- arousal pleasant images (Benning et al., 2004).

Even so, specific categories of emotional stimuli within the same valence kind were found to modulate differently the startle eyeblink reflex. Eyeblink reflex attenuation was found to be maximal for pleasant erotic pictures and potentiation was found to be maximal for directly threatening aversive pictures (Benning et al., 2004; Bradley, Codispoti, Cuthbert, & Lang, 2001; Levenston, Patrick, Bradley, & Lang, 2000).

Eyeblink modulation was also tested in response to different odors (Ehrlichman, Brown, Zhu, & Warrenburg, 1995; Ehrlichman, Kuhl, Zhu, & Warrenburg, 1997; Miltner et al., 1994) and it was found that unpleasant odors potentiate the eyeblink reflex. One study (Ehrlichman et al., 1997) additionally found an attenuation of the startle eyeblink reflex to pleasant odors (Schneider & Spanagel, 2008).
Factors that influence the eyeblink reflex pattern other than emotional states, valence, arousal, and category of the emotional stimuli, include attention and habituation (Grillon & Baas, 2003). While the modulatory effects of habituation can be controlled by regulating the presentation of the experimental conditions, the modulatory effects of attention are more difficult to control (Grillon & Baas, 2003). Engagement of attention resources away from the acoustic startle probe can lead to an attenuation of the startle reflex (Grillon & Baas, 2003).

Moreover, animal models suggested that the eyeblink reflex potentiation is dependent on projections from the amygdala to the PnC (Fendt & Fanselow, 1999; Koch, 1999) whereas attenuation from the intact nucleus accumbens (Koch, 1999). More recently, Andreatta and Pauli (2015) applied a concomitant appetitive and aversive differential conditioning paradigm, in which they presented a neutral stimulus (avCS+) associated to an aversive US (electric shock), a second neutral stimulus (appCS+) associated to an appetitive US (sweet or salty food) and a third neutral stimulus (CS-) not associated to any US. They used startle eyeblink modulation, SCR and subjective valence and arousal ratings as measures of the conditioned response. All measures indicated successful aversive and appetitive conditioning (except that the CS+ was not rated as more arousing than the CS-). More significantly, they found that the eyeblink reflex was potentiated in response to an aversive conditioned stimulus (avCS+) and attenuated in response to an appetitive conditioned stimulus (appCS+) compared to the CS-, replicating results obtained in rats (e.g., Koch, Schmid, & Schnitzler, 1996). They therefore seem to support the use of startle eyeblink reflex as a psychophysiological indicator of human appetitive conditioning.

Nevertheless, the role of the eyeblink reflex as index of appetitive responding is still under debate (see Dillon & LaBar, 2005; Grillon & Baas, 2003; Jackson, Malmstadt, Larson, & Davidson, 2000). It is argued that the eyeblink reflex might principally be an index of defensive responding rather than of appetitive responding per se (Dichter, Benning, Holtzclaw, & Bodfish, 2010; Dichter & Tomarken 2008), meaning that the attenuation of the eyeblink reflex in response to pleasant stimuli may be a result of the inhibition of defensive responding rather than of the direct activation of appetitive responding. Further investigations are therefore needed in order to validate the role of startle eyeblink reflex as index of appetitive conditioning.

Postauricular reflex

On the other hand, besides its interest in attention-modulation studies (Hackley et al., 1987; Patuzzi & O’Beirne, 1999; Sollers & Hackley, 1997), the postauricular reflex has been proposed to be a valid index of appetitive responding (Benning et al., 2004; Hess et al., 2007; Sandt et al., 2009). Previous studies have indeed underlined its potentiation during positive emotional states induced by
viewing of pleasant stimuli relative to neutral or unpleasant stimuli (Aaron & Benning, 2016; Benning, 2011; Benning et al., 2004; Dichter et al., 2010; Gable & Harmon-Jones, 2009; Hackley et al., 2009; Hebert, Valle-Inclán, & Hackley, 2015; Johnson et al., 2012; Sandt et al., 2009), providing evidence for its sensitivity to affective modulation. According to Lang’s motivational priming hypothesis (Lang et al., 1990), opposite to the eyeblink reflex pattern which appears to be enhanced by negative emotional induced defensive priming (Lang et al, 1990), the PAR would be potentiated while someone is experiencing positive emotions due to the priming of appetitive reflexes, fitting with the expected pattern of an appetitive reflex (Benning et al., 2004).

Actually, in their pioneer study on affective modulation of the PAR, Benning and colleagues (2004) presented pictures that varied systematically in emotional valence, content and intensity. They found a linear valence modulation effect, with pleasant pictures potentiating (although only marginally) and unpleasant pictures inhibiting the post-auricular reflex in comparison with neutral pictures, opposite to the startle eyeblink reflex pattern. Picture content was not found to modulate the PAR but PAR was most robust for highly intense emotional pictures, as shown by a higher postauricular reflex magnitude to high arousal pleasant pictures compared to high arousal unpleasant pictures (Benning et al., 2004, Gable & Harmon-Jones, 2009). Interestingly, the magnitude of the PAR during picture viewing was usually smaller than during intertrial intervals (ITIs), suggesting that perceptual engagement with a foreground stimulus generally inhibits the reflex (Benning, 2011; Hackley et al., 1987).

In the same vein, Hess and colleagues (2007) replicated the finding of Benning et al.’s study (2004). Intriguingly, they showed that facial emotional stimuli produced different PAR and startle eyeblink reflex responses depending on the sex of the expresser. More precisely, they demonstrated a greater PAR magnitude to happy expressions displayed by a woman (i.e., an appetitive stimulus) relative to angry expressions displayed by a man (i.e., a threatening stimulus) (Hess et al., 2007). They suggest that this modulatory effect is due to the added social signal value of human emotional expressions (Hess et al., 2007).

Notably, the study of Gable and Harmon-Jones (2009), indicated that, opposite to the eyeblink reflex, pleasant stimuli induced larger postauricular reflex activation than neutral and unpleasant emotional stimuli, regardless of the arousal level (Gable & Harmon-Jones, 2009). In discrepancy with previous studies, they used a block-type design in which affective pictures of one type were only pitted against neutral pictures. These authors inferred that PAR is a measure of valence, independent of arousal, and thus may represent a reflex purely modulated by affective valence rather than by motivation (Gable & Harmon-Jones, 2009).
Further, Hackley et al. (2009) found that the PAR is potentiated prior to response-contingent rewards compared to punishments, whereas the startle eyeblink reflex is enhanced prior to punishments relative to rewards. However, the study of Hebert et al. (2015) contradicted the previous findings of Hackley and colleagues (2009) by showing that the PAR and startle eyeblink reflexes were influenced by valence only during the consumption of the reward (i.e., picture viewing) and not during anticipation, suggesting that they index hedonic impact (liking) rather than incentive salience (wanting).

Interestingly, Dichter et al. (2010) assessed startle eyeblink and PAR reflexes in autism spectrum disorder. They replicated previous findings on affective eyeblink and postauricular reflex modulation in control participants whereas they showed that the ASD group displayed exaggerated startle eyeblink responses to pleasant images and exaggerated postauricular responses to unpleasant images, despite similar subjective ratings.

Ultimately, Sandt and colleagues (2009) further replicated the findings of Benning et al. (2004) and Hess et al. (2007), showing that PAR magnitude was greater to pleasant images relative to neutral and unpleasant images. Of greater relevance, Sandt and colleagues (2009) also showed that the PAR magnitude was found to be potentiated during the presentation of pleasant appetitive-related images (e.g., food, erotica) compared to pleasant non-appetitive-related images (e.g., nature scenes, adventure) and to unpleasant (e.g., human attack, animal attack, mutilation) and neutral images (e.g., kitchen utensils). These findings thus provide additional evidence for the PAR enhancement during appetitive context (Sandt et al., 2009) supporting the use of the PAR as a psychophysiological measure of appetitive conditioning in human subjects.

Nonetheless, the question why the postauricular reflex acts as an index of appetitive responding remains unclear. One possible explanation proposed by Benning and colleagues (2004), is exemplified by the PAR’s ancestral function as pinna-orienting component that served to focus the attention to novel salient stimuli. Indeed, during appetitive contexts, it may be necessary to engage toward a relevant appetitive stimulus, thus entailing a PAR potentiation (Sandt et al., 2009). Johnson et al. (2012) however argued that animals do not pull the pinnae backward when attending to what is happening in front of them. In an analogous manner, the human PAR is only activated when attention is directed behind and laterally, but not forward (Stekelenburg & van Boxtel, 2002). Moreover, the direction of PAR affective modulation is inconsistent with the fact that aversive and appetitive stimuli attract attention in an equal way (Johnson et al., 2012).

Another valuable explanation might be proposed by the nursing hypothesis, which is based on the fact that most infant mammals retract their pinnae during nursing in order to find a comfortable position that supports suckling (Johnson et al., 2012). This hypothesis states that the ear-retraction
reflex might have survived through natural selection even in higher primates in which the pinna movements are vestigial (Johnson et al., 2012). These authors explain that the PAR might be primed by appetitive emotions through the activation of the appetitive motivational system directly or through the suppression of defensive reflexes during appetitive states. If an intense, abrupt sound is presented during this primed state, the PAR is facilitated. As evidence for this hypothesis, Johnson and colleagues (2012) showed greater potentiation of PAR amplitude by appetitive emotion when subjects pursed their lips, simulating suckling by an infant. The nursing hypothesis is also supported by the findings of Sandt and colleagues (2009), which found that within the different thematic picture categories, the two associated with the largest PAR magnitudes were erotica (which included exposed breasts) and appetizing food.

Overview of the study: objectives and hypotheses

As a result of the previously reviewed literature, the purpose of the present study was to determine whether the postauricular and the startle eyeblink reflexes represent appropriate psychophysiological measures of human appetitive conditioning. More precisely, we wanted to confirm Andreatta and Pauli (2015)’s findings on the startle eyeblink reflex modulation during human appetitive conditioning. In addition, we wanted to test whether the use of the postauricular reflex as a measure of appetitive processing could be suitable within the framework of human appetitive conditioning, which represents the most innovative aspect of our study.

To test their efficiency as human appetitive conditioning indices, we conducted a study using a differential appetitive conditioning paradigm. Two neutral geometrical figures acted as conditioned stimuli (CSs), one of which (CS+) was contingently paired with a pleasant odor (US), whereas the other (CS-) was never paired with any odor. More precisely, during the initial habituation phase the two neutral stimuli (neutral geometrical figures) were presented without being reinforced. During the acquisition phase, the CS+ was systematically followed by the presentation of the US while the CS- was never reinforced. During the final extinction phase, only the CSs were presented and no US was delivered. To assess the participants’ appetitive conditioning, psychophysiological measures, including electromyography (EMG) signals of the startle eyeblink and postauricular reflexes, as well as skin conductance response (SCR), and subjective measures of CS-US contingency and CS liking were collected during the course of the experimental procedure, thus allowing a comparison thereof.
We hypothesized that the PAR magnitude would be specifically potentiated, and the startle eyeblink magnitude specifically attenuated, in response to the CS+ compared to the CS- during the acquisition phase. In addition, we expected the CS+ to be evaluated as more predictive of the US and more pleasant compared to the CS- and we expected a greater SCR in response to the CS+ compared to the CS-.
MATERIALS AND METHODS

Participants

This study was accepted by the Ethics committee of the Faculty of Psychology and Educational Sciences of the Geneva University. Sixty-three volunteers took part to the study and provided informed consent. Participants participated in exchange for either research course credit or monetary compensation. Based on previous related studies (Gable & Harmon-Jones, 2009; Hebert et al., 2015; Sandt et al., 2009), a sample size of around 60 participants was selected before the start of data collection.

According to the experiment’s requirements, healthy participants, aged between 18 and 40, were asked to come to the study without wearing any deodorant or perfume (this condition was also respected by the experimenter), without having any kind of olfactory problems and by refraining from eating and drinking (except of water) for 4 hours prior to the start of the experimental session. These precautions were adopted in order to optimize the participants’ sensitivity to the odors used in our study, thereby trying to improve the appetitive conditioning procedure.

In total, 8 participants were excluded from the conditioning analysis due to technical problems. In the end, 55 participants (21 men) aged between 18 and 40 (M = 25.27, SD = 5.56) were taken for the analysis and had no history of psychiatric or neurological disorder (except for four participants) and no olfactory problems that could interfere with proper odor perception. From this final sample, four participants (1 man) had to be additionally excluded from the SCR analysis due to technical problems with the SCR recordings.

Stimuli and apparatus

Two neutral geometrical complex figures (see Figure 2) as the ones typically used in human conditioning studies (Gottfried et al., 2002, 2003; Pool, Brosch, et al., 2014; Pool, Brosch, Delplanque, & Sander, 2015) served as conditioned stimuli (CSs). The same geometrical figures were presented to all participants and each figure served both as positively conditioned stimulus (CS+) and

1 Two participants had untreated neurological disorders, one of whom has had a light traumatic brain injury in 2013, while the other has had a concussion almost 20 years ago. The two other participants both presented a depression, only one participant was using Effexor as an antidepressant. Nonetheless, all four were kept in the analyses because they did not influence or change our results.
negatively conditioned stimulus (CS-), counterbalanced across participants. The CS+ always predicted the release of the US (i.e., the selected odor) during acquisition, while the CS- never predicted the release of the US (see Figure 3A). The CSs were presented using MATLAB (The MathWorks, Inc., Natrick, Massachusetts) with the Psychophysics Toolbox extensions (Brainard, 1997; Pelli, 1997).

A pleasant odor, chosen by each participant for its preference in terms of liking and intensity ratings, out of a set of 17 odors (Firmenich SA, Geneva, Switzerland; see Table 1), served as unconditioned stimulus (US). The odor was delivered through a computer-controlled olfactometer, with an airflow fixed at 1 L/min through a nasal cannula. We chose to adopt a pleasant odor as US, because it represents an effective primary reward that is able to trigger appetitive conditioning in humans (Gottfried et al., 2002, 2003; Pool, Brosch, et al., 2014; Pool et al., 2015).

An acoustic startle probe (white noise burst of 50ms and 105dB) was delivered binaurally through two loudspeakers placed at a fixed and equal distance from the participant and was necessary to elicit the startle eyeblink and postauricular reflexes.

Figure 2. Two neutral geometrical figures used as conditioned stimuli (CSs).
Figure 3. Experimental design. (A) Experimental conditions. (B) Conditioning phases. During habituation and extinction, only the two CSs were presented, without any US (odor) delivered. (C) Illustration of a trial during the acquisition phase of the delay appetitive conditioning procedure.

Table 1
Odors used during the unconditioned stimulus (US) selection procedure.

<table>
<thead>
<tr>
<th>Odorant name</th>
<th>Odor family</th>
<th>Concentration (% in di-propylene glycol)</th>
<th>Mean liking (SD)</th>
<th>Mean intensity (SD)</th>
<th>Number of times selected as the US</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aladinate</td>
<td>Floral</td>
<td>50</td>
<td>32.95 (19.92)</td>
<td>63.49 (22.45)</td>
<td>0</td>
</tr>
<tr>
<td>Ariana</td>
<td>Detergent</td>
<td>20</td>
<td>64.69 (22.26)</td>
<td>66.96 (14.58)</td>
<td>10</td>
</tr>
<tr>
<td>Caramel</td>
<td>Sweet food</td>
<td>20</td>
<td>39.94 (25.01)</td>
<td>60.43 (19.27)</td>
<td>3</td>
</tr>
<tr>
<td>Chocolate</td>
<td>Sweet food</td>
<td>20</td>
<td>39.65 (26.38)</td>
<td>69.36 (20.88)</td>
<td>3</td>
</tr>
<tr>
<td>Galbex®</td>
<td>Floral</td>
<td>50</td>
<td>57.23 (21.69)</td>
<td>52.69 (22.04)</td>
<td>3</td>
</tr>
<tr>
<td>Geraniol</td>
<td>Floral</td>
<td>50</td>
<td>39.32 (22.17)</td>
<td>59.32 (22.81)</td>
<td>2</td>
</tr>
<tr>
<td>Green tea</td>
<td>Floral green</td>
<td>50</td>
<td>50.72 (15.16)</td>
<td>33.43 (24.65)</td>
<td>1</td>
</tr>
<tr>
<td>Perfume</td>
<td>Type</td>
<td>Sample Size</td>
<td>US 1</td>
<td>US 2</td>
<td></td>
</tr>
<tr>
<td>-------------------------</td>
<td>------------</td>
<td>-------------</td>
<td>------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>Lavender</td>
<td>Floral</td>
<td>20</td>
<td>46.14 (23.78)</td>
<td>61.74 (20.14)</td>
<td>1</td>
</tr>
<tr>
<td>Linalol</td>
<td>Floral</td>
<td>50</td>
<td>50.85 (20.89)</td>
<td>49.55 (24.40)</td>
<td>2</td>
</tr>
<tr>
<td>Magnolia grandiflora</td>
<td>Floral</td>
<td>50</td>
<td>53.29 (23.91)</td>
<td>60.91 (20.18)</td>
<td>4</td>
</tr>
<tr>
<td>Peach</td>
<td>Fruity</td>
<td>50</td>
<td>56.05 (21.35)</td>
<td>45.39 (21.40)</td>
<td>1</td>
</tr>
<tr>
<td>Pine</td>
<td>Woody</td>
<td>33</td>
<td>48.88 (19.88)</td>
<td>48.64 (24.09)</td>
<td>1</td>
</tr>
<tr>
<td>Pipol</td>
<td>Herbal</td>
<td>20</td>
<td>29.63 (20.79)</td>
<td>65.19 (24.76)</td>
<td>0</td>
</tr>
<tr>
<td>Speculaas</td>
<td>Sweet food</td>
<td>20</td>
<td>39.42 (22.85)</td>
<td>61.74 (19.24)</td>
<td>1</td>
</tr>
<tr>
<td>Strawberry</td>
<td>Fruity</td>
<td>20</td>
<td>58.88 (19.30)</td>
<td>60.27 (21.30)</td>
<td>4</td>
</tr>
<tr>
<td>Tiare</td>
<td>Floral</td>
<td>50</td>
<td>48.97 (22.02)</td>
<td>51.76 (24.26)</td>
<td>3</td>
</tr>
<tr>
<td>Tutti frutti</td>
<td>Fruity</td>
<td>20</td>
<td>64.69 (25.24)</td>
<td>62.48 (23.42)</td>
<td>16</td>
</tr>
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### Design and procedure

The experiment took place at the Brain and Behaviour Laboratory (BBL) at the University Medical Center (CMU) of the University of Geneva. The experimental sessions were hold always at the same daytime (between 9 am and 10.30 am and between 11 am and 12.30 pm). This decision, in addition to the request of a fasting state, meant to make the participants feel hungrier in order to increase the probability of the US (at least when it consisted in a food-related odor) to be perceived as rewarding. Once the participants arrived, they had to read and sign an informed consent form and to fill out a questionnaire. In this latter, they were asked to answer some questions regarding their age, their gender, the number of accomplished school years, their level of French, whether they were wearing any perfume at that moment, the time of their last meal, their hunger level (on a Likert scale ranging from 1 = *not hungry at all* to 10 = *very hungry*), their dominant hand, if they ever suffered from neurological disorders (such as cerebrovascular accidents, epilepsy, cerebral hemorrhages, brain tumors, traumatic brain injuries, concussions, etc.) or psychiatric disorders (if it was the case, to specify which type and for how long) and to indicate if they were currently taking psychotropic medications or the contraceptive pill.

Consequently, they were asked to sit comfortably in a chair located in the test room. The chair’s height was adjusted in order that the participant’s gaze coincided with the center of the

30
computer screen that was located in front of them. Participants were then informed about the general procedure of the experiment and were instructed about their task. The skin conductance electrodes and the nasal cannula were then attached to them.

During the experiment, the experimenter was in a parallel room and could see the participants though a webcam. No video recordings were however collected during the course of the experiment.

The whole study was subdivided in three different parts. At first, participants performed a US selection task. They then started the appetitive conditioning task and finally, they performed ratings of the CS-US contingency and the CS liking.

US selection

Participants had to rate 17 different odors (Firmenich SA, Geneva, Switzerland, see Table 1), which were all reported to be positive and which were delivered to them along with odorless air, on a pleasantness and intensity visual analog scale (VAS). Participants were recommended to rate each odor by itself without comparing them one to another. The presentation of each odor occurred only once, in a counterbalanced order: first the 9 odors positioned on the left side and then the 10 odors on the right side of the olfactometer (including two odorless air presentations, one for each side) always in a fixed order for half of the participants, the reverse for the other half of the participants.

More precisely, participants were asked to stare at the center of the computer screen. At the beginning of each new trial, a countdown of 3 seconds appeared at the center of the screen suggesting the participants to breathe out evenly and to prepare for the subsequent inspiration. At the end of the countdown, a small asterisk (inspiration cue) appeared at the same place advising the participant to breathe in evenly. The odors were released from the computer controlled olfactometer 0.5 s before the small asterisk for a total duration of 1.5 s.

Participants were next asked to rate each odor on a pleasantness and intensity VAS that was presented to them in a separate order. The pleasantness VAS ranged from 0 = extremely unpleasant to 100 = extremely pleasant and the intensity VAS ranged from 0 = not perceived to 100 = extremely strong. After each trial, an intertrial interval (ITI) consisting of a black fixation cross placed at the center of the gray computer screen, of a duration adapted to the participants’ time to rate (15 s minus the time the participant took to rate, with a minimal duration of 0.5 s) was presented.

The odor that was rated as the most pleasant and the most intense was chosen to be the unconditioned stimulus. To be more precise, the odor that was rated as the most pleasant was selected as US if its intensity was evaluated not lower than 50 on the intensity VAS. Otherwise, the second most pleasant odor was selected if and only if its intensity was rated as higher than the most pleasant
odors’ intensity and the difference in pleasantness between the most pleasant and second most pleasant odor was equal or less than 10.

In this way, each participant chose its favorite odor and we could avoid problems due to the high interindividual variability of affective responses to odors (e.g., Ferdenzi et al., 2013). Moreover, through this odor rating procedure, we could ensure that the selected odor was evaluated as more pleasant and intense than the odorless air, thereby representing an appropriate appetitive US.

During the US selection part, SCR was measured but was eventually not analyzed.

Appetitive conditioning

The EMG electrodes were placed on participants and the lights of the experimental room were turned dim to facilitate the acoustic startle responses (Grillon, Pellowski, Merikangas, & Davis, 1997). The schematic representation of the experimental setup is reported below in Figure 4.

Before starting the actual appetitive conditioning procedure, participants were exposed to 10 acoustic startle probes randomly distributed every 10 to 20 s over time. This first phase was intended to reduce the initial high reactivity of the reflex responses to the acoustic startle probe and to bring the postauricular and the startle eyeblink responses to a baseline level. Subsequently, we proceeded with the three contiguous phases of the delay differential appetitive conditioning paradigm. The initial habituation phase consisted of 4 unreinforced presentations of each CS. During the acquisition phase (9 presentations of each CS), one stimulus (CS+) was systematically paired with the selected odor (US), while the other stimulus (CS-) was never reinforced. During the final extinction phase (9 presentations of each CS), no US was delivered (see Figure 3B).

The illustration of a trial during the acquisition phase of the delay appetitive conditioning procedure is reported in Figure 3C. The CS was presented at time point 0. After 5 to 6 seconds an acoustic startle probe was released and at 6.5s the valve of the olfactometer opened and triggered the odor release (in response to a CS+) or no odor release (in response to CS-) whose presentation co-terminated with the CS presentation. Half of a second after, a small asterisk on the center of the screen (inspiration cue) gave the instruction to the participant to breathe in evenly in order to detect the odor. One trial lasted 8 s during which the CS was presented and there were 12 to 15 seconds between each trial (ITI, which again consisted in a black fixation cross placed at the center of the gray computer screen). The startle probes were delivered randomly between 5 to 6 seconds after CS onset, or between 6 and 7.5 s after CS offset during ITIs, in order to reduce their predictability. More precisely, the same number of acoustic startle probes were delivered for each one of the two CS (2 out of 4 trials throughout habituation, 6 out of 9 trials throughout acquisition, and 6 out of 9 trials throughout extinction) and extra startle probes were delivered during ITIs (2 throughout habituation, 6 throughout
acquisition, and 6 throughout extinction). Startle probe presentation occurred outside of the analysis window of SCR in order to avoid the artifacts of the startle probe on SCR.

Subjective ratings

Once the appetitive conditioning task terminated, a subjective rating collection was done to determine if participants were aware of the reinforcement contingencies and to check for the evulative effects of appetitive conditioning. To this purpose, participants answered randomized-order questions regarding the CS-US contingency (i.e., they had to answer the question: “to what extent was the stimulus predictive of the pleasant odor delivery?” ranging from 0 = never to 100 = always) and CS liking (i.e., they had to answer the question: “to what extent was the stimulus unpleasant or pleasant?” ranging from 0 = very unpleasant to 100 = very pleasant) on a VAS following the randomized presentation of both CSs.

Finally, participants were debriefed.
Physiological measures and response definition

To measure appetitive conditioning and to assess our a priori hypotheses, we analyzed the startle eyeblink reflex magnitude and the postauricular reflex magnitude during the presentation of the CS+, the CS- and the ITI, across the different phases of conditioning. Moreover, we analyzed the SCR in response to the CS+ and the CS- across the different phases of appetitive conditioning, and the subjective ratings collected at the end of the appetitive conditioning procedure.

Skin conductance response

More precisely, the skin conductance response (SCR) was measured with two 6-mm contact diameter Ag-AgCl electrodes filled with 0.5% NaCl electrolyte gel and attached to the external phalanges of the index and medial finger of the participants’ non-dominant hand. Participants were instructed to keep the recorded hand as still as possible to reduce artifacts’ formation. Data was continuously recorded at 2000 Hz through a BIOPAC MP150 system (Santa Barbara, California). An offline analysis of the SCR was performed with AcqKnowledge 4.2 software (BIOPAC Systems Inc., Goleta, California). We downsampling to 1000 Hz and low-pass filtered (1Hz) the SCR before starting the analysis. We then scored the SCR for each trial as the peak-to-peak amplitude difference in skin conductance to the biggest response occurring in the 0.5-4.5s temporal window following the CS onset, to capture the response to the CS+, respectively CS-. The threshold for the minimal response was 0.02 µS. Responses below that threshold were scored as “0” and were taken into the analysis. SCRs were detected automatically with an AcqKnowledge routine and checked for artifacts and misdetections manually. The raw SCR scores were square-root-transformed to reduce positive skew distribution, and scaled according to each participant’s maximal SCR (Lykken & Venables, 1971). The mean SCR magnitude during habituation comprised the first four presentations of each CS. The mean SCR magnitude during acquisition comprised the nine presentations of each CS after the first pairing between the CS+ and the US. Finally, the mean SCR magnitude during extinction were composed of the last eight presentations of each CS after the first US omission.

Startle eyeblink and postauricular reflexes

The startle eyeblink and postauricular reflexes were measured by applying four 4-mm contact diameter Ag-AgCl electrodes filled with electrolyte gel and attached respectively upon the left orbicularis oculi muscle sited under the lower eyelid of the left eye (one electrode placed below the lower eyelid in line with the pupil while the subject was looking in front of him, a second electrode placed approximately 1-2 cm beside the first one, with reference to the guidelines described in Blumenthal et al., 2005) and behind the left pinna on each side of the tendon of insertion for the PAR
(by pulling the pinna forwards, positioning one electrode directly posterior to the tendon on the pinna surface, and the other electrode over the postauricular muscle (Sollers & Hackley, 1997). Two additional reference and ground signal electrodes were positioned on the forehead very near to the hairline, which represents a relative electrically inactive site (Blumenthal et al., 2005).

The positioning of the EMG electrodes for the recording of the muscle activity related to the postauricular and the startle eyeblink reflexes is shown in Figure 5 below. Data was continuously recorded at 2048 Hz though a BIOSEMI Active-Two amplifier system (BioSemi Biomedical Instrumentation, Amsterdam, the Netherlands). An offline analysis of the EMG was performed with Brain Vision Analyzer software (version 2.1; Brain Products GmbH, Gilching, Germany).

Furthermore, we extracted the EMG signals of startle eyeblink and postauricular responses by calculating conventional bipolar montages from electrode pairs by subtracting the recorded activity of one electrode from the adjacent one. Further, we bandpassed (10-400 Hz) and notch filtered (50 Hz) the PAR signal, and we bandpassed (20-400 Hz) and notch filtered (50 Hz) the startle eyeblink reflex signal. We then rectified the PAR signal and we low-pass filtered (40 Hz) the eyeblink reflex signal before rectifying it, according to the instructions of Blumenthal et al., 2005. The filtered and rectified EMG signals were segmented into epochs from 100 ms prior to the acoustic startle probe onset to 250 ms after probe onset. The baseline was corrected to 50 ms prior to the startle probe onset. The average signal of all rectified waveforms comprising all the different conditions (i.e., CS+, CS- and ITI during habituation, acquisition and extinction) for each subject was found.

Afterward, we separated the average signal for each participant condition by condition (Hab-ITI, Hab-CS+, Hab-CS-, Acq-ITI, Acq-CS+, Acq-CS-, Ext-ITI, Ext-CS+, Ext-CS-) and we obtained the signal average of the rectified waveforms across trials within conditions. Each segment was then visually examined and segments having artifacts were removed from the analyses (4.16% of the trials for the PAR, and 4.16% of the trials for the eyeblink reflex). Subsequently, the PAR and the startle eyeblink reflex magnitude were scored from the aggregate waveforms as the baseline-to-peak amplitude for each condition and peaks were extracted. The peaks were calculated as the maximum EMG activity, occurring within 5-35 ms after startle probe onset for the PAR (Gable & Harmon-Jones, 2009; Sandt et al., 2009) and within 21-120 ms after startle probe onset (Blumenthal et al., 2005) for the eyeblink reflex and peaks information (latency and amplitude) were extracted, in order to do the statistical analyses.

Since the PAR is a microreflex with a low signal-to-noise ratio, it was necessary to score it solely after signal averaging of the rectified waveforms across trials within conditions (Aaron & Benning, 2016; Benning, 2011; Benning et al., 2004; Hackley et al., 1987, 2009; Hebert et al., 2015; Hess et al., 2007; Sollers & Hackley, 1997). To have a more straightforward comparison of the two
measurements, we decided to do the same kind of analysis for both the PAR and startle eyeblink reflex, even though the most common method of analysis of the latter is by means of a single-trial analysis (Blumenthal et al., 2005).

In order to make a graphical representation of the eyeblink reflex and PAR modulation, we obtained the grand-averaged of the PAR and eyeblink reflex waveforms across trial within conditions.

![Image of electrodes positioning site for the (A) startle eyeblink reflex and the (B) postauricular reflex. Figures adapted from Blumenthal et al. (2005) and Hackley et al. (2015).]

**Figure 5.** Electrodes positioning site for the (A) startle eyeblink reflex and the (B) postauricular reflex. Figures adapted from Blumenthal et al. (2005) and Hackley et al. (2015).

**Statistical analysis**

One-way repeated measures analyses of variance (rmANOVAs) with CS condition (CS+ vs. CS- vs. ITI) as a within-participant factor, were conducted to determine whether there was a statistically significant difference in magnitude of the postauricular reflex, respectively the eyeblink reflex, as a function of stimulus condition (ITI, CS+ and CS-) for each different phase of the appetitive conditioning paradigm (habituation, acquisition and extinction) separately.

To test our a priori hypotheses, follow-up pairwise comparisons on significant main effects for PAR and startle eyeblink reflex magnitudes in response to the different conditions across the different phases were made. More specifically, a planned contrast of PAR magnitude, respectively startle eyeblink reflex magnitude, to the CS+ vs. the CS- during the acquisition phase was conducted. A stringent Bonferroni correction of the pairwise comparisons was performed in order to adjust the p-value for multiple testing (i.e., for each comparison made, we multiplied the p-value by 3).

In addition, we performed paired t-tests on: (1) the SCR amplitudes to the CS+ vs. the CS-
during the different appetitive conditioning phases, (2) the subjective ratings that were collected at the end of the appetitive conditioning procedure, and (3) the pleasantness and intensity ratings collected during the US selection procedure in order to verify the efficacy of the olfactory US vs the odorless air.

The alpha level was set at .05. We report the Bonferroni corrected $p$-value and the Huyhn-Feldt correction value ($\varepsilon_{HF}$) for the one-way repeated measures ANOVAs. In order to report the estimates of effect size, we indicate the partial $\eta^2$ or the Hedges’ $g_{av}$ and their 90% or 95% confidence interval (CI), respectively.

For the sake of simplicity and clarity, we will refer to the two neutral figures as CS+ and CS- also during habituation, even if any association with the US has yet been made.
RESULTS

Hunger level

Participants reported a mean hunger level of 5.75 ($SD = 2.44$) on the Likert scale.

Olfactory US ratings

With regard to the odor ratings, we found that the odor selected as the US was rated as more pleasant ($M = 83.84, SD = 13.53$) than the odorless air ($M = 47.56, SD = 14.99$), $t(54) = 14.76, p < .001, g_{av} = 2.506, 95\% \text{ CI} = [1.952, 3.122]$ (see Figure 6A). Similarly, the odor selected as US was evaluated as more intense ($M = 70.19, SD = 16.59$) than odorless air ($M = 24.46, SD = 22.18$), $t(54) = 12.82, p < .001, g_{av} = 2.302, 95\% \text{ CI} = [1.764, 2.896]$ (see Figure 6B).

**Figure 6.** Mean (A) liking ratings and (B) intensity ratings of the US and odorless air. Error bars represent ± 1 standard error of the mean. **$p < .001$.**
Postauricular reflex

Habituation

A statistically significant main effect of CS condition emerged during the habituation phase, $F(2, 108) = 5.31, p = .007$, partial $\eta^2 = .090$, 90% CI = [.016, .173]. Pairwise follow-up comparisons showed that there was no statistical difference between PAR magnitude to the CS+ compared to the CS-, $t(54) = -0.11, p > .99$ (Bonferroni corrected), $g_{av} = -0.010$, 95% CI = [-0.184, 0.164] (see Figure 7A). Interestingly, we found that the PAR magnitude was greater to the ITI compared to the CS+, $t(54) = 3.01, p = .012$ (Bonferroni corrected), $g_{av} = 0.239$, 95% CI = [0.077, 0.406], and to the CS-, $t(54) = 2.48, p = .048$ (Bonferroni corrected), $g_{av} = 0.224$, 95% CI = [0.042, 0.411] (see Figure 7A).

Acquisition

During the acquisition phase, we observed a statistically significant main effect of CS condition, $F(2, 108) = 6.87, p = .003$, partial $\eta^2 = .113$, 90% CI = [.029, .201]. Planned contrast showed a statistically significant enhancement of the PAR magnitude to the CS+ compared with the CS-, $t(54) = 2.97, p = .013$ (Bonferroni corrected), $g_{av} = 0.095$, 95% CI = [0.030, 0.161], which was in line with our a priori hypothesis (see Figure 7B). In addition, we found no statistical difference between the PAR magnitude to the ITI compared with the CS+, $t(54) = 1.47, p = .444$ (Bonferroni corrected), $g_{av} = 0.074$, 95% CI = [-0.027, 0.177], while we found that the PAR magnitude was still greater to the ITI compared to the CS-, $t(54) = 3.33, p = .005$ (Bonferroni corrected), $g_{av} = 0.166$, 95% CI = [0.063, 0.271] (see Figure 7B), analogous to the habituation phase.

Extinction

During the extinction phase, the one-way repeated measures ANOVA revealed a statistically significant main effect of CS condition with, $F(2, 108) = 6.34, p = .004$, partial $\eta^2 = .105$, 90% CI = [.024, .192]. Pairwise follow-up comparisons showed that there was no longer a statistical difference between the PAR magnitude to the CS+ compared with the CS-, $t(54) = 0.95, p > .99$ (Bonferroni corrected), $g_{av} = 0.043$, 95% CI = [-0.047, 0.134] (see Figure 7C). Moreover, the PAR magnitude was greater to the ITI compared with the CS-, $t(54) = 3.35, p = .004$ (Bonferroni corrected), $g_{av} = 0.184$, 95% CI = [0.071, 0.301], and marginally greater compared with the CS+, $t(54) = 2.28, p = .080$ (Bonferroni corrected), $g_{av} = 0.135$, 95% CI = [0.016, 0.257] (see Figure 7C).
Figure 7. Grand-averaged postauricular reflex waveforms as a function of stimulus condition (CS+ vs. CS- vs. ITI) across (A) habituation, (B) acquisition and (C) extinction.

Eyeblink reflex

Habituation

During the habituation phase, we found a statistically significant main effect of CS condition, $F(2, 108) = 3.04, p = .053$, partial $\eta^2 = .053$, 90% CI = [.000, .125]. Pairwise follow-up comparisons showed that there was no statistical difference between eyeblink reflex magnitude to the CS- relative to the CS+, $t(54) = -0.433, p > .99$ (Bonferroni corrected), $g_{av} = -0.038, 95\%$ CI = [-0.215, 0.138] (see Figure 8A). The eyeblink reflex magnitude was however greater to the CS+ compared to the ITI, $t(54) = 2.570, p = .039$ (Bonferroni corrected), $g_{av} = 0.216, 95\%$ CI = [0.046, 0.389], while there was no statistical difference between the eyeblink reflex magnitude to the CS- compared to the ITI, $t(54) = 1.736, p = .265$ (Bonferroni corrected), $g_{av} = 0.192, 95\%$ CI = [-0.029, 0.417], (see Figure 8A).

Acquisition

During the acquisition phase, a statistically significant main effect of CS condition was observed, $F(2, 108) = 7.20, p = .002$, partial $\eta^2 = .118$, 90% CI = [.029, .212]. Planned contrast showed that there was no statistically significant difference between eyeblink reflex magnitude to the CS- compared to the CS+, $t(54) = 0.293, p > .99$ (Bonferroni corrected), $g_{av} = 0.015, 95\%$ CI = [-0.087, 0.118], which contrasts with our a priori hypothesis (see Figure 8B). We however found that the eyeblink reflex magnitude was greater in response to the CS- relative to the ITI, $t(54) = 3.133, p = .008$ (Bonferroni corrected), $g_{av} = 0.259, 95\%$ CI = [0.090, 0.434] and that eyeblink reflex magnitude
was greater to the CS+ compared with the ITI, $t(54) = 2.911, p = .016$ (Bonferroni corrected), $g_{av} = 0.226$, 95% CI = [0.068, 0.389] (see Figure 8B), analogous to the habituation phase.

**Extinction**

During the extinction phase, the one-way repeated measures ANOVA showed a statistically significant main effect of CS condition, $F(2, 108) = 5.161, p = .007$, partial $\eta^2 = .087$, 90% CI = [.014, .170]. Pairwise follow-up comparisons showed that there was no statistical difference between the eyeblink reflex magnitude to the CS- compared with the CS+, $t(54) = -1.673, p = .301$ (Bonferroni corrected), $g_{av} = -0.106$, 95% CI = [-0.234, 0.020] (see Figure 8C). Moreover, there was no statistical difference between the eyeblink reflex magnitude to the CS- compared to the ITI, $t(54) = 1.655, p = .311$ (Bonferroni corrected), $g_{av} = 0.113$, 95% CI = [-0.023, 0.251] (see Figure 8C). Finally, we still found a larger eyeblink reflex magnitude in response to the CS+ compared to the ITI, $t(54) = 3.014, p = .012$ (Bonferroni corrected), $g_{av} = 0.204$, 95% CI = [0.066, 0.347] (see Figure 8C).

![Figure 8](image.png)

**Figure 8.** Grand-averaged eyeblink reflex waveforms as a function of stimulus condition (CS+ vs. CS- vs. ITI) across (A) habituation, (B) acquisition and (C) extinction.

**Skin conductance response**

With regard to the skin conductance responses, we found no statistical difference in SCRs to the CS+ ($M = 0.07$, $SD = 0.11$) compared with the CS- ($M = 0.06$, $SD = 0.09$) during habituation, $t(50) = 0.71, p = .479$, $g_{av} = 0.097$, 95% CI = [-0.173, 0.369] (see Figure 9A). Likewise, we found no statistical difference in SCRs to the CS+ ($M = 0.03$, $SD = 0.05$) compared with the CS- ($M = 0.02$, $SD = 0.06$) during acquisition, $t(50) = 0.71, p = .479$, $g_{av} = 0.097$, 95% CI = [-0.173, 0.369] (see Figure 9B).
During acquisition, $t(50) = 0.88, p = .381, g_{av} = 0.113, 95\%\ CI = [-0.141, 0.369]$ (see Figure 9B). In addition, SCRs to the CS+ ($M = 0.03, SD = 0.05$) were not statistically different from SCRs to the CS- ($M = 0.03, SD = 0.05$) during the extinction phase, $t(50) = -0.52, p = .606, g_{av} = -0.073, 95\%\ CI = [-0.352, 0.206]$. (see Figure 9C).

Subjective ratings

With regard to the CS-US contingency ratings, we found that the CS+ was evaluated as more predictive of the US than the CS- after the extinction phase, $t(54) = 4.78, p < .001, g_{av} = 0.944, 95\%\ CI = [0.522, 1.386]$ (see Figure 10A). Furthermore, analysis of the CS liking ratings showed that the CS+ was evaluated as more pleasant compared to the CS- after extinction, $t(54) = 2.77, p = .008, g_{av} = 0.584, 95\%\ CI = [0.155, 1.024]$ (see Figure 10B).
Figure 10. Mean (A) CS-US contingency ratings and (B) CS liking ratings to the CS+ and the CS-. Error bars represent ± 1 standard error of the mean. ***p < .001, **p < .01.
DISCUSSION

The present study sought to determine whether human appetitive conditioning may be measured with the postauricular and startle eyeblink reflexes. To this end, we applied a differential appetitive conditioning paradigm, in which one neutral figure (CS+) was contingently paired with a pleasant odor (US), whereas another neutral figure (CS-) was never paired with any odor. We hypothesized that the PAR magnitude would be specifically potentiated, and the eyeblink magnitude specifically attenuated, to the CS+ compared with the CS- during the acquisition phase. Our results provide initial evidence that the postauricular reflex represent a valuable and more sensitive psychophysiological measure of appetitive conditioning relative to the startle eyeblink and skin conductance response.

First of all, in order to check for the effectiveness of our experimental design, we analyzed the olfactory US ratings. We found that the odor that was selected as US was evaluated as more pleasant and more intense than the odorless air. Hence, the selected odor represented a suitable unconditioned stimulus.

Secondly, with respect to the CS-US contingency and CS liking ratings that were collected after the extinction phase of the appetitive conditioning procedure, we found that the CS+ was evaluated as more predictive of the US and more pleasant than the CS-. Primarily, these results reflect learning at the subjective level and indicate that the overall appetitive conditioning procedure was successful. Furthermore, these findings show that participants were aware of the appetitive contingencies as they rated the CS+ to be more predictive of the US than the CS-, even after the extinction phase. Also, since the CS+ was rated as more pleasant than the CS- after the extinction phase, it indicates that appetitive conditioning successfully influenced the subjective valence of the CS+ and it suggests that these evaluative effects may be resistant to extinction. Nevertheless, we cannot be sure that the observed evaluative effects are actually resistant to extinction, as we did not do a trial-by-trial online rating. It could indeed be possible that our liking results were due to the memories of the CSs related to the acquisition phase rather than to the real subjective pleasantness of the CSs after extinction.

Third and more significant were our results regarding the postauricular reflex. During habituation, where the two neutral stimuli were presented alone without reinforcement, we observed no pre-existing difference between the two neutral stimuli, in that there was no statistical difference in PAR magnitude to the CS+ compared to the CS-. Moreover, the larger PAR magnitude to the ITI compared with the CS+ and the CS- is in line with previous studies showing that the PAR magnitude
is smaller during stimulus presentation than during intertrial intervals (ITIs), due to the inhibition of the postauricular reflex response by perceptual engagement in response to a foreground stimulus (Benning, 2011; Benning et al., 2004; Hackley et al., 1987). During acquisition and consistent with our a priori hypothesis, the PAR was specifically enhanced in response to the CS+ compared with the CS-, showing learning at the psychophysiological level. During extinction, there was no longer a difference between the PAR magnitude to the CS+ compared with the CS-. Taken that the PAR is potentiated only during acquisition and no longer during extinction where none of the CSs is reinforced, this indicates that the PAR is indeed sensitive to the appetitive contingencies. In other words, it suggests that the PAR potentiation is specific in response to the stimulus that is associated to the pleasant odor (US) delivery.

Taken together, our results indicate that the PAR is a sensitive and reliable measure of human appetitive conditioning as it was potentiated specifically in response to an appetitive conditioned stimulus only during the acquisition phase. Our findings are consistent with previous studies on the PAR modulation in response to pleasant stimuli (Aaron & Benning, 2016; Benning, 2011; Benning et al., 2004; Dichter et al., 2010; Gable & Harmon-Jones, 2009; Hackley et al., 2009; Hess et al., 2007; Johnson et al., 2012; Sandt et al., 2009), thus aligning with the view that the PAR is a reliable index of appetitive processing.

Moreover, based on our findings that the PAR was potentiated specifically during the acquisition phase but not during extinction, and on the fact that the evaluative effects conversely may have persisted after extinction, it seems that the PAR modulation does not merely reflect the subjective valence of the conditioned stimuli. From this perspective, our results were inconsistent with previous authors, Gable & Harmon-Jones (2009), who claimed that the PAR is a reflex purely modulated by the affective valence of a stimulus. Disparately, taken that the PAR potentiation was specific to the appetitive conditioned stimulus during the acquisition phase, we think that this potentiation was conditioned to the pleasant odor (US) delivery. As a consequence, based on our results, we rather think that the PAR is sensitive to the predictive or current reward value of the conditioned stimulus. Expressing it differently, we do not think that the PAR modulation is purely dependent on the affective valence of the conditioned stimulus but rather on its predictive or current reward value, which is not limited to the dimension of valence solely but probably depends on the interaction of the multiple psychological components of the reward system.

Conversely, with regard to the arousal dimension, one might argue that the PAR may be modulated exclusively by the arousal level of the conditioned stimulus. Indeed, it might be possible that the specific PAR potentiation to the CS+ compared to the CS- during the acquisition phase was due to arousal value of the US that followed the presentation of the CS+ and not the CS-, rather than
to the current or predictive reward value of the CS+. Unfortunately, since we did not measure the subjective arousal of the US, we can not draw any conclusions. However, we do not think that the PAR was modulated simply by the arousal level of the CS+ since it has been reported that the PAR modulation is independent from the arousal level of pleasant and unpleasant stimuli (Gable & Harmon Jones, 2009). In addition, Sandt et al.’s (2009) findings that the PAR was potentiated specifically in response to pleasant appetitive-related images, argue against the view that the PAR is affected by the arousal value, in that similar arousal ratings for pleasant non-appetitive related images and appetitive-related images were found. Further evidence may be provided by our SCR results. As SCR is considered as a measure of arousal (Andreatta & Pauli, 2015; Grillon & Baas, 2003; Lang et al., 1990), the fact that no statistical difference in SCR was reported during the habituation phase might indeed suggest that the arousal level did not account for the PAR modulation.

On the other hand, our results concerning the startle eyeblink and skin conductance response were incongruent with our expectations. During habituation, we found no statistically significant difference in the startle eyeblink reflex magnitude to the CS+ compared to the CS-, which shows that we observed no pre-existing difference between the two neutral stimuli. Strangely, we found that the startle eyeblink reflex magnitude was greater to the CS+ compared to the ITI while no difference was observed to the CS- compared to the ITI. During the acquisition phase, there was no statistically significant difference between eyeblink reflex magnitude to the CS+ compared to the CS-, which contrasts with our a priori hypothesis. We also found that the eyeblink reflex magnitude was greater in response to the CS- and to the CS+ compared to the ITI. During the extinction phase, no statistical difference between the eyeblink reflex magnitude to the CS+ compared to the CS- was found. Moreover, there was no statistical difference between the eyeblink reflex magnitude to the CS- compared to the ITI, but we still found a larger eyeblink reflex magnitude in response to the CS+ compared to the ITI.

Likewise, we found no effect of appetitive conditioning on SCR, since we did not find any difference in SCR to the CS+ compared to the CS- during the acquisition phase of the appetitive conditioning procedure.

Our startle eyeblink reflex and SCR findings are therefore inconsistent with the results of Andreatta & Pauli (2015)’s study, which successfully highlighted conditioning with both the startle eyeblink reflex and SCR. More generally, our results are in contradiction with the literature on startle eyeblink modulation (e.g., Andreatta & Pauli, 2015; Dichter et al., 2010) and SCR modulation (e.g., Andreatta & Pauli, 2015; Klucken et al., 2013) by positive emotional stimuli. Possible reasons for the discrepancy between Andreatta and Pauli’s (2015) study and ours, might be found in the use of a different study design. In fact, in the present study we used a differential appetitive conditioning
whereas Andreatta and Pauli used a concurrent aversive and appetitive conditioning paradigm. This choice of design could have induced a contrast effect, which refers to the observation of a greater response to appetitive conditioning and to aversive conditioning during their simultaneous exposition. This reciprocal boost in responses is due to the comparison between the appetitive and the aversive CS+ when appetitive and aversive conditioning occur simultaneously. The resulting contrast effect could in turn explain why they obtained a greater magnitude of the eyeblink reflex and SCR to the appetitive CS+ compared to the CS- during the acquisition phase. To clarify the reliability of the eyeblink reflex as a measure of appetitive conditioning and to identify potential influences of the contrast effect, future studies should try to replicate a concurrent aversive and appetitive paradigm using an appetitive and an aversive odor as US.

Moreover, Andreatta and Pauli (2015) presented neutral geometrical stimuli as CSs, namely a neutral stimulus (avCS+) associated to an aversive US (electric shock), a second neutral stimulus (appCS+) associated to an appetitive US (sweet or salty food) and a third neutral stimulus (CS-) not associated to any US. However, during the acquisition phase only, they additionally associated the different conditioned stimuli with images of the US. Notably, a lightning bolt as a symbol for the electric shock together with the avCS+, an image of Smarties or a salty pretzel together with the appCS+ and a ban symbol together with the CS–. Pictures of flashes, chocolate/salty pretzels, or bans were not anymore presented in conjunction with the different CSs during extinction. This is an arguable choice since it is not sure whether their findings were due to the presentation of the CSs or to the presentation of the images themselves.

Also, the use of a pleasant odor as appetitive US in the present study compared to the use of food in the study of Andreatta and Pauli (2015), represents another potential cause of disparity. Indeed, although both are primary rewards (Gottfried, 2011), odors might be less powerful in eliciting appetitive responses as compared to other USs (see Hermann et al., 2000).

A further explanation for the fact that we did not observe the predicted results for the startle eyeblink reflex might be that this psychophysiological measure, being a defensive reflex, is thought to be principally an index of defensive responding rather than of appetitive responding per se (Dichter et al., 2010; Dichter & Tomarken 2008). That is, that the attenuation of the startle eyeblink reflex in response to pleasant appetitive stimuli may be a result of the inhibition of defensive responding rather than of the direct activation of appetitive responding. Therefore, it would not be ideally suited as index of human appetitive conditioning. Further investigations are thus needed in order to validate the role of startle eyeblink reflex as index of appetitive conditioning.

A final issue regarding the eyeblink reflex findings concerns the quantification method of the startle eyeblink reflex modulation. We here decided to analyze the PAR and the startle eyeblink reflex
in the same way (i.e., by using signal averaging rather than analyzing trial-by-trial), in order to compare these two measures in a more straightforward way. This choice of analysis was made to avoid the problem of low signal-to-noise ratio usual for the PAR. However, as explained by Grillon and Baas (2003) the quantification method can largely influence the outcome of a study and the current methods of preference are percentage scores and within-subject Z- or T-standardizations of raw scores. Indeed, Andreatta and Pauli (2015) used within-subject T-standardizations of the raw data, which may have accounted for their startle eyeblink reflexes significant results. However, to rule out this possibility, we also performed the same kind of analysis as Andreatta and Pauli (2015) and we did not find any statistically significant differences.

With regard to the SCR, by being a measure of arousal, it might be particularly sensitive to the US intensity. Taken that appetitive conditioning is a very repetitive paradigm and that SCR is subject to habituation, it is possible that SCR probably fails to systematically detect slight changes induced by appetitive conditioning. In addition, an odor used as US could represent an insufficiently strong activator of arousal in comparison to other stimuli such as food, which would have probably led to different results in SCR. Unfortunately, we did not collect arousal ratings of the US, therefore we do not have the means to verify this speculation.

Taken together, our results reveal that human appetitive conditioning may be reliably measured with the postauricular reflex, which was specifically potentiated in response to an appetitive conditioned stimulus. We also showed that the postauricular reflex may represent a more suitable and sensitive psychophysiological indicator than the startle eyeblink reflex and skin conductance response.

Nevertheless, we should acknowledge some limitations of our study. As already mentioned, we did not test for the arousal level of the US but only for the valence and the intensity level, which in our case was referring to the perceptual odor intensity. Therefore, we could not demonstrate that the PAR modulation does not depend on the arousal level of the conditioned stimulus but on the current or predictive reward value of the CS+ as in our opinion. Future studies will thus have to clarify the effect of arousal on the PAR modulation during human appetitive conditioning. Moreover, another limitation of our study was that we were not able to determine whether acquisition and extinction of the postauricular reflex potentiation to the CS+ happened right at the beginning of habituation and extinction phases or more progressively during these two phases, since we used a signal averaging and not a trial-by-trial analysis, due to the low signal-to-noise ratio of the PAR.

Possible directions for future studies on this subject may include the use of the PAR for assessing and disentangling the distinct components wanting, liking, and reward learning of the reward system (Berridge & Robinson, 2003; Pool et al., 2016) applying however an appropriate
concept operationalization that takes into account the relevance of the affective stimulus for the organism’s ongoing concerns and potential confounds such as the expected pleasantness (Pool et al., 2016). It would be interesting to investigate the above mentioned topic in order to understand the specificity of the PAR modulation to any of these psychological components and to further be able to study pathological systems in which wanting and liking dissociate, such as pathological gambling, addiction or overeating (see, e.g. Pool et al., 2016).

Moreover, as Benning and colleagues (2004) suggested, it would be interesting to assess the specificity of the postauricular reflex modulation during an emotional state where liking and wanting components act in an opposite way, as in a negatively valent approach state (e.g., anger; Harmon-Jones & Sigelman, 2001).

Furthermore, because of its different potential clinical applications, it would be of major interest to assess the postauricular reflex use on clinical populations, in order to increase the understanding of these disorders and ultimately improve the therapy and outcome (as for instance, the study of Dichter et al. (2010) on autism spectrum disorder). Indeed, if the utility of the postauricular reflex as investigative tool of human appetitive conditioning would be confirmed, it may be conveyed into clinical practice.

Grillon and Baas (2003) previously illustrated the great potential of the startle eyeblink reflex as diagnostic tool for the understanding of the psychopathology of mood and emotion disorders, the therapy prognosis and outcome, the treatment evaluation, the screening of at risk population, the identification of risk-markers as well as for the screening of various drugs such as potential anxiolytic compounds or antidepressants. Indeed, since the startle eyeblink reflexes is sensitive to emotional states, abnormalities in its modulation to the presentation of emotionally salient stimuli may relate to disturbances in the underlying motivational states (Grillon & Baas, 2003). That is why using the acoustic startle probe methodology may help assessing the emotional reactivity related to the etiology and maintenance of different psychopathologies (Grillon & Baas, 2003). The startle eyeblink reflex may be used to investigate deviant aversive states (e.g., anxiety disorders, depression and psychopathy), to study the impact of trauma, and to assess the abnormal learning, expression and regulation of affect characterized by these disorders (Grillon & Baas, 2003). In addition, startle eyeblink reflex may have a prognostic potential as for example in the case of posttraumatic stress disorders (Grillon & Baas, 2003). Moreover, startle eyeblink reflex could be a useful tool for the assessment of treatment outcome. On one hand, pharmacological and behavioral treatments should bring the startle eyeblink modulation back to normality, on the other hand startle eyeblink reflex could help identify subjects who are more likely to respond to a given treatment (e.g., the presence of a high startle reactivity in smokers prior to quitting together with a reduction in startle eyeblink reflex during
the abstinence state predicted smokers’ ability to quit) (Grillon & Baas, 2003). The screening of at risk population for several psychopathologies or the identification of risk-markers for subsequent development of the disorder is another potential use of the startle eyeblink reflex, which could be done by detecting associations between negative emotional traits and eyeblink startle modulation or by evaluating family history to identify premorbid risk and protective factors, as well as early signs of expression of these disorders (e.g., affective modulation of eyeblink startle may discriminate between individuals at low and high risk for alcoholism or substance abuse) (Grillon & Baas, 2003). Finally, areas where eyeblink startle could make great contribution include the study of motivational factors (e.g., drug addiction) and psychopharmacology (e.g., anxiolytics) because these studies could rely on solid parallel preclinical research, due to the cross-species application of the acoustic startle probe methodology (Grillon & Baas, 2003).

Similarly, as the PAR is sensitive to emotional states like the startle eyeblink reflex, abnormalities in its modulation may relate to disturbances in the underlying motivational states as well. The PAR may therefore own a similar clinical potential and involve analogous applications. Especially in the context of appetitive conditioning, related pathologies such as addiction, eating-disorders and depression for instance, might take advantage of the PAR reflex. The PAR could hence provide useful tools for the identification of context conditioned cues and for their correct reconditioning during treatment. Moreover, the PAR could be used in order to assess normal or pathological emotional learning.

To the best of our knowledge, we investigated for the first time the use of the postauricular reflex as learning index of human Pavlovian appetitive conditioning. With this study, we therefore made a contribution to the study of emotional learning, opening the door for new avenues of research on appetitive conditioning in humans.
CONCLUSION

To conclude, we investigated the modulation of the postauricular and startle eyeblink reflexes during a Pavlovian differential appetitive conditioning procedure with the purpose of assessing their role as psychophysiological measures of human appetitive conditioning. We showed that the postauricular reflex possibly is a useful and more sensitive psychophysiological index of human appetitive conditioning than the startle eyeblink reflex and skin conductance response. The reliability of this psychophysiological measure lies in its sensitivity to the appetitive contingencies, which suggests that the modulation of the PAR depends on the reward value of the conditioned stimulus.

Our findings hence indicate that the postauricular reflex may represent a suitable measure of human appetitive conditioning and that it might constitute a valuable tool for further investigating the basic mechanisms underlying appetitive conditioning in humans, as well as their dysfunctions in related pathologies, such as depression, addiction and eating disorders.
REFERENCES


