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Yacine Tensaouti. Contribution of the rat insular cortex to stimulus-guided action. Neurobiology. Université de Bordeaux, 2024. English. NNT: . tel-04696586v1

HAL Id: tel-04696586

<https://hal.science/tel-04696586v1>

Submitted on 13 Sep 2024 (v1), last revised 18 Nov 2024 (v2)

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THESE PRESENTEE
POUR OBTENIR LE GRADE DE
DOCTEUR DE
L'UNIVERSITE DE BORDEAUX

ECOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTE
SPECIALITE NEUROSCIENCES

Par **YACINE TENSAOUTI**

Contribution du cortex insulaire dans les actions guidées
par stimulus chez le rat

Contribution of the rat insular cortex to stimulus-guided action

Sous la direction de : **Shauna PARKES** (CR, CNRS)

Soutenue le **17 Octobre 2024**

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ACKNOWLEDGEMENTS

Je tiens tout d'abord à exprimer ma profonde gratitude aux membres du jury pour avoir accepté d'évaluer mon travail de thèse. Je remercie sincèrement Claire Rampon, Sébastien Carnicella et Fabien Naneix pour le temps précieux que vous avez consacré à la lecture de ce manuscrit et pour votre participation à ma soutenance.

Je souhaite également remercier chaleureusement les membres de mon comité de mi-thèse. Merci à Anna Beyeler, qui a également été ma tutrice et qui s'est toujours montrée disponible pour échanger tout au long de ce parcours. Je remercie également Pierre Trifilieff, Nicolas Mallet et Kevin Guilhem pour les discussions enrichissantes et leurs précieux conseils qui ont grandement contribué à l'évolution de ce projet.

Je tiens également à remercier Aline Desmedt qui m'a donné l'opportunité de donner un cours magistral de deuxième année de master en neurosciences. Merci de m'avoir accordé votre confiance, ce fut une expérience extrêmement stimulante et enrichissante.

Je tiens également à remercier Olivier Raineteau, qui a été le premier à m'accueillir dans un laboratoire, ainsi que Steven Kernie qui m'a donné l'opportunité d'acquérir de nombreuses compétences et m'a accordé sa confiance pendant de longues années.

Enfin, j'aimerais exprimer ma reconnaissance à Stéphanie Trouche pour avoir rendu possible ma rencontre avec Shauna Parkes, qui m'a offert l'exceptionnelle opportunité de réaliser un doctorat sous sa supervision, en croyant en mon potentiel malgré mon manque d'expérience en neurosciences comportementales.

Bien sûr, j'aimerais remercier Shauna Parkes, sans qui cette thèse ne serait pas ce qu'elle est aujourd'hui. En effet, Shauna a été *instrumentale* à la réussite de ce projet.

Shauna, working under your supervision has been an immense pleasure. You inspired in me a passion for experimental psychology and behavioral neuroscience, and through your mentorship, I developed a genuine interest in this field of research. You have not only imparted invaluable technical and theoretical skills but you have also been a role model of integrity and scientific rigor. You created an environment where I felt supported and challenged in equal measure. Your high expectations pushed me to bring out the best in myself throughout my PhD, all while you offered thoughtful guidance whenever I needed it. Thank you for always being available and approachable- your flexibility and patience allowed me to explore my ideas with autonomy and confidence, as you trusted me to manage my time and work as I saw fit. Your unwavering support and “bienveillance” for your students is truly admirable. I sincerely appreciate all the time you spent performing surgeries with me, as well as your sense of humor which always lightened the atmosphere. I will miss our impromptu meetings that often turned into long, intellectually stimulating discussions. Thank you for the encouragement you provided that helped me increase confidence in my scientific abilities. These four years have been incredibly rewarding, and this work would not have been possible without your leadership. I feel so fortunate to have had the opportunity to grow as a researcher under your wing. I wish you the very best in both your professional career and personal life, and I have no doubt that you will achieve whatever you set your mind to. Your dedication, passion, and commitment to science will undoubtedly pave the way for continued success.

Thank you, Shauna, for everything.

Je tiens à exprimer ma profonde gratitude à toute l'équipe DECAD qui m'a accueillie avec tant de bienveillance et de gentillesse. Vos qualités humaines et intellectuelles ont fait de cet environnement un lieu idéal pour s'épanouir professionnellement et personnellement – on se sent vraiment chouchouté dans cette équipe. Merci Mathieu Wolff d'avoir gardé ta porte toujours ouverte, que ce soit pour discuter science ou carrière professionnelle. Tu t'assures constamment que les membres de l'équipe puissent travailler dans les meilleures conditions possibles, et tu n'hésites jamais à défendre nos intérêts. Ton expertise sur le thalamus a été d'une aide précieuse pour mes travaux, et c'était un réel plaisir de travailler dans une équipe dirigée par quelqu'un comme toi.

Merci Etienne Coutureau pour ta bienveillance. Tu as toujours su apporter une touche d'humour ou un mot encourageant, et c'est toujours un plaisir d'être en ta compagnie. Merci aussi pour tous les chocolats que tu as apportés dans notre bureau, de véritables boosts glycémiques pour mes neurones fatigués en période de rédaction ! Au-delà de tes qualités humaines, ta grande expertise en comportement a rendu ces quatre années passées à tes côtés d'autant plus enrichissantes. Merci d'avoir toujours gardé ta porte ouverte pour discuter, et tu t'es toujours assuré que nous ne manquions de rien au labo.

"Je ne joue pas aux jeux vidéo, je ne veux pas d'un joystick sur le microscope" – et pourtant, merci Catherine Le Moine d'avoir finalement cédé à ma demande de modifier la configuration du microscope pour automatiser la prise d'images ! Cela m'a grandement aidé et m'a fait gagner un temps précieux. Merci aussi pour tes conseils et ton expertise en histologie, ainsi que tes retours et encouragements. Je suis ravi d'avoir fait ta rencontre et j'ai apprécié chaque moment, tant scientifique que social, que nous avons eu la chance de partager au cours de ces dernières années.

Merci également à Nicole Mons pour ton expertise notamment en histologie ainsi que pour ta gentillesse. Nous n'avons malheureusement pas eu l'occasion de passer beaucoup de temps ensemble, mais notre installation dans le BBS nous aura au moins permis de nous côtoyer pendant les derniers mois de ma thèse.

Merci à Martine Cador et à Stéphanie Caillé-Garnier (le S), avec qui j'ai partagé le quotidien à Charles Perrens. Votre bonne humeur constante a toujours rendu nos moments ensemble particulièrement agréables, et j'ai adoré nos discussions autour d'un verre où nous refaisions le monde. L'époque Perrens restera gravée dans ma mémoire, et c'est en grande partie grâce à vous.

Merci à Yoan Salafranque pour t'être occupé avec soin de la commande, de la réception et du maintien de mes animaux durant ces quatre années. Tu t'es toujours montré flexible et professionnel. Merci aussi pour tous les moments partagés, les discussions intéressantes et parfois cocasses que nous avons eues. Ce fut un réel plaisir de travailler à tes côtés, d'échanger à chaque rencontre et de plaisanter autour d'un verre chaque fois que l'occasion se présentait. Je suis très heureux d'avoir fait ta connaissance et je ne doute pas que nous resterons en contact.

Je tiens à remercier également Angélique Faugère pour l'aide que tu m'as apporté tout au long de ma thèse. Tu as toujours su trouver le temps de m'épauler, et adhérer à ma requête de faire de la DAB et des perfusions le Vendredi au risque de retarder l'arrivée du weekend. Merci aussi pour les moments partagés en dehors de la salle d'histologie, nos discussions durant les pauses, ainsi que pour ta sensibilité constante à ma situation capillaire.

Je tiens à remercier également Marie-Line Fournier et Florence Darlot (le F), merci pour votre contribution essentielle au bon fonctionnement du labo, tant humain que scientifique. J'ai apprécié le peu de fois où nous avons collaboré, ainsi que tous les moments conviviaux passés ensemble, surtout durant la grande époque Perrens. Votre bonne humeur et votre sens de l'humour ont été rafraichissants, et je me considère chanceux d'avoir bénéficié de votre compagnie. Tu ne me verras plus au CIRCE avec ma caquette le F, et tes blagues vont me manquer !

Je remercie également l'ensemble des chercheurs et techniciens de l'INClA que j'ai eu l'opportunité de rencontrer suite au déménagement au BBS, lesquels se sont toujours montrés courtois, et avec lesquels j'ai pu développer certaines affinités.

Ces quatre années de thèse n'auraient pas été si facilement supportable sans le soutien constant de mon ami Alessandro. Alé et moi sommes arrivés à peu près en même temps dans l'équipe DECAD, et nous en prenons congés relativement au même moment. Nous nous sommes installés dans le même bureau, et nous avons rapidement développer une forte complicité. Quelques mois ont passé, et Hadrien nous a rejoint, faisant souffler un vent de jeunesse dans le bureau de trentenaire. Ce fut alors le début d'une dynamique en trio, et je ne peux compter les fous-rires et délires. Nous fûmes bientôt rejoints par Lachlan, qui a su apporter une touche rafraichissante d'humour et de cocasserie. Enfin, Florence Pontais rejoint l'équipe, faisant de la dream-team ce qu'elle est aujourd'hui. Merci à vous quatre pour tous ces moments conviviaux passés ensemble (aux différents QG notamment), les conversations scientifiques, la constante entraide mutuelle, vos mots doux, encourageants, réconfortants, et surtout vos bêtises. Vous allez me manquer, mais je suis sûr qu'on se reverra bientôt ! Où ? J'ai ma petite idée...

Je tiens aussi à exprimer ma profonde gratitude à Louis, qui a participé activement à la réussite de ce projet, grâce à son implication en comportement et histologie notamment. Tu t'es toujours montré prêt à m'aider et à me soulager de certaines tâches pour faire avancer notre projet, je suis extrêmement reconnaissant pour tous les efforts que tu as fournis. Ce fut un plaisir de travailler en ta compagnie, tant sur le plan humain que scientifique, et je n'ai aucun doute que tu arriveras à mener ton projet à bien. Tu dois être soulagé de mon départ et de ne plus entendre parler de PIT ou d'insula ! Je compte sur toi pour me tenir au courant de l'avancée de tes expériences.

Je tiens à remercier également tous les autres collègues avec qui j'ai pu partager d'agréables moments. Je pense d'abord aux autres membres de l'équipe DECAD, Juan Ma, Emma, Mélie et Louise, ainsi qu'aux anciens doctorants, Robin et Sarah. J'ai apprécié ta gentillesse et ton humour Juan Ma, et je te souhaite bien du courage Louise pour tes expériences de PIT ! Je remercie également Mari Carmen et Nuria pour leur gentillesse et leurs encouragements, c'est toujours un immense plaisir que de vous croiser. Je remercie également Alice, Kevin, Anna-Chiara, Renan et Claudia pour les quelques moments conviviaux récemment partagés au BBS. Merci également à Léandre et Maëlle d'avoir participé à rendre la vie au laboratoire plus agréable et conviviale.

Je tiens à remercier tous mes frères d'une autre mère, qui n'ont jamais cessé de me démontrer leur soutien malgré mon indisponibilité constante. Merci à Adam, Alex, Anisse, Ben, Bleu, David, Vins, Lomb, Mathieu (x2), Mooss, Stan, Tib, Tom, Wal, j'espère qu'on aura bientôt le temps de rattraper un peu du temps perdu !

Merci également à mon petit frère Yani, ainsi que mes deux petites sœurs Dia et Inès. Merci pour votre soutien indéfectible et pour les moments de complicité. J'espère vous rendre fière autant que vous me rendez fière. Je regrette que nous n'ayons pas eu l'occasion de passer autant de temps ensemble que nous l'aurions voulu durant ces dernières années, mais je sais qu'on n'y manquera pas d'y remédier prochainement.

Merci à ma femme Lorena, qui a, sans aucun doute, vécu ces années de doctorat au moins autant que moi. Tu as toujours été à mes côtés, prête à faire des sacrifices, même lorsque cela signifiait mettre mon bien-être avant le tien. Je sais combien ces dernières années ont été exigeantes pour toi, et je te suis infiniment reconnaissant pour ton soutien indéfectible, ta patience et ta compréhension, même dans les moments les plus difficiles. Ta présence a été une ancre, ta force une source d'inspiration constante, et ton amour un moteur essentiel pour moi. Merci d'avoir cru en moi, même quand je doutais, et d'avoir illuminé ce chemin parfois ardu avec ta lumière et ton sourire. Je n'aurais jamais pu accomplir ce travail sans toi, et je suis éternellement reconnaissant de t'avoir à mes côtés.

Enfin, je tiens à remercier mes parents, sans qui je ne serais pas là aujourd'hui. Votre amour et votre soutien inconditionnels m'ont permis de poursuivre et d'atteindre mes objectifs. Vous avez toujours été un modèle d'exemplarité, tant par votre humilité que par votre travail acharné. Les mots me manquent pour exprimer toute la gratitude que je ressens à votre égard. Je vous serai éternellement reconnaissant pour tous les sacrifices que vous avez consentis afin d'assurer la réussite de vos enfants. J'espère de tout cœur que nous vous rendons fiers et que nous aurons encore de nombreuses années à partager ensemble.

A mes oncles et tantes, à mes cousins et cousines, à toute ma grande famille.

ABSTRACT (ENGLISH)

Every day, individuals are faced with numerous decisions that shape their behavior. The factors influencing these choices are multifaceted and encompass a range of considerations. Immediate needs and desires often play a significant role in action selection, guided by the value of the outcome. However, it is crucial to recognize the impact of environmental stimuli. For instance, stimuli associated with food can not only direct us toward nourishment but also trigger cravings, even in the absence of hunger. To uncover the role of the rat insular cortex (IC) in stimulus-guided actions directed towards obtaining food outcomes, we used the Pavlovian-to-Instrumental Transfer (PIT) paradigm. Given the well-established role of IC in encoding and tracking general and specific outcome-expectancies, and its critical contribution in choice guided by specific-outcome values, we hypothesized a role of the IC during the PIT transfer test where actions are influenced by reward-predictive stimuli. Using chemogenetics, we demonstrated that IC inhibition during both general and specific transfer tests abolished the ability of Pavlovian reward-predictive stimuli to energize instrumental responding, and to specifically bias action selection towards the same outcome as the presented predictive stimulus, respectively. These results demonstrated for the first time the critical role of the IC in stimulus-guided choice, encompassing both the general motivational properties acquired by Pavlovian stimuli and their ability to specifically bias action selection towards specific outcomes. Moreover, preliminary results suggest that the latter may critically depend on an intact cortico-thalamic pathway involving the mediodorsal part of the thalamus. In conclusion, we provide the first evidence that the GC is required for both general and specific forms of PIT, with the latter depending on an intact cortico-thalamic pathway.

ABSTRACT (FRENCH)

Chaque jour, nous sommes confrontés à de nombreuses décisions qui façonnent nos comportements. Les facteurs influençant ces choix sont multiples. Les besoins et désirs immédiats jouent souvent un rôle important dans la sélection des actions, guidés par la valeur du but. Cependant, il est essentiel de reconnaître l'impact des stimuli environnementaux. Par exemple, les stimuli alimentaires peuvent non seulement nous orienter vers la nourriture, mais aussi déclencher des envies, même en l'absence de faim. Afin d'identifier le rôle du cortex insulaire (CI) du rat dans les actions guidées par des stimuli et visant à obtenir des aliments, nous avons utilisé le paradigme du transfert Pavlovien-Instrumental (PIT). Étant donné le rôle bien établi du CI dans l'encodage et la surveillance des attentes générales et spécifiques, et sa contribution critique dans le choix guidé par la valeur spécifique d'un but, nous avons émis l'hypothèse d'un rôle du CI pendant le test de transfert PIT où les actions sont influencées par des stimuli prédictifs de récompense. Grâce à une approche chimiogénétique, nous avons démontré que l'inhibition du CI pendant les tests de transfert généraux et spécifiques abolissait la capacité des stimuli prédictifs de récompense pavloviens à stimuler la réponse instrumentale et à orienter spécifiquement le choix de l'action vers la même récompense que le stimulus prédictif présenté, respectivement. Ces résultats démontrent pour la première fois le rôle critique du CI dans le choix guidé par stimulus, englobant à la fois les propriétés motivationnelles générales acquises par les stimuli pavloviens et leur capacité à orienter spécifiquement la sélection de l'action vers des résultats spécifiques. De plus, nos résultats préliminaires suggèrent que cette dernière peut dépendre de façon critique d'une voie cortico-thalamique intacte impliquant la partie médiodorsale du thalamus.

PUBLICATIONS AND COMMUNICATIONS

This thesis was financed by the French National Research Agency (Cocochoice ANR-19-CE37-0004-07). Chapter II is currently under review.

Publication list:

1. Yu, TS., **Tensaouti, Y.**, Stephanz, E.P., Chintamen, S, Elizabeth, E. Rafikian, E.E., Yang, M. & Kernie, S.G. (2021) Astrocytic ApoE underlies maturation of hippocampal neurons and cognitive recovery after traumatic brain injury in mice. *Communications Biology*. 4, 1303.
2. **Tensaouti Y**, Yu TS, Kernie SG (2020) Apolipoprotein E regulates the maturation of injury-induced adult-born hippocampal neurons following traumatic brain injury. *PLOS ONE* 15(3): e0229240. 8
3. **Tensaouti Y**, Stephanz EP, Yu TZ, and Kernie SG (2018). ApoE regulates the development of adult newborn hippocampal neurons. *eNeuro*. July/August, 5(4) e0155-18.2018 1-15. Accompanied by press release from the Society of Neuroscience.
4. Yu TS, **Tensaouti Y**, Bagha ZM, Davidson R, Kim A, and Kernie SG. (2017) Adult newborn neurons interfere with fear discrimination in a protocol-dependent manner. *Brain and Behavior*. Aug 2;7(9): e00796. PMID 28948089.

In submission

1. **Tensaouti Y**, Morel L, and Parkes SL. Contribution of rat insular cortex to stimulus-guided action (2024). *Cerebral Cortex*.

Scientific talks

1. 2022: Symposium speaker at The Animal Cognition Research Center (CRCA), Toulouse, FRANCE. Insular cortex is required to guide action selection in outcome-specific devaluation and Pavlovian-to-Instrumental Transfer.

Poster presentations:

1. **FENS**, Paris, France, July 9-13, 2022.
2. **NeuroCompare**, Bordeaux, France, September 26-28, 2023.
3. **SFN**, Washington DC, Etats-Unis, November 11-15, 2023.

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2021 – 2024: Student representative to the INCIA Unit Council (Conseil de Labo).

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Mentoring:

2023: Louis Morel, intern (M2 student) - INCIA, University of Bordeaux, Bordeaux, FRANCE.

Scholarships:

2023: GPR Brain 2030 PhD extension grant – 6 months.

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ABBREVIATIONS

Af: Affective	MDc: Central part of the MD
ACC: Anterior cingulate cortex	MDD: Major depressive disorder
AI: Agranular insular cortex	MDI: Lateral part of the MD
A-O: Action – Outcome association	MDm: Medial part of the MD
AUD: Alcohol use disorder	MO: Medial orbitofrontal cortex
BLA: Basolateral amygdala	mPFC: Medial prefrontal cortex
CEA: Central nucleus of the amygdala	NAc: Nucleus accumbens
CR: Conditioned response	OFC: Orbitofrontal cortex
CS: Conditioned stimulus	PBN: Parabrachial nucleus
CTA: Conditioned taste aversion	PIT: Pavlovian-to-instrumental transfer
DI: Dysgranular insular cortex	PL: Prelimbic cortex
DLS: Dorsolateral striatum	PrCm: Medial precentral cortex
DMS: Dorsomedial striatum	Rew: Reward
Em: Emotional	Rnf: Reinforcement
fMRI: Functional magnetic resonance imaging	RR: Random ratio
GC: Gustatory cortex	S-A: Stimulus – Action association
GI: Granular insular cortex	SAP: Social affective preference
IC: Insular cortex	SNc: Substantia nigra pars compacta
IL: Infralimbic cortex	SNPc: Substantia nigra pars compacta
LO: Lateral orbitofrontal cortex	SNr: Substantia nigra pars reticula
mAG: Medial agranular area	S-O: Stimulus – Outcome association
MD: Mediodorsal thalamus	S-R: Stimulus – Response association
	SZ: Schizophrenic patients

UR: Unconditioned response

US: Unconditioned stimulus

VA: Ventral anterior thalamus

vIOFC: Ventrolateral orbitofrontal cortex

VO: Ventral orbitofrontal cortex

VPm: Medial ventral pallidum

VTA: Ventral tegmental area

PREFACE

The ability to accurately anticipate rewards in our environment is fundamental for adaptive decision-making. While our immediate needs and desires often play a significant role in action selection, it is crucial to recognize the impact of environmental cues. Indeed, we often rely on external stimuli to infer the availability of rewards and make predictions about future outcomes. These predictions are based on learned associations, formed through experiences that guide our behavior and enable us to make advantageous choices. For example, encountering a fast-food advertisement when you're hungry can lead you to visit the nearest restaurant of the chain to enjoy a burger, or more generally, trigger a craving for food. Similarly, the honk of a car horn while you're crossing the street, distracted by your smartphone, can make you freeze and avoid an accident. These scenarios illustrate how we learn that specific cues or stimuli in our environment precede certain events and guide us in deciding which actions to take. However, these stimuli can also lead us to pursue goals that are detrimental for our health. For instance, cue-triggered motivation for food, drugs, or gambling may induce cravings and relapses in individuals battling addiction. Therefore, understanding how environmental stimuli interact with action selection is critical in unraveling the mechanisms behind flexible and adaptive decision-making that promote health and survival, as well as the development of maladaptive behaviors.

CHAPTER I

GENERAL INTRODUCTION

1. ASSOCIATIVE LEARNING UNDERPINS COMPLEX BEHAVIOR

The brain is a remarkable organ, constantly processing a vast array of information from our surroundings and experiences. At the core of our cognitive processes lie the formation of associations, whereby connections between stimuli, actions, and outcomes are forged. These associations serve as the building blocks of our understanding of the world, allowing us to predict and control events in our environment (Heyes, 2012). Pioneer work by Pavlov (Pavlov, 1927) and Thorndike (Thorndike, 1898) laid the groundwork for our understanding of how associations shape behavior and cognition. Through the complex network of associations within our minds, we are not only able to anticipate future events but also to exert control over our surroundings. Instrumental behavior, guided by the value of outcomes, is a manifestation of this control, as individuals learn to perform actions that lead to desirable outcomes (or to the avoidance of aversive outcomes). Moreover, instrumental processes can interact with Pavlovian stimuli that predict the availability of these outcomes, shaping what is known as stimulus-guided behavior. The mechanisms underlying associative learning are not exclusive to humans but are conserved across species (Macphail, 1982; Pearce, 1997). The formation of associations thus emerges as a fundamental aspect of cognition, shaping our perceptions, behaviors, and ultimately, our survival.

1.1. Pavlovian predictions: Anticipating future events

Our ability to infer the availability of rewards in our environment can stem from environmental cues or stimuli that reliably predict those rewards, a phenomenon commonly referred to as Pavlovian or classical conditioning (Pavlov, 1927). In classical conditioning, a stimulus (conditioned stimulus; CS) becomes linked with either an innately

rewarding or aversive outcome (unconditioned stimulus; US), and a CS-US association is formed. These associations are contingent upon the conditional relationship between the CS and US, and will shape subsequent behavioral responses (conditioned response, CR) such as approach or avoidance (Fanselow & Wassum, 2016; Rescorla, 1968). Once a CS becomes associated with a US, the CS alone can elicit a CR similar to, or even distinct from, the innate response originally elicited by the US, known as the unconditioned response (UR).

This phenomenon was illustrated in Pavlov's classical experiment with dogs (**Figure 1**). Initially, the dogs naturally salivated (UR) upon encountering an ecologically relevant stimulus, such as food (US). Through repeated pairings of food delivery with a previously neutral stimulus, such as the sound of a metronome (CS), the dogs learned to associate the clicking metronome with the impending delivery of food. Subsequently, the mere presentation of the metronome (CS) alone was sufficient to induce salivation (CR), demonstrating that the CS had acquired predictive value. This anticipatory response enabled the animals to prepare physiologically for the forthcoming reward delivery, showcasing the power of associative learning in shaping behavior.

In Pavlov's experiment, was the dog salivating to the metronome? At first glance, it may seem so. However, further investigation uncovered a more nuanced understanding. It appears that the metronome (CS) was not directly eliciting salivation (CR). Instead, it was serving as a stimulus that prompted the mental representation of the food (US), which in turn triggered salivation. Essentially, the metronome was facilitating a CS-US association, an association between the stimulus and its outcome (S-O) rather than a direct stimulus-response one (S-R).

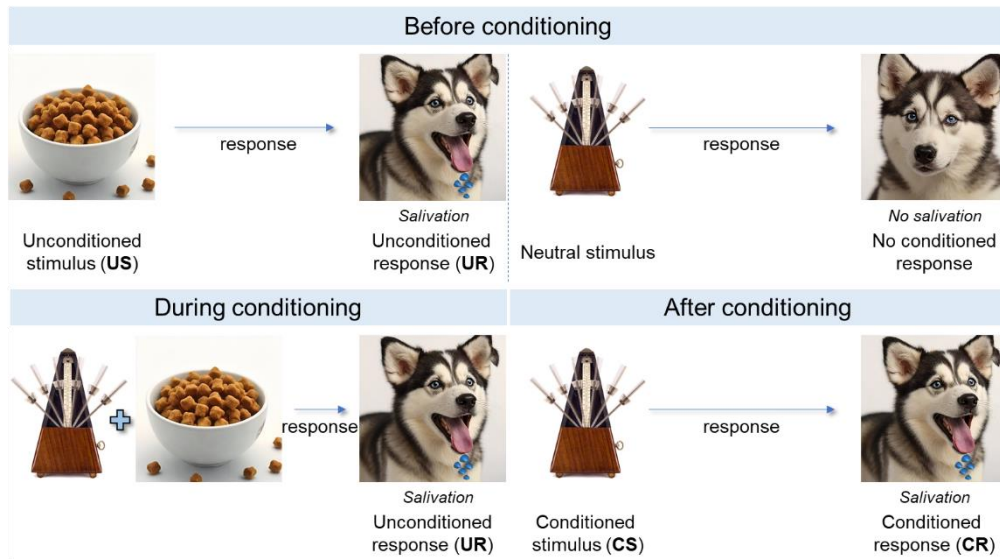


Figure 1. Classical conditioning: Pavlov's dog. Before conditioning, the unconditioned stimulus (US), food, induces an unconditioned response (UR) of salivation in the dog, while the neutral stimulus, a ticking metronome, induces no response. During conditioning, the ticking metronome is repeatedly paired with food delivery, inducing the salivation response. After conditioning, the ticking metronome alone, now the conditioned stimulus (CS), is sufficient to induce the conditioned response (CR) of salivation.

Empirical evidence such as the sensory-preconditioning task strengthen the idea that Pavlovian learning is primarily governed by associations between stimuli and outcomes (Rizley & Rescorla, 1972). In this task, two initially neutral stimuli S1 and S2, such as a light and a tone, are initially paired together. Subsequently, one of these stimuli, S2 (e.g., the tone), is paired with a US, such as food reward, eliciting a CR, like approaching the food port. In a third phase, when the other stimulus (e.g., the light) is presented alone, it proves sufficient to elicit the food port approach despite never having been directly paired with the food reward. The S-R account alone cannot explain these results, as it would predict a CR only when the tone is presented, not the light. Instead, the observed response suggests that the subject has formed an internal representation of the S1-S2-US chain, which in turn motivates the CR. Accordingly, it's proposed that Pavlovian conditioning forms S-O associations, linking the US with the CS with which it was paired.

This implies that Pavlovian CRs arise from a cognitive expectation of the anticipated US rather than automatic responses ([Bolles, 1972](#); [Fanselow & Wassum, 2016](#)).

Further empirical evidence supports the notion that Pavlovian learning relies upon the specific sensory properties of the US, encoded and retrieved through S-O associations. For example, post-conditioning devaluation of a US through taste aversion or satiety protocols can diminish both food port approach and food consumption triggered by the associated CS ([Holland & Straub, 1979](#); [Holland & Rescorla, 1975](#)). This suggests that the subject possesses an internal representation linking the CS and its associated outcome, the value of which is retrieved to guide the CR.

Moreover, an initially aversive CS can become desired upon reencounter in a different physiological state, a phenomenon proposed to play a role in addiction pathogenesis ([Robinson & Berridge, 2013](#)). In the "deadly salt" experiment, rats were trained to develop an aversion to an extremely salty solution, akin to the salinity of the Dead Sea (three times saltier than sea water). Cannulas were used for oral delivery of either sucrose or the highly concentrated NaCl solution. The rats were conditioned to associate a distinct Pavlovian stimulus (CS_{Salt}) with the salty solution, signaled by the sudden extension of a lever accompanied by a tone. Another cue (CS_{Sucrose}) predicted the infusion of palatable sucrose, signaled by the insertion of a different lever located on the opposite wall, accompanied with a white noise. Notably, interactions with the levers had no effect; they were simply part of the Pavlovian stimuli indicating the imminent passive delivery of one of the two solutions. Sucrose reliably elicited positive hedonic reactions, while rats avoided the salt lever, displaying retreat behaviors. After training, rats were injected with compounds mimicking sodium deficiency, inducing a strong salt appetite. Upon re-encountering CS_{Salt} without actual salt delivery, rats exhibited approach behaviors, such

as grasping, sniffing, and nibbling the lever, indicating a sudden attraction to the previously aversive stimulus. This persisted even when rats received the intensely salty solution in their sodium-depleted state, suggesting a persistent “liking” of the previously aversive stimulus.

This sudden shift in behavior suggests that CS_{Salt} became a potent "motivational magnet," even though the salty solution was previously perceived as disgusting. However, once returned to normal sodium levels, the value of CS_{Salt} decreased to pre-conditioning levels, indicating conditioned alliesthesia. This suggests that the behavior was driven by an S-O (i.e., CS-US) association rather than a simple S-R automatism, as the response varied based on the rats' physiological state. But under which conditions do these S-O associations develop?

The establishment of S-O associations was originally conceptualized in the framework of “contiguity theory”, emphasizing the crucial role of temporal contiguity between the CS and the US (Guthrie, 1930; Kimble, 1947). However, subsequent research revealed that while contiguity is important, it alone is insufficient in driving conditioned responses; instead, the concept of contingency emerged as pivotal (Kamin, 1967; Rescorla, 1968). These later investigations demonstrated that degrading the contingency between CS presentations and US delivery significantly decreased the strength of conditioning. Further analyses revealed that the degree of conditioning was positively correlated with the probability of the US occurring during CS presentation and negatively correlated with the probability of US occurrence in the absence of the CS. A positive contingency, coupled with a low probability of US in the absence of the CS, enhances the strength of the conditioned response upon CS presentation, as the CS becomes a reliable predictor of the US. Conversely, when US delivery occurs exclusively in the absence of the CS

(negative contingency), the CS becomes a strong conditioned inhibitor of the organism's response, as the CS reliably predicts the absence of US.

In conclusion, the examination of Pavlovian predictions provides insight into the intricate processes of associative learning. The evidence underscores the development of detailed outcome representations within associative memory (**Figure 2**). Emphasizing the pivotal role of S-O associations, these findings reveal the complexity of Pavlovian learning, where cognitive expectations of outcomes shape behavioral responses. In the next section we will explore instrumental behavior, which allows animals to not simply predict events in their environment but to exert control over that environment in the service of their needs and desires.

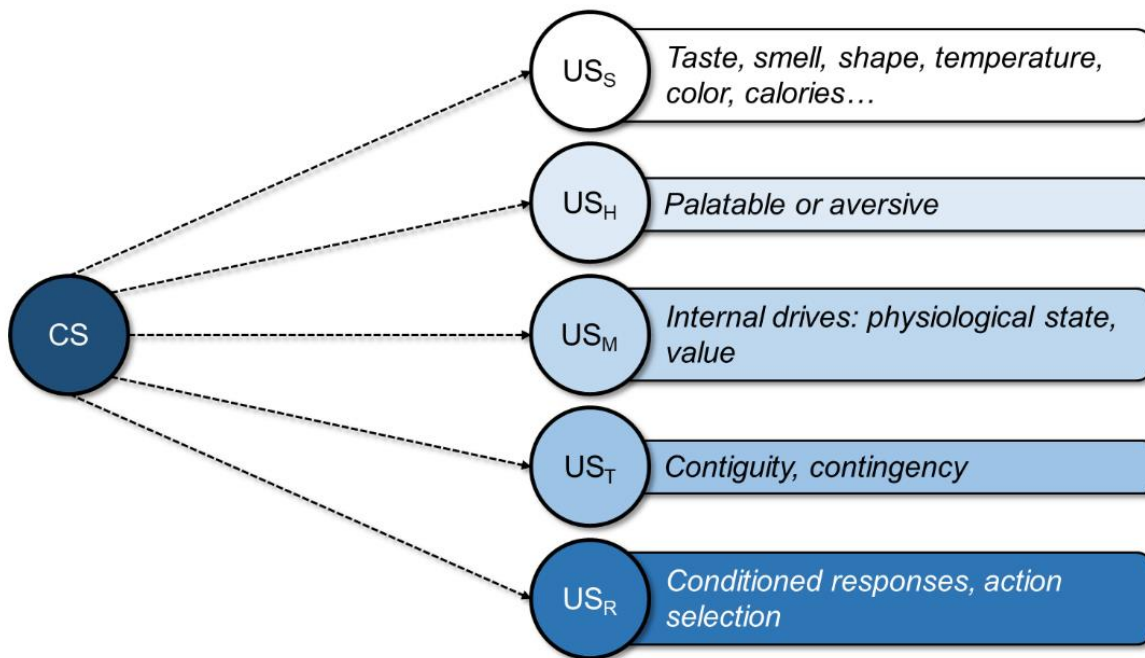


Figure 2. Possible components of Pavlovian US representations, and possible associative links between the CS and those US components. The CS may form associations with different reinforcer attributes such as its specific sensory-properties (US_S), its hedonic features (US_H), its general motivational properties (US_M), its temporal occurrence (US_T) and its specific response-eliciting characteristics (US_R). These associations may participate to the elaboration of a detailed representation of the stimulus-evoked US. Adapted from [Delamater & Oakeshott, 2007](#)

1.2. Instrumental action: Controlling our environment

Instrumental or operant behavior stands in contrast to Pavlovian conditioning in that it requires active engagement. Unlike Pavlovian conditioning, where the availability of the outcome is independent of the subject's actions, in operant behavior, performing an action becomes *instrumental* in obtaining the outcome.

In the early 20th century, Thorndike pioneered the study of associations between actions and outcomes by designing puzzle boxes for cats ([Thorndike, 1898](#)). These boxes were spacious enough for the cats to move around in and allowed them to see through to where food was placed. Confined in these boxes, the cats would attempt to escape, inadvertently activating the mechanism to open the door and gain access to the food. Through repeated trials and errors, the cats became more proficient at escaping the box, as indicated by decreased latency. Thorndike formulated the Law of Effect, which posited that any behavior followed by a rewarding outcome is likely to be repeated, while behaviors followed by negative outcomes are less likely to recur, introducing the notion of reward as a driving force in learning. This perspective gained further support through Skinner's experiments several decades later ([Skinner, 1932](#)). Skinner designed operant boxes, where rats learned to press a lever to receive a reward. The rate of lever pressing increased over time, indicating that conditioning was being strengthened. Skinner's work introduced the concept of reinforcement learning, demonstrating how actions are influenced by their immediate consequences. An alternative perspective, proposed by Hull, posited that when exposed to biological needs, such as hunger or thirst, a subject experiences discomfort ([Hull, 1943](#)). According to this *Drive theory*, the desire to restore homeostasis motivates engagement in behaviors aimed at reducing these drives to achieve a state of satisfaction.

The pioneering work of Thorndike, Skinner, and Hull highlighted fundamental principles of learning. However, these theories primarily view outcomes as mere reinforcers of associations between the environment and subject responses, such that the response is automatically elicited by the stimulus (S-R). In the upcoming paragraphs, we will explore research challenging this perspective, suggesting that actions can actually be driven by the representation of outcomes and their values.

The notion that instrumental actions are driven by the pursuit of specific outcomes or goals, such as obtaining a food reward, has gained prominence in contemporary research. In order to attain its goal, a subject must understand the causal relationship between the action (A) performed and its consequence or outcome (O), and possess the internal drive that motivates them to pursue their goal. These two criteria, *belief* and *desire*, have been proposed to underlie what is known as goal-directed instrumental action (Balleine & Dickinson, 1998). Hence, the goal-directed nature of an instrumental response or action can be assessed by manipulating the motivation to perform the responses by altering the current value of the outcome (*desire*), or by degrading the causal relationship between actions and outcomes (*belief*).

Manipulating the current value of an outcome is often achieved in the literature using outcome devaluation. In this paradigm, hungry subjects are trained to perform instrumental actions to obtain food rewards and, then, the value of one of these rewards is decreased (**Figure 3**). This decrease in value (or devaluation) can be achieved by sensory specific satiety, which induces a temporary reduction in the value of the sated outcome, or by pairing the reward with an aversive event, which produces a more long-lasting aversion to the reward (Adams & Dickinson, 1981; Colwill & Rescorla, 1985). The subjects are then tested and, typically, we observe a decrease in the performance of the

action that earns the now devalued outcome, relative to the performance on an action that earns a non-devalued (or valued) outcome. This pattern of responding reflects the subject's sensitivity to the current outcome value and their ability to use this feature of the outcome to guide their action selection. This observation cannot be explained by the S-R theory, which posits that reinforcers merely strengthen S-R associations without being encoded within the associative structure itself (Colwill & Rescorla, 1985). Therefore, the S-R account predicts that subjects would not reduce their performance of the action associated with the devalued outcome. Instead, the outcome devaluation paradigm highlights the ability of subjects to encode and retrieve A-O associations, guiding them to specifically select the action leading to the most desirable outcome.

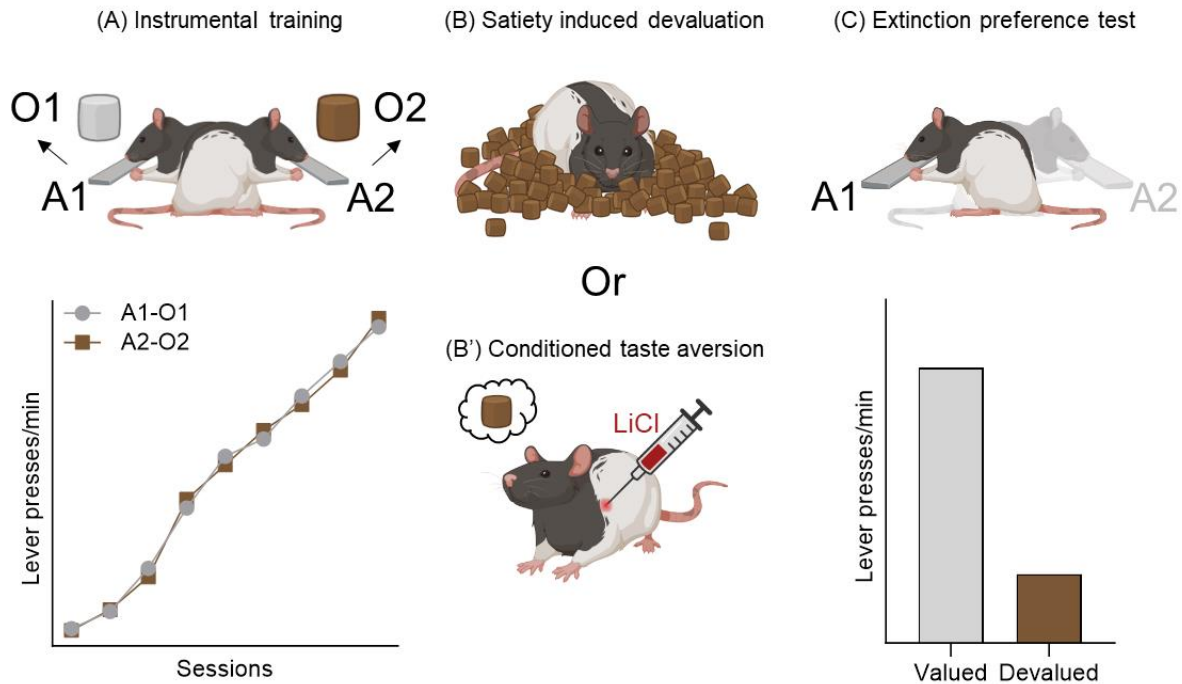


Figure 3. Instrumental outcome devaluation. (A) Subjects first undergo instrumental conditioning where they learn that specific actions A1 and A2 (e.g., pressing a right or left lever) earn distinct outcomes O1 and O2 (e.g., sucrose or grain pellets). Then, one of the outcomes is intentionally overconsumed to induce its devaluation (B) or paired with an aversive event, such as an intraperitoneal injection of Lithium Chloride (LiCl), which induces digestive malaise in the subject (B'). (C) Subsequently, preference for the outcomes is assessed in a non-rewarded test session. During this test, it is anticipated that responding on the lever associated with the devalued or aversively paired outcome will be diminished compared to the lever associated with the still-valued outcome. In both scenarios, the ability to preferentially perform on the lever associated with the non-devalued outcome indicates the subject's ability to use a representation of the outcome to guide their behaviour. Note that outcome devaluation can also be conducted under non-choice situations using a single A-O design.

As previously mentioned, instrumental actions can be considered goal-directed when the subject is sensitive to the current outcome value, and can accurately predict the consequences of its actions. In instrumental learning, much like Pavlovian conditioning, both contiguity and contingency are important for learning the causal relationship between an action and its associated outcome. For example, the rate of lever pressing in rats to obtain food rewards decreases with increasing delays between actions and outcomes,

indicating the importance of contiguity (Coutureau et al., 2012). To assess the subject's sensitivity to the causal relation between the action (A) and its associated outcome (O), a method can be used to degrade the A-O association and assess the extent to which the subject's behavior is affected by this degradation (Crimmins et al., 2022; Dickinson & Mulatero, 1989; Hammond, 1980). In this contingency degradation method, following instrumental conditioning with two distinct rewards, it is arranged that delivery of one of the two rewards is no longer contingent of the performance of its associated action, resulting in "free" rewards that are delivered whether or not the action is performed. After degradation training, animals undergo a choice test where responses no longer lead to reinforcement. During this preference test, subjects typically reduce their performance on the degraded action, indicating that they are aware of the change in the causal relationship between this action and its outcome, while performance of the non-degraded action is maintained. Importantly, because the test is conducted in extinction, subjects do not benefit from sensorial feedback when performing actions, and have therefore to retrieve their mental representations of the A-O associations. Once again, the S-R theory cannot explain this result, as it would predict that subjects will continue performing the degraded action by pure automatism or habit. Instead, it appears that subjects are able to flexibly adapt to changes in contingencies, reflecting the encoding of A-O associations during instrumental learning and their retrieval at test.

An insensitivity to changes in outcome value or instrumental contingencies reflect a loss of goal-directed control of actions. Hence, together, the devaluation and degradation paradigms allow for the assessment of the subject's ability to encode and recall specific A-O associations, enabling them to preferentially choose the optimal action

to take, i.e. the non-devalued or non-degraded lever over the devalued or degraded one respectively, reflecting the goal-directed nature of instrumental actions.

In summary, the examination of Pavlovian processes and instrumental behavior reveals fundamental mechanisms underlying the ability to both predict outcomes in our environment and to engage in actions to obtain desired outcomes. Pavlovian expectancies, driven by S-O associations, allow organisms to forecast and prepare for future events using environmental stimuli. In contrast, instrumental behavior driven by A-O associations, enables individuals to regulate their actions in pursuit of valued outcomes. Up to this point, we have considered Pavlovian and instrumental processes as distinct mechanisms. However, there is an extensive literature describing the interaction of these two forms of associative learning ([Dayan et al., 2006](#); [Guitart-Masip et al., 2014](#); [Rescorla & Solomon, 1967](#)).

1.3. *Interaction of Pavlovian predictions and instrumental actions*

In our everyday lives, environmental stimuli exert a remarkable influence, pushing us toward certain behaviors or discouraging us from others. Consider the busy streets of Paris: the aroma of freshly baked baguettes floating from a nearby bakery (stimulus) may tempt passersby to indulge in a warm loaf (action) satisfying their craving (outcome). The sound of a passing ambulance (stimulus) compels pedestrians to swiftly clear the way (action) promoting their safety (outcome). This exemplifies how environmental stimuli can trigger behavior resulting in particular consequences. Yet, the influence of these Pavlovian stimuli extends beyond mere convenience; they can subtly shape our decisions, sometimes misleading us. For instance, the allure of a charming café terrace decorated with tempting advertisements for pastries might prompt us to partake in sweets even when

we're not hungry. Moreover, these stimuli can exert a profound impact on our health and well-being, contributing to the development of pathologies such as addiction by triggering drug-seeking behavior and inducing relapse in abstinent individuals.

In the laboratory, the influence of stimulus-guided predictions on action control can be carefully assessed using Pavlovian-to-Instrumental Transfer (PIT). PIT experiments comprise three stages. Firstly, Pavlovian conditioning is utilized to establish a predictive relationship between a stimulus and a rewarding outcome (S-O). Next, the subject learns to perform an action to obtain the same, or similar, reward (A-O). Finally, during the critical PIT test phase, the predictive stimulus (S) is presented concurrently with the availability of the action (A), allowing for the assessment of the stimulus' influence on action performance. It's important to note that the action and the predictive stimulus are presented together for the first time in this phase, with no prior direct associative link between them. However, because both are associated with a common (or similar) outcome, instrumental responses are typically increased when the predictive stimulus is presented compared to baseline, thus demonstrating the transfer of response control from the Pavlovian prediction to instrumental performance as shown in

Figure 4. Notably, this transfer effect has been demonstrated in rodents, monkeys, and humans ([Cartoni et al., 2016](#); [Colagiuri & Lovibond, 2015](#); [Holmes et al., 2010](#)), as well as in equines ([Lansade et al., 2013](#)), pigeons ([Foree & LoLordo, 1973](#); [Herrnstein & Morse, 1957](#); [Overmier et al., 1983](#)) and rabbits ([Lovibond, 1983](#)).

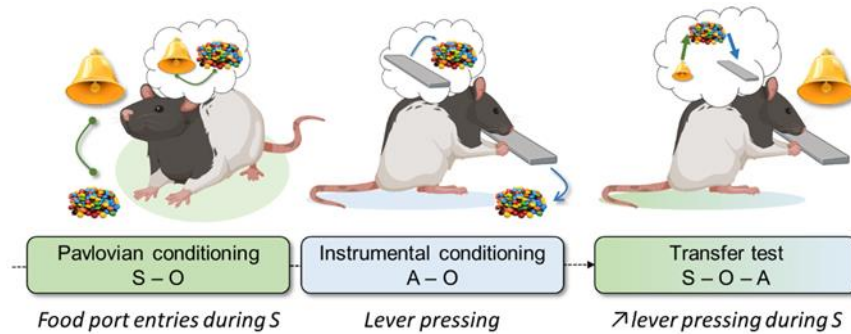


Figure 4. Conceptual framework of PIT in rodents. Classically, PIT experiments are conducted in three phases. First, hungry rats are trained to associate an auditory stimulus with a food outcome through Pavlovian conditioning, thereby forming an S-O association. The strengthening of this conditioning is reflected by an increasing rate of food port entries during stimulus presentations compared to periods without the stimulus. Secondly, rats are trained to press a lever to obtain the food reward, in the absence of any stimulus, thereby forming an A-O association. Finally, the influence of the Pavlovian stimulus on instrumental action is assessed during a non-rewarded PIT test. Although the Pavlovian stimulus and the instrumental action have never been directly paired together, it is typically observed that the baseline rate of lever pressing increases during stimulus presentation and returns to baseline after the stimulus ends, reflective of the transfer effect. Current PIT theories suggest that separately learned A-O and S-O associations stored in associative memory can interact. Through an S-O-A associative chain, the Pavlovian stimulus primes a mental representation of the associated outcome, which in turn triggers a representation of the action associated with that outcome, leading to an increase in the execution of the instrumental action.

1.3.1. Two forms of Pavlovian-to-instrumental transfer

The origins of PIT studies can be traced back to the 1940s. During this period, pioneering studies reported that stimuli paired with food were capable of enhancing instrumental actions directed towards obtaining food (Estes, 1943; Walker, 1942). Initial findings suggested that this transfer effect was modulated by primary motivational processes, a phenomenon commonly referred to as **general PIT**. For instance, Dickinson and Dawson, demonstrated that a stimulus predicting a sugar solution would enhance an instrumental action both when rats were hungry and thirsty whereas a stimulus associated with dry food pellets would only elevate performance when hungry, and not when thirsty (Dickinson & Dawson, 1987). However, this view was challenged by studies showing that “*reinforcer-derived expectancies are specific to the conditions under which they develop*”,

rather than universally motivational (Baxter & Zamble, 1982). Indeed, a CS signaling food delivery increased lever pressing for food, while a CS signaling rewarding electrical brain stimulation did not in a different group of animals.

This was further refined by Kruse and colleagues, who employed a more sophisticated experimental design. In their task, food-restricted rats initially learned to associate different stimuli with distinct appetitive rewards (e.g., food pellet or sugar solution) and subsequently performed actions to obtain these outcomes, e.g., pressing a right or left lever (Kruse et al., 1983). In the third phase, animals were subjected to a choice situation (unrewarded), where the effect of each stimulus on action selection could be assessed. Their results revealed that animals specifically biased their choice toward the action sharing the exact same outcome as the Pavlovian stimulus that was presented, indicating that the transfer effect was elicited by a reinforcer-specific expectancy state rather than a general drive state, a phenomenon referred to as **specific PIT**.

It is now well-established that Pavlovian-to-Instrumental transfer effects can be observed in two forms (**Figure 5.A-B**). General PIT illustrates how a stimulus can invigorate instrumental responses primarily through a motivational drive state, without the need for a detailed representation of the outcome (**Figure 5.A**). Indeed, this effect is evident even when animals are trained with two different outcomes of the same nature, such as two different food rewards, which are equally desired and distinguished only by taste (Aitken et al., 2016; Corbit et al., 2007; Corbit & Balleine, 2005, 2011a; Laurent et al., 2021; Lingawi et al., 2022; Panayi & Killcross, 2022). This suggests that the stimulus activates a broad motivational drive, invigorating instrumental behaviors that have been reinforced by an appetitive outcome of a similar nature (Dickinson & Dawson, 1987).

Conversely, specific PIT illustrates how stimuli can bias choice between specific actions. In specific PIT, Pavlovian stimuli trigger the expectancy of a specific reward, thereby enhancing the likelihood of choosing the action associated with that reward, through an outcome-selective expectancy state (Baxter & Zamble, 1982; Cartoni et al., 2016). In the specific PIT paradigm, stimuli presentations fall into two conditions: in the “same” condition, subjects perform the action sharing the same outcome as the presented stimulus, whereas in the “different” condition, subjects perform the action associated with a different outcome from the one predicted by the presented stimulus (**Figure 5.B**). Hence, the specific PIT effect is observed when subjects perform the action in the *same* condition at a higher rate than in the *different* condition or in the absence of any stimulus (baseline). This requires subjects to discriminate between different outcomes and their associated stimuli. Thus, in contrast with the general form, specific PIT requires subjects to possess and retrieve a more detailed and precise representation of the sensory-specific properties of the outcomes to effectively use Pavlovian stimuli to choose between competing actions.

While general and specific PIT are often studied separately, it is also possible to observe both general and specific transfer effects in a single paradigm. Corbit and Balleine designed the “full-transfer paradigm,” which assesses both general and specific influences of Pavlovian stimuli on action selection within the same subjects during a single session (Corbit & Balleine, 2005). As illustrated in **Figure 5.C**, subjects are trained during Pavlovian conditioning to associate three distinct rewards with three different auditory stimuli (i.e. S1-O1, S2-O2, S3-O3). Subsequently, two A-O associations are trained exactly as in the specific PIT paradigm (i.e. A1-O1, A2-O2). During the test phase, the three auditory stimuli are presented concurrently with the two available actions. Subjects are expected to demonstrate both specific and general transfer effects. Specifically, during

S1 and S2 they should selectively perform actions that share the same outcome as the presented stimulus, reflecting specific transfer (higher responding in the *same* condition than in the *different* one). Additionally, it is expected that subjects will perform both actions, above baseline, equally during the presentation of the third stimulus (S3), for which the reward has not been associated with an action, reflecting general transfer.

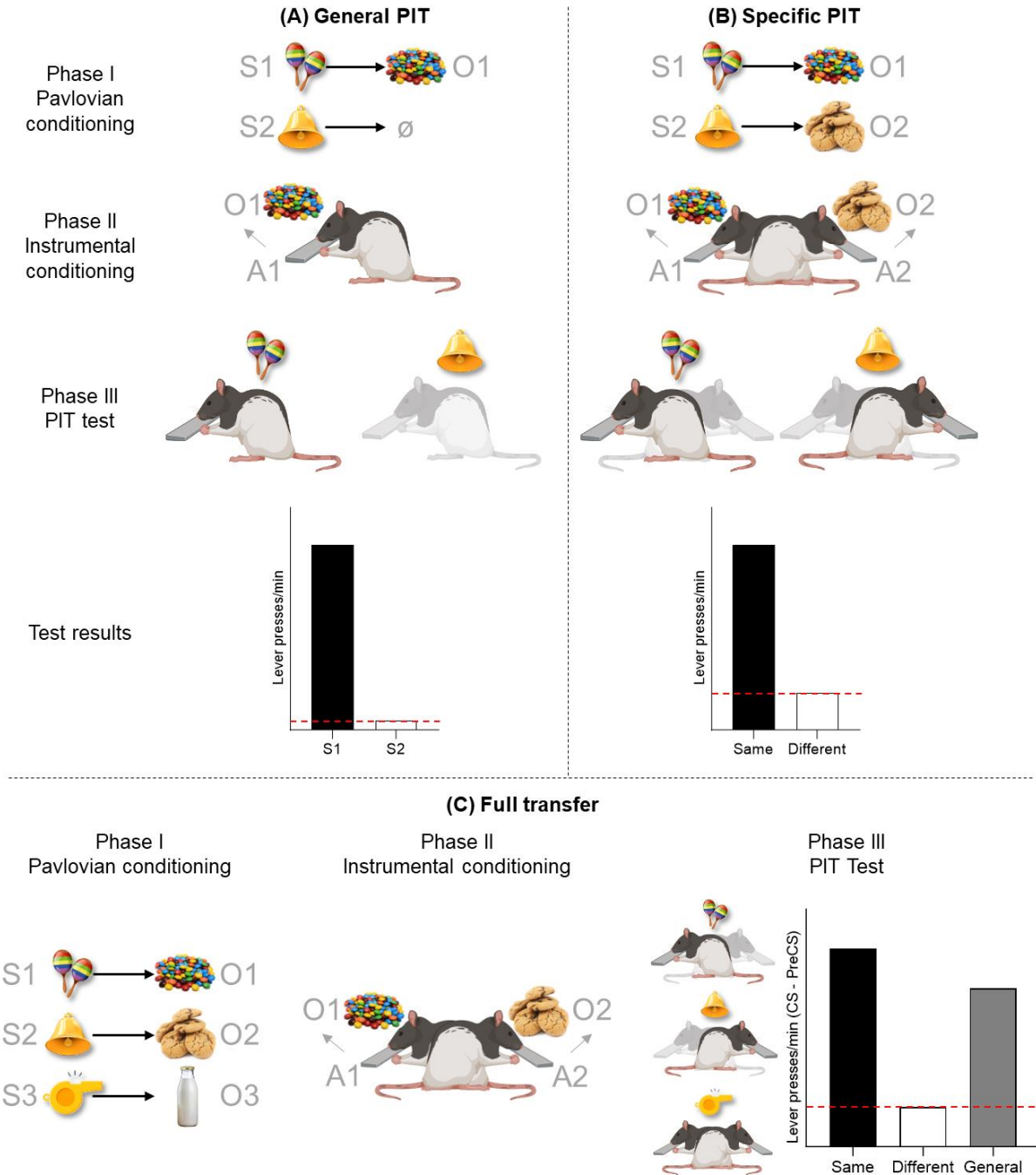


Figure 5. Pavlovian-to-instrumental transfer. (A) In general PIT, subjects learn that one stimulus (S1; maracas) predicts an appetitive outcome (O1; candies) while another stimulus (S2; bell) is unrewarded (∅). Then, they learn that an instrumental action (e.g., left lever press) earns the same outcome (O1; candies), or an equivalently desired one. A subsequent unrewarded test reveals that the stimulus predicting the reward delivery (S1) will invigorate responding on the rewarded lever, while the other stimulus (S2) does not. **(B)** In specific PIT, subjects learn two Pavlovian associations S1-O1 (maracas-candies) and S2-O2 (bell-cookies) and are then trained to perform two different actions A1 and A2 to obtain the rewards O1 and O2 respectively. During the transfer test, Pavlovian cues

bias responding towards the lever associated with the common outcome, S1 invigorating responding on A1 and S2 on A2 (i.e., the “same” condition); while pressing A1 during S2 and A2 during S1 corresponds to the “different” condition. Expected results are presented as the difference in action performance during the stimulus versus an equal period of time preceding the stimulus (PreCS). **(C)** In the full transfer paradigm, subjects learn three Pavlovian associations S1-O1 (maracas – candies), S2-O2 (bell – cookies) and S3-O3 (whistle – milk). Subsequently they are trained to perform two different actions A1 and A2 to obtain the rewards O1 and O2 respectively, while O3 is not earned during this training phase. At test, a higher level of responding in the “same” condition compared to the “different” condition reflects specific transfer. Additionally, responding above baseline equally on both levers during the presentation of S3 reflects general transfer. Baseline levels of responding are indicated by a red dotted line.

1.3.2. Theoretical aspects of transfer

The most comprehensive model of transfer to date is the associative-cybernetic model, illustrated in **Figure 6** (Balleine & Ostlund, 2007; Cartoni et al., 2013, 2016). This model explains both specific and general transfer effects through three distinct modules: an associative memory, a stimulus-action (S-A) memory, and a reward memory module, with outcome representations embedded in each. The associative memory stores S-O and A-O associations learned during Pavlovian and instrumental conditioning, respectively. According to Balleine and Ostlund (2007), both A-O and O-A associations are learned during instrumental training: that is, an outcome O not only results from pressing its associated lever A, but it also precedes the next lever press. It results that outcome O serves thus as both a consequence of A (A-O) and an antecedent for subsequent actions (O-A). Hence, in the S-A memory, outcome O is treated as a stimulus (S^O) that precedes action A, thereby forming S^O -A associations.

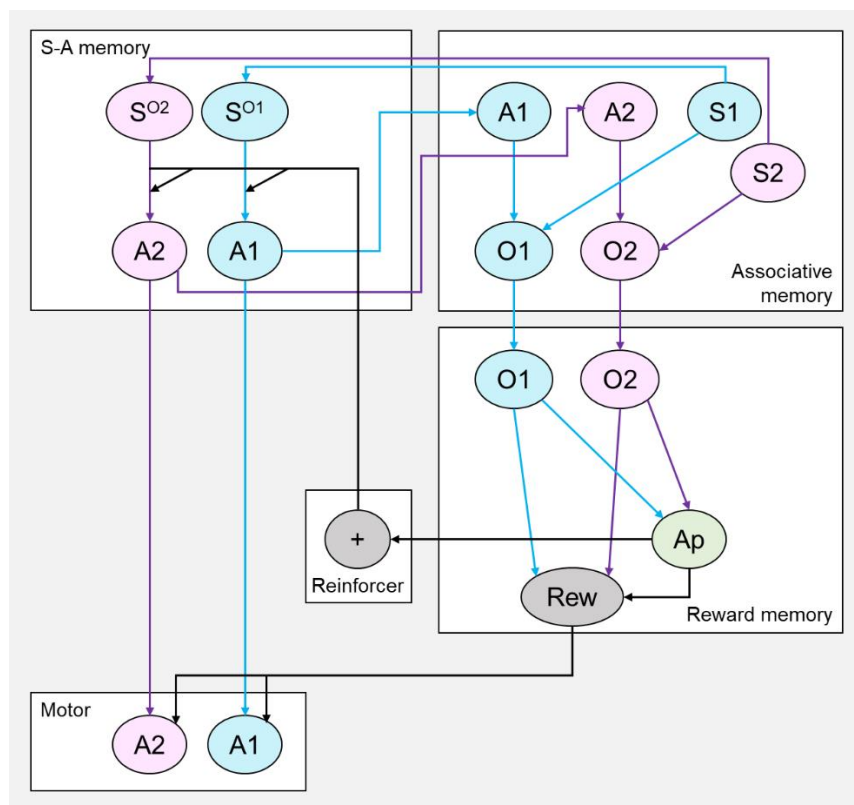


Figure 6. Associative-cybernetic model by Balleine and Dickinson adapted from Balleine & Ostlund (2007). The model comprises three distinct modules: An S-A memory containing S-A associations, including the feedforward O-A association; an associative memory including feedback S-O and A-O associations involved in Pavlovian and instrumental conditioning; and a reward memory responsible for encoding the appetitive (Ap) value of outcomes, with separate functions for rewarding (Rew) and reinforcing (+) aspects: the Rew node can be seen as “value expectation” which can energize instrumental actions aspecifically, while the Ap node has reinforcer-specific effects. As a result, action execution is influenced by both the S-A memory and associative memory modules, with the latter's impact on the motor system regulated by the incentive memory. In this framework, a CS prompts the representation of its associated outcome in the reward memory, where its affective value generates an expectancy of reward, thereby enhancing motor responses (general transfer). Alternatively, each CS primes the representation of its associated reward, acting as an antecedent for a specific response: for example, S1 activates the representation of O1 as a stimulus S^{O1} in the S-A memory, which then activates A1's representation and then the execution of this specific motor response (specific transfer).

Upon encountering a Pavlovian stimulus, its representation is activated in the associative memory (e.g. S1), which in turn activates its associated outcome as a stimulus in the S-A memory (S^{O1}), which then triggers the corresponding instrumental action (A1). This pathway explains how specific PIT operates through an S^O -A chain. A second

pathway contributes to the transfer effect. In addition to the S-A and associative memories, a Pavlovian stimulus S1 can also activate the representation of O1 in the reward memory, where the appetitive value of outcomes is encoded. This activation leads to an “expectation of value”, by activating the reward node, which can exert excitatory influences on all motor responses: a previously rewarded Pavlovian stimulus can evoke a generalized expectation of value, thereby aspecifically facilitating instrumental actions associated with a different food, providing a theoretical basis for general transfer.

Originally, the associative-cybernetic model was intended to be a general model of Pavlovian and instrumental learning, but it also offers a mechanism to understand the interaction(s) between these processes ([Balleine & Ostlund, 2007](#); [Cartoni et al., 2013](#)). However, while this model provides a strong theoretical base for understanding PIT, it is incomplete and requires a more complex explanation to fully account for all PIT effects. For example, according to this model, a Pavlovian stimulus is expected to produce both specific (S1-S^{O1}-A1) and general (S1-O1-O1-Rew-A1) effects simultaneously (see **Figure 6**). However, as will be discussed in the following sections, that is not the case. Indeed, experimental data exploring the neural bases of the different forms of transfer effects have challenged this assumption. Another issue with the model is its inability to account for the lack of elevated responding in the *different* condition in specific PIT, corresponding to the performance of the instrumental action associated with an outcome (A1-O1) different from the one predicted by the presented Pavlovian stimulus (S2-O2). According to the associative-cybernetic model, a general transfer effect should still occur in this scenario, yet such an effect is not typically observed in the empirical evidence. This suggests that the non-reinforcer specific effects of the “general PIT pathway” are inhibited in the different condition, a phenomenon for which the associative-cybernetic model does not currently

provide an explanation. Therefore, PIT cannot be fully explained solely by the S⁰-A associative chain and a nonspecific excitatory process. Instead, at least a third process, possibly an inhibitory mechanism, appears to be involved and requires further investigation to be understood (Cartoni et al., 2013, 2016).

1.3.3. PIT in humans

The PIT paradigm has been increasingly used in human studies during the last decade, revealing both general and specific forms of transfer effects much as in rodents. For example, Morris and colleagues adapted the full transfer paradigm developed by Corbit and Balleine in rodents (Corbit & Balleine, 2005; R. W. Morris et al., 2015). In this study, participants were trained to liberate two snack foods from a virtual vending machine by pressing either a right or left button to tilt the machine in one direction or the other (A1-O1, A2-O2) as illustrated in **Figure 7.A**. Participants were provided with the earned food and allowed to consume it. During the subsequent Pavlovian phase, participants learned the relationship between three colors illuminating the vending machine and three snacks (S1-O1, S2-O2, S3-O3). A fourth stimulus, which was never reinforced, served as CS- (S4-∅). Participants were again allowed to eat each snack when it appeared. Importantly, the Pavlovian stimuli and the actions were never directly paired. Lastly, participants underwent a transfer test in which they could tilt the vending machine to the right or left in the presence or absence of the Pavlovian stimuli. The results revealed a significant specific transfer effect, with more pressing during the *same* condition compared to the *different* condition and baseline period (**Figure 7.B**). Additionally, a general transfer effect was observed, with significantly more responding on both left and right buttons during the CS+ (S3) compared to the CS- (S4) as shown in **Figure 7.C**.

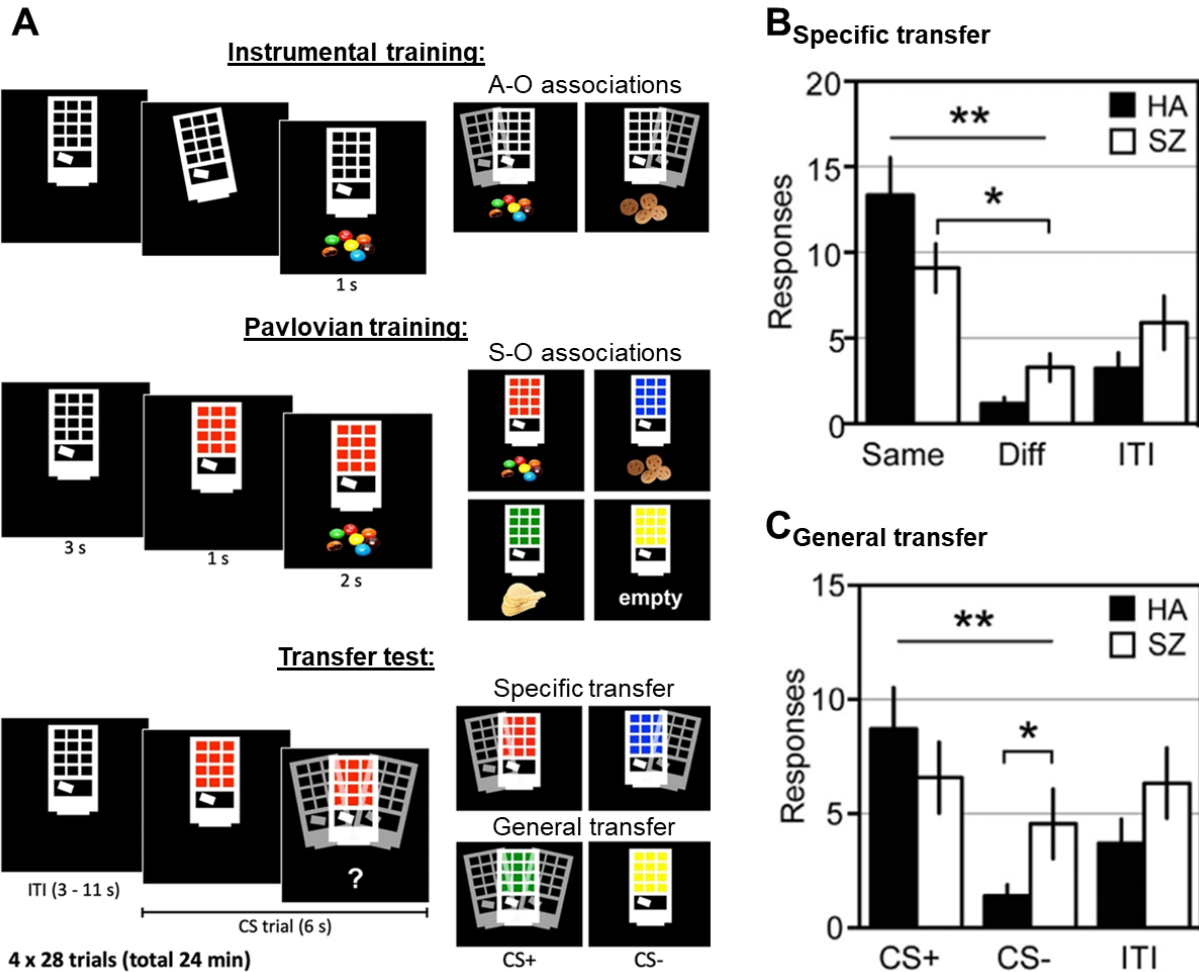


Figure 7. The virtual vending machines. (A) Participants are first trained to tilt a virtual vending machine either to the left or to the right to liberate two distinct snack foods, smarties and cookies (A1-O1, A2-O2). Then, participants learn to associate four distinct stimuli (vending machine illuminated with one of four colors) with the delivery of one of three snack foods, (S1-O1, S2-O2, S3-O3) or with no reward (S4- \emptyset , i.e., CS-). O3 was never delivered during the instrumental phase, such that S3 serves as a CS+ to assess general transfer relative to the CS-. Participants, healthy adults (HA) and schizophrenic patients (SZ), were then given a transfer test in an fMRI scanner. (B) Both groups of participants showed a specific transfer effect, with responding during the “same” condition higher than in the “different” condition (Diff), albeit a weaker transfer effect in SZ patients. (C) While healthy participants showed a significant general transfer effect, SZ patients responded equally during both CS+ and CS- presentations, and were unable to withhold responding during presentation of the never rewarded stimulus (S4). ITI: inter trial interval (baseline). * $p < 0.05$, ** $p < 0.01$. Adapted from [Morris et al., \(2015\)](#) and [van Timmeren et al., \(2020\)](#).

The development of PIT protocols in humans also provides an opportunity to use non-invasive imaging to identify the neural correlates of the transfer effects across various health conditions. In Morris and colleagues' research described above, neural hemodynamic responses were assessed during testing with functional magnetic resonance imaging (fMRI) in both healthy adults and patients suffering from schizophrenia (R. W. Morris et al., 2015). Behaviorally, schizophrenic (SZ) patients showed a weaker specific transfer than healthy controls (**Figure 7.B**), and the general transfer was abolished in SZ patients due to a high level of responding during the unrewarded Pavlovian stimulus CS- (**Figure 7.C**). Hence, while Pavlovian stimuli were able to specifically bias action selection in SZ patients, they showed a deficit in withholding responding during presentations of an unrewarded stimulus, which was interpreted as an aberrant incentive motivation by the authors of the study. Moreover, fMRI revealed aberrant hemodynamic responses in SZ patients relative to controls, with bilateral hypoactivity in the amygdala during specific transfer, and hyperactivity in the medial orbitofrontal cortex during CS- presentations. In the second part of this chapter (**Neural bases of stimulus-guided actions**), we will assess how these neural correlates relate to findings from the rodent literature.

In a different example in humans, Nadler and colleagues used a gamified version of the full transfer paradigm (Corbit & Balleine, 2005; Nadler et al., 2011). Participants were asked to defend their fictitious country against enemy attacks by firing missiles at potentially attacking vessels (**Figure 8**). During the instrumental phase, subjects learn that pressing the P button fires missiles at oncoming warplanes (A1-O1), while pressing the S button fires missiles at oncoming warships (A2-O2) as shown in **Figure 8.A**. During the Pavlovian phase, subjects observed their imaginary country being defended by allies from

enemy attacks. In this phase, two of four colored squares were each shown with one of the previously reinforced instrumental outcomes (S1-O1 for black square-warplane, S2-O2 for purple square-warship) as shown in the right part of **Figure 8.A**. A third, unexperienced outcome, a tank, was associated with one of the remaining colored squares (S3-O3 for yellow square-tank), and the last colored square was not reinforced serving as CS- (red square- \emptyset). Again, it is important to note that instrumental actions and Pavlovian stimuli were never made concurrently available during these training phases: the black stimulus was never directly paired with pressing the P button, nor the purple stimulus with the S button.

In the subsequent test phase, subjects assisted other commanders in defending the imaginary country. They were told that the other commanders would send coded information, in the form of colored squares, to alert them of the attack type (air, sea or ground), and they would have to press the buttons to fire missiles and help defend against the enemies. The transfer results showed that subjects specifically biased their actions when presented with the stimulus sharing the same outcome (i.e. pressing the warplane-directed missile button P when shown the black square, pressing the warship-directed missile button S when shown the purple square), indicating a specific transfer (**Figure 8.B**). The results also highlighted a general transfer effect, evidenced by equal responding above baseline on both S and P buttons when presented with the third Pavlovian stimulus (yellow square), which was predictive of the tank outcome. Additionally, the CS- (red square) had no excitatory influence on responding. This highlights that the PIT effect can be demonstrated in humans in a variety of experimental setups.

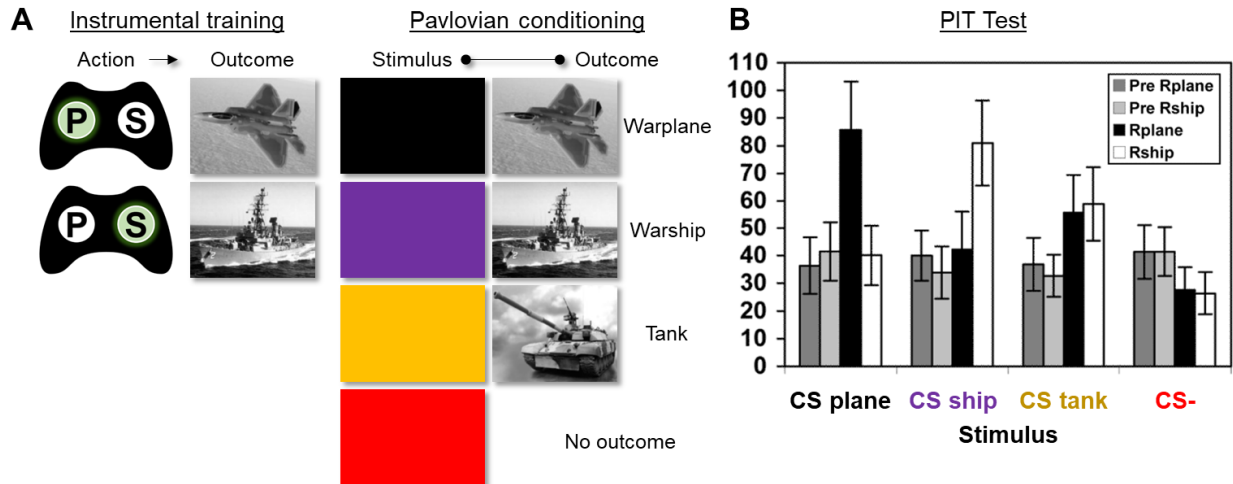


Figure 8. Example of a gamified human Pavlovian-to-instrumental transfer design. (A) Participants learn to perform two responses R_{plane} and R_{ship} (A1 & A2 or press buttons P & S respectively) to fire missiles towards different targets (O1: warplane, O2: warship) during the instrumental training, on a computer. Subsequently, participants learn to associate three colored squares with three distinct outcomes (S1-O1 for black square-warplane, S2-O2 for purple square-warship and S3-O3 for yellow square-tank), with O_3 never experienced during the instrumental phase. Additionally, a fourth stimulus served as CS- (red square-no reinforcement). Panel (B) shows the results of the transfer test. It is clear from figure inspection that participants selectively biased their responses towards the CS sharing the same outcome (R_{plane} or P button during “CS plane” and R_{ship} or S button during “CS ship”, above baseline and different conditions), reflective of the specific transfer effect. During “CS tank” presentations, participants performed both responses equally above baseline. Because the tank outcome was never associated with an instrumental action, this performance is reflective of the general transfer effect, while the CS- had no excitatory influence over responding. Adapted from *Nadler et al., (2011)*.

1.3.4. Translational relevance of PIT

Studies on Pavlovian-instrumental transfer also have clear clinical implications, as illustrated with the assessment of PIT effects in schizophrenic patients ([R. W. Morris et al., 2015](#)). Indeed, the clinical relevance of the transfer effects has been investigated across various health conditions in humans, such as in the context of maladaptive food-seeking behaviors, drug abuse such as tobacco and alcohol, and neuropsychiatric disorders such as schizophrenia and major depressive disorders, as briefly discussed in the next subsections.

Maladaptive food-seeking behaviors and PIT

We are constantly reminded of the availability of food in our environment, notably through advertising and other pervasive stimuli—a phenomenon often referred to as an "obesogenic environment." These food-associated stimuli can lead to over-consumption even when sated. In this context, the investigation of transfer effects has gained prominence as a mean to understand the mechanisms underlying maladaptive food-seeking behaviors.

In a study by Watson and colleagues, healthy human participants were subjected to the full transfer paradigm that was originally developed in rodents ([Corbit & Balleine, 2005](#); [Watson et al., 2014](#)). Following instrumental and Pavlovian conditioning, participants were sated on one reward or left in a non-sated state, before undergoing an appetitive PIT test. During the test, evidence of outcome-specific and general PIT effects was observed in both sated and non-sated participants. Notably, in the absence of food-associated stimuli, sated participants exhibited normal value-guided behavior, selectively pressing more on the button associated with the non-devalued outcome compared to the devalued one. However, this behavior was overridden by the presentation of food-associated stimuli, leading to increased performance of the devalued response even when participants were sated. These findings suggest that the biological mechanism of satiety, which typically serves to inhibit eating, can be overridden by food-associated stimuli. This override can result in increased food-seeking behavior and potential over-consumption.

Similarly, the impact of a motivational downshift on the expression of general PIT in healthy human participants has been investigated ([Colagiuri & Lovibond, 2015](#)). Participants underwent both training and test phases under reinforcement with chocolate rewards, a first in the literature as tests are traditionally conducted unrewarded. A

facilitatory PIT effect was observed in sated participants, indicated by increased responding to the chocolate-associated stimulus (CS+) and enhanced chocolate consumption. This finding suggests that the general PIT effect observed was independent of the current reward value, implying that food-related stimuli may contribute to over-consumption by eliciting motivation to obtain food. Again, this phenomenon holds potential to override biological mechanisms such as satiety. Conversely, the study found that when participants had low satiation and high motivation to obtain food, inhibitory stimuli (CS-) effectively reduced baseline responding, with excitatory stimuli having minimal impact. Hence, establishing an inhibitory link between stimuli and food constitute a potential approach to reduce stimulus-induced motivation for food.

Stimulus-induced drug-seeking behavior

In addiction theory, drug-seeking can manifest as either habitual, where drug expectancy plays no direct role in seeking behavior, or as goal-directed, where drug expectancy plays a causal role in the selection and performance of drug-seeking behaviors ([Hogarth et al., 2007](#)). Hogarth and colleagues conducted a series of experiments to explore the relative contributions of goal-directed (e.g. sensitivity to outcome value) and habitual (reflexes, automatisms) behaviors to nicotine dependence using PIT. They first showed that outcome-specific PIT was intact in smokers, with cigarettes and money serving as outcomes ([Hogarth et al., 2007](#)). This finding indicated that cigarette-associated stimuli controlled drug seeking through an expectation of the drug rather than habitual responses. Hence, authors concluded that smokers' drug-seeking behaviors were driven by goal-directed mechanisms, where the expected outcome representation played a key role in motivating the behavior.

In a subsequent study, the effect of a motivational downshift on the expression of specific PIT was assessed in adult smokers ([Hogarth & Chase, 2011](#)). Participants were trained with chocolate and cigarettes, with one of the rewards later devalued through satiety and health warnings. First, an unrewarded test was conducted in the absence of stimuli, revealing a decrease in the performance of the action associated with the devalued reward, suggesting normal goal-directed behavior. However, specific PIT test results revealed no effect of devaluation on the transfer effect, suggesting habitual responding. This dissociation between outcome-specific devaluation and specific PIT suggests a complex interplay between habitual and goal-directed processes in addiction. The authors proposed that a phenomenon of stimulus-induced reward hyper-valuation was at play, which could enhance vulnerability to dependence. Contrary to earlier findings of intact specific PIT in smokers linked to goal-directed behavior ([Hogarth et al., 2007](#)), the perseverance of the PIT effect following a motivational downshift suggest that drug-associated stimuli can undermine the goal-directed control of actions, driving habitual responses instead. This suggests that both habitual and goal-directed mechanisms can coexist and influence drug-seeking behaviors in different contexts.

Finally, Hogarth and colleagues investigated the impact of alcohol expectancy on goal-directed control over tobacco-seeking behavior ([Hogarth et al., 2013](#)). Young adult smokers first learned to perform two actions to earn either cigarettes or chocolate. After establishing these associations, tobacco was devalued through health warnings and smoking satiety. Participants were then divided into two groups: one group was presented with a glass of beer or wine (alcohol expectancy), while the other group received water (water expectancy). They were informed that they could consume the beverage after completing the task. Choice between the tobacco- and chocolate-associated actions were

then assessed in an instrumental extinction choice test and in a PIT test. The results revealed that alcohol expectancy selectively abolished goal-directed control over tobacco-seeking behavior in the extinction test but did not affect stimulus control in the PIT test. Specifically, after the devaluation of cigarettes, participants in the alcohol expectancy group continued to perform the action associated with cigarettes more frequently than the action associated with chocolate. In contrast, participants in the water expectancy group demonstrated normal devaluation effects, avoiding the tobacco-associated action. Importantly, the specific PIT effect remained intact and similar across groups. Authors suggested that not only goal-directed and habitual control mechanisms coexist in the regulation of drug-seeking behavior, but also that transitions to habitual control can occur phasically, driven by competing cognitive demands (here alcohol as an alternative reinforcer), which may play a significant role in the relapse of addictive behaviors.

The PIT paradigm has also been used to study the influence of stimuli on actions in patients suffering from alcohol use disorder (AUD). A pilot study involving recently detoxified alcohol-dependent individuals revealed a heightened propensity to general PIT, and a stronger response suppression during non-rewarded CSs in AUD patients when compared to healthy controls ([Garbusow et al., 2014](#)). These findings suggest that individuals with AUD might exhibit increased sensitivity to environmental stimuli. In a follow up study, Garbusow and colleagues investigated the neural substrates underlying the transfer effect in individuals with AUD, coupling PIT with fMRI ([Garbusow et al., 2016](#)). The study not only confirmed the previous observations of an enhanced general PIT effect in AUD patients but also revealed a significant increase in functional activation of the Nucleus Accumbens (NAc) among those who subsequently relapsed, as opposed to those who remained abstinent or were healthy controls. This increased NAc activation in

response to PIT suggests that heightened neural responsiveness in this region may be predictive of relapse in AUD patients. In the following section of this chapter, we will delve deeper into the role of the rat NAc in PIT, which has been a focal point of rodent research over the past two decades ([Cartoni et al., 2016](#)).

Neuropsychiatric disorders and PIT

The interaction between Pavlovian and instrumental processes has been explored in patients with various psychiatric conditions using the PIT paradigm. Notably, in patients with schizophrenia, studies have revealed an intact but weaker specific PIT effect, along with an abolished general PIT effect, as previously discussed ([R. W. Morris et al., 2015](#)). These findings correlated with distinct neural activity patterns: amygdalar hypoactivity during specific transfer and hyperactivity in the medial orbitofrontal cortex during presentations of non-rewarded conditioned stimuli.

The PIT paradigm has also been applied to study patients with major depressive disorders or MDD ([Huys et al., 2016](#)). Results of the study revealed that patients with depression did not exhibit a specific transfer effect when compared to healthy control subjects. A follow-up assessment conducted four months later aimed to evaluate the progression of depression in these patients, revealing that while some patients showed improvement, others did not. Upon re-analysis, the data indicated that those who improved displayed a specific PIT effect, whereas non-improvers did not. This association between greater PIT effect and better recovery over the follow-up period suggests that PIT may serve as a predictive tool for treatment outcomes in patients with MDD.

The studies reviewed here demonstrate that both forms of PIT can be observed in humans using paradigms similar to those employed in rodent research. This cognitive

process is evident across a variety of setups, including monetary, food, cigarette and alcohol rewards, indicating its pervasive influence on behavior. The observed effects of PIT underscore its significant role in driving actions and highlight its clinical relevance, particularly in understanding and potentially helping to treat maladaptive behaviors, substance abuse disorders and psychiatric conditions. These findings underscore the critical importance of understanding the neural substrates of PIT, which can provide invaluable insights into the mechanisms driving maladaptive behaviors and inform the development of effective diagnostics and therapeutic strategies.

1.4. Factors influencing transfer effects

The magnitude of the PIT effect exhibits considerable variability across different studies, especially in specific PIT experiments. In some cases, the effect manifests as a suppression of responding on the lever associated with a different reward from the presented CS ([Delamater & Holland, 2008](#)), while in others, it results in increased responding on the lever associated with the same outcome as the presented stimulus (**Figure 9**). Although the precise factors influencing these differential effects remain incompletely understood, several potential contributors will be discussed in the following section.

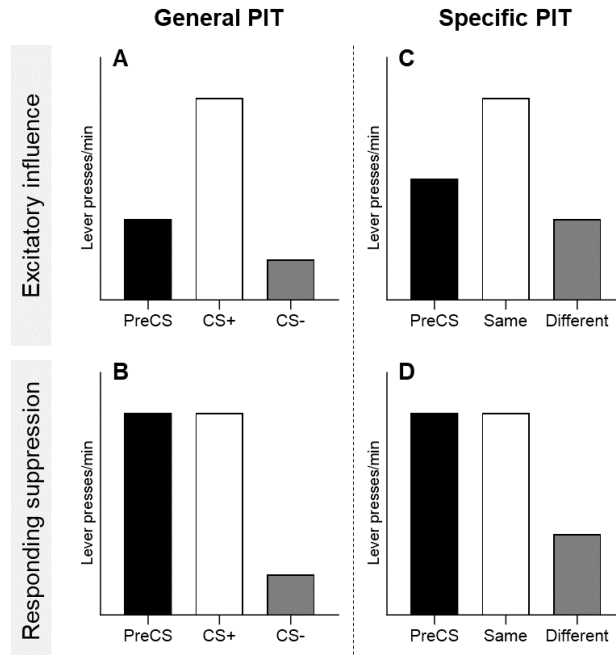


Figure 9. Different patterns of PIT expression. The PIT effect, which traditionally refers to the excitatory influence of a reward-predictive stimulus on action performance, is measured against the period preceding the stimulus onset, known as the PreCS, which serves as the baseline. The durations of the CS and PreCS periods are equal. In general PIT, the transfer effect can manifest as an increased lever-pressing rate during the rewarded CS (CS+) compared to the PreCS and the non-rewarded CS (CS-) periods (**A** – excitatory influence). However, when the baseline level of lever pressing is potentially too high, indicating a ceiling effect, performance during the PreCS and CS+ periods remain equal, but responding is suppressed during the CS- period (**B** – responding suppression). Similarly, in specific PIT, the transfer effect can appear as an increased performance in the same condition compared to the PreCS and different conditions (**C** – excitatory influence), or as a suppression of responding during the different condition in comparison to the other two conditions (**D** – responding suppression).

1.4.1. Instrumental and Pavlovian response control

The effect of the specific parameters used during instrumental training on the expression of the PIT effect has been investigated. Holland demonstrated that a greater amount of instrumental training enhances both general and specific forms of PIT (Holland, 2004). Both variable interval (reinforcement after an unpredictable amount of time has elapsed) and random ratio schedules (reinforcement after an unpredictable amount of actions) have been shown to evoke robust PIT effects (Holmes et al., 2010). Additionally,

it is worth noting that a high level of baseline responding can obscure the excitatory PIT effect, which can manifest in the form of responding suppression during non-rewarded stimulus presentations instead (cf. **Figure 9**). To mitigate this, instrumental extinction sessions are typically conducted before testing to reduce baseline responding levels against which the PIT effect is assessed.

Regarding Pavlovian conditioning parameters, the duration of the CS has also been identified as a crucial factor influencing the PIT effect with CS durations shorter than 60 seconds tending to suppress instrumental responding, whereas CS durations longer than 60 seconds elevate responding ([Holmes et al., 2010](#)). Additionally, variations in the number of Pavlovian conditioning trials and the interval between the CS and US have been shown to impact PIT ([Delamater & Holland, 2008](#)). It has been found that an increased number of food port entries during stimulus presentation at test correlated with an increasing number of Pavlovian sessions. This suggests that Pavlovian (food port entries) and instrumental (lever pressing) responses may compete during testing, such that a high level of Pavlovian CR will reduce the instrumental responses.

Research has also investigated how contingency degradation procedures affect the expression of PIT effects in rats. Studies have shown that degrading the causal relationship between an action and its outcome leaves the specific PIT effect intact, while the effect of instrumental contingency degradation on general PIT remains unexplored ([Colwill, 2001](#); [Rescorla, 1992](#)). This suggests that degradation procedures inhibit the targeted A-O association but do not eliminate it (also see [Laurent et al., 2021](#)). Pavlovian stimuli could reinstate actions previously paired with a common reward, potentially by releasing this inhibitory link, or by triggering reward-representation in S-A memory serving as a stimulus preceding the associated action, which in turn activates specific motor

responses, as illustrated by the $S_1-S^{O1}-A_1$ pathway of the associative-cybernetic model (Balleine & Ostlund, 2007). Therefore, degradation procedures seem to extinguish instrumental performance while preserving the underlying association, but see Crimmins et al., (2022).

Conversely, Pavlovian contingency degradation influences specific PIT effects while its effect on general PIT remains unexplored. Following Pavlovian conditioning and instrumental training, Park and colleagues assessed the effects of four different Pavlovian degradation treatments on the expression of specific PIT (Park et al., 2024): (1) a “random” treatment during which the two outcomes O1 and O2 could be delivered during their associated stimuli S1 and S2 as well as in their absence (intertrial interval), and during the non-contingent CS in rare occasions (i.e. O1 during S2 and O2 during S1); (2) a “negative-contingency” treatment during which outcomes are delivered exclusively in the absence of their associated stimulus; (3) a “zero-contingency” treatment during which outcomes are delivered equally in the presence and absence of their respective associated stimulus; (4) a “mixed” treatment, where both outcomes are delivered equally during both CSs. Test results revealed that only the random and the mixed treatments, during which outcomes were delivered during both CSs, even on rare occasions, disrupted the specific PIT effect which was spared by the two other treatments. These findings collectively indicate that the presence of S-O associations is essential for the expression of specific PIT. Particularly, the specific transfer effect is nullified only when outcomes are delivered during both CSs, underscoring the significance of intact S-O associations in driving the specific PIT effect.

1.4.2. Conditioning order and amount of training

To explore the interplay between Pavlovian and instrumental training during testing, Holmes and colleagues conducted a meta-analysis investigating the impact of conditioning order and training intensity on the PIT effect (Holmes et al., 2010). Their findings revealed that the extent of instrumental and Pavlovian conditioning influences PIT, with outcomes varying based on the sequence of training. Generally, they observed that Phase One training (either Pavlovian or instrumental training) could disrupt PIT, but this disruption could be mitigated by increasing Phase Two training (complementary training phase). Furthermore, they examined the effect of Pavlovian training on PIT through two experiments. First, they investigated the impact of Pavlovian over-training and found that it led to an increase in food port entries compared to moderately trained groups, with no evidence of a transfer effect. This supports the notion of competition between Pavlovian and instrumental responses. In a subsequent experiment, they demonstrated that Pavlovian extinction training in over-trained rats was able to restore the PIT effect, suggesting that Pavlovian extinction training enhanced PIT expression by reducing competition between Pavlovian and instrumental responses.

These studies reveal that the PIT effect is subject to various influences that can significantly impact its manifestation. Factors such as the amount and order of training, training conditions including duration of CSs, and the presence of competing Pavlovian-instrumental responses can all affect the magnitude and direction of the PIT effect. Additionally, high levels of baseline responding can obscure the detection of the transfer effect. Understanding these influences is crucial for accurately interpreting experimental results and advancing our knowledge of how environmental stimuli influence behavior.

1.4.3. Other factors influencing PIT

While beyond the scope of this thesis, it is worth briefly discussing other factors that influence PIT studies in rodents, particularly from a translational perspective. These factors include the interaction between motivation and stimulus-guided behaviors, the impact of stress, diet and obesity, as well as substances of abuse. Understanding how these elements influence PIT outcomes can provide valuable insights into decision-making processes and their clinical and transversal relevance to various behavioral and health conditions.

Motivational downshift effect

Several studies have explored how motivational shifts affect PIT expression. For instance, through taste aversion paradigms, Rescorla and Holland demonstrated that specific PIT remains intact despite outcome devaluation, indicating that the learned signaling properties of a stimulus can operate independently of the current motivational value of the expected outcome ([Holland, 2004](#); [Rescorla, 1994](#)). Furthermore, general PIT was also found to be insensitive to outcome devaluation by taste aversion in Holland's research. However, subsequent studies revealed nuances in this relationship. It has been shown that a shift from hunger to satiety alone can abolish general PIT while sparing specific transfer ([Corbit et al., 2007](#); [Lingawi et al., 2022](#)). Conversely, Sommer and colleagues found that specific PIT remained unaffected by both general satiety and outcome-specific sensory devaluation, albeit with a reduced transfer effect magnitude after devaluation ([Sommer et al., 2022](#)). These findings initially suggested that specific PIT was impervious to changes in current outcome value or general motivational states, in contrast with general PIT. However, recent work by Panayi and Killcross challenged

this perspective, revealing that specific PIT can indeed be sensitive to satiety-induced outcome devaluation ([Panayi & Killcross, 2022](#)), an effect that was masked in the two precedent reports due to differences in baseline responding ([Lingawi et al., 2022](#); [Sommer et al., 2022](#)).

Hence it seems that both forms of PIT are sensitive to outcome devaluation treatments in rats, contrasting with human research traditionally showing no effect of devaluation on transfer effects (see previous section **Translational relevance of PIT**). This apparent discrepancy between rodent and human literature may arise from procedural differences between experiments, such as the efficacy of the devaluation protocol ([Seabrooke et al., 2017](#)). Indeed, some studies demonstrated sensitivity of human specific PIT effect to devaluation using instructions ([Allman et al., 2010](#); [Eder & Dignath, 2016a](#)) or by making an outcome taste unpleasant ([Eder & Dignath, 2016b](#)).

Stress and PIT

The impact of both acute and chronic stress on PIT has also been assessed in rodents. Acute stress, induced prior to testing, has been shown to leave general PIT intact in rats, indicating that stress in the short term does not significantly disrupt the ability of predictive stimuli to exert their excitatory influence on action performance ([Pielock et al., 2013](#)). While acute stress effects on specific PIT have not been investigated, research by Morgado and colleagues found that chronic stress abolished specific PIT in rats ([Morgado et al., 2012](#)). Interestingly, this effect was transient, disappearing after a period of six weeks without exposure to stressing stimuli. More research is needed to understand how stress can shape the PIT effect.

Diet and obesity vulnerability

Diet-induced obesity has been found to significantly impact Pavlovian conditioning and the motivation for instrumental actions in rodents ([Gladding, Bradfield, et al., 2023](#); [Harb & Almeida, 2014](#); [Shipman & Corbit, 2022](#); [Tantot et al., 2017](#); [Tracy et al., 2015](#)). Studies utilizing obese-prone and obese-resistant strains, where phenotype is contingent on exposure to an obesogenic diet, have revealed intriguing findings. Notably, food-restricted obese-prone rats maintained on standard chow exhibited an increased magnitude of general PIT ([Derman & Ferrario, 2018, 2020](#)). Moreover, post-testing, obese-prone rats switched to a "junk-food" diet showed a positive correlation between post-diet weight and the magnitude of general PIT, but not specific PIT, suggesting a potential link between incentive motivation and obesity vulnerability. Similarly, mice with a history of high-fat diet displayed general PIT under both hunger and satiety conditions, unlike control mice where satiety alone abolished general PIT ([Gladding, Lingawi, et al., 2023](#)). Interestingly, body weight differences were not observed between groups at the commencement of Pavlovian and instrumental training.

This persistence of general PIT in high-fat diet-fed mice despite changes in motivational state implies a long-term alteration in food-seeking behavior, independent of excess body weight and adiposity. Such findings suggest that a loss of control over stimulus-driven food-seeking may contribute to eating in the absence of hunger and subsequent weight gain. Additionally, rats on a high-fat diet have been shown to be insensitive to satiety-induced outcome devaluation when trained on a variable interval schedule, suggesting a promotion of habitual responding ([Tantot et al., 2017](#)). Altogether, these findings suggest that alterations in general PIT can manifest prior to obesity development and persist after consuming an obesogenic diet. However, the influence of

diet and obesity vulnerability on specific PIT remains to be fully elucidated. While mice switched from a high-fat diet to normal chow did not show impairment in specific PIT ([Gladding, Lingawi, et al., 2023](#)), rats fed a junk-food diet for six weeks exhibited abolished specific PIT effects ([Kosheleff et al., 2018](#)). Further studies are warranted to unravel the intricate relationship between diet, obesity vulnerability, and specific PIT.

Substances of abuse

Pavlovian learning is a key component in various addiction theories, particularly in understanding relapse dynamics ([Lamb et al., 2016](#)). The traditional hypothesis suggests that encountering drug-associated stimuli could trigger relapse by intensifying the motivation to consume substances. If a stimulus associated with a drug does amplify the motivation to consume drugs, it follows that a drug-paired CS would likely elevate drug consumption. Three studies investigated how drug-associated stimuli in self-administration of cocaine in rats would influence responding, but did not find evidence of increased drug seeking elicited by the stimuli ([Kruzich et al., 2001](#); [LeBlanc et al., 2012](#); [Takahashi et al., 2019](#)). Both Glasner et al. and Corbit and Janak have reported that ethanol-paired stimuli increase responding for ethanol as well as responding on a different lever previously reinforced by a different reward ([Corbit & Janak, 2007a](#); [Glasner et al., 2005](#)). This suggests that ethanol-associated stimuli exert general motivational effects rather than outcome-specific effects. Krank et al. conducted a study demonstrating that alcohol-associated stimuli enhance operant responding for alcohol as well as seeking behaviors ([Krank et al., 2008](#)). Similarly, Milton and colleagues showed that an ethanol-paired stimulus, but not an unpaired stimulus, elicited instrumental responding in a general PIT test ([Milton et al., 2012](#)). However, none of the previous studies provide evidence that

drug-paired stimuli actually increase drug-taking. Hence the role of PIT in the development of addiction and relapse remains unclear.

1.5. Summary

In this first section, we have explored how Pavlovian predictions influence and interact with action selection. Specifically, our focus has been on PIT, which manifests in two distinct forms, each susceptible to different influencing factors. The next chapter will explore the neural substrates underlying the expression of stimulus-guided actions. Indeed, evidence showing that the general and specific forms of PIT can be dissociated at the behavioral level have promoted investigations into whether parallel dissociations also exist at the neural level. We will thus consider studies that have directly compared the involvement of different regions or pathways in both general and specific PIT, as well as studies that have focused on only one form of PIT.

2. NEURAL BASES OF PIT

In the preceding section, we examined the multifaceted nature of the PIT effect, discerning between its general and specific manifestations. In this section, our focus shifts towards unraveling the neural bases of these transfer effects. Interestingly, it appears that, beyond the behavioral delineation, a functional dichotomy also exists at the neural level. While this section is not exhaustive, the objective here is to provide an overview of some of the key studies that have informed our understanding of the neurobiology of stimulus-based choice. Moreover, I hope to illustrate that the PIT paradigm itself is a powerful tool to better understand the precise contribution of different brain regions to Pavlovian and instrumental conditioning themselves. The findings of the studies discussed in the following sections are summarized in **Appendix I**.

2.1. *Contribution of amygdala subregions to PIT*

The amygdala has long been implicated in PIT, which is perhaps unsurprising given its well-established role in the acquisition and retrieval of S-O associations ([Holland & Gallagher, 1999](#); [Petrovich, 2011](#)). However, interestingly, amygdala subregions contribute differently to the general and specific forms of PIT. Hall and colleagues first demonstrated that pretraining lesions of the central nucleus of the amygdala (CeA) prevented a reward-predictive stimulus (CS+) to increase lever responding above baseline at test, while lesions of the basolateral amygdala (BLA) left the general transfer intact ([Hall et al., 2001](#)). This specific involvement of the CeA in the invigoration of instrumental responses by Pavlovian stimuli was subsequently confirmed in studies employing pretraining lesions ([Corbit & Balleine, 2005](#); [Holland & Gallagher, 2003](#); [Lingawi & Balleine, 2012](#)). By contrast, perturbation of the BLA impairs the expression of

specific PIT, with subjects pressing both levers equally (above baseline responding) during both CS presentations (Blundell et al., 2001). This result indicates that while BLA-lesioned rats were sensitive to the motivational properties of the CSs, reflecting reward expectation, they were unable to bias their choice towards the action sharing the same reward as the presented CS, indicative of a non-reinforcer specific PIT effect. This double dissociation at the level of the amygdala was further illustrated by an elegant study from Corbit and Balleine using the full transfer paradigm (cf. **Figure 5.C**). In a single experiment (**Figure 10**), they showed that BLA-lesions impaired specific but not general PIT, while CeA lesions impaired general but not specific PIT (Corbit & Balleine, 2005). A similar dissociation at the level of the amygdala has also been observed in humans undergoing general and specific PIT tasks combined with functional imaging (Prévost et al., 2012).

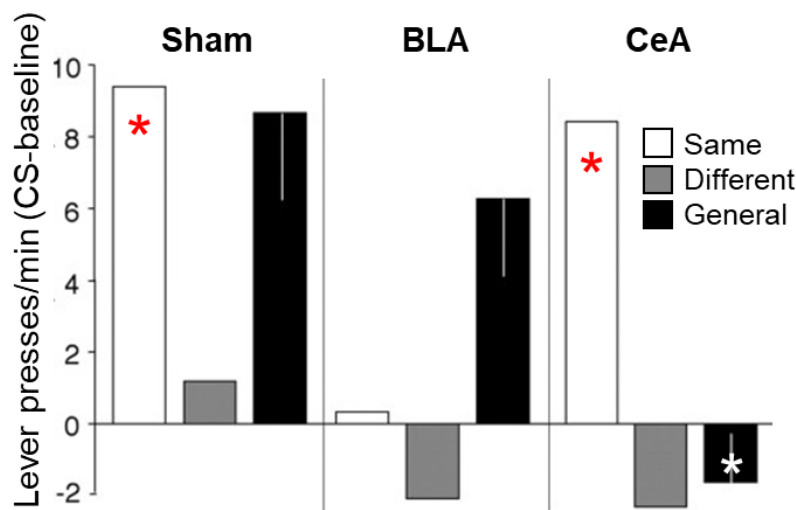


Figure 10. Double dissociation of BLA and CeA in the general and specific PIT. Corbit & Balleine (2005) trained sham-, BLA- and CeA-lesioned rats in a full transfer PIT procedure. The transfer was measured by obtaining the difference of lever presses per minute during CS presentations and baseline period (i.e., the net excitatory effect of the stimuli over baseline). The results

showed that specific PIT is abolished in BLA-lesioned rats, while it is intact in sham- and CeA-lesioned rats, as indicated by the significant difference between performance in the same and different conditions (red stars). Moreover, CeA-lesioned rats' performance in the general condition was lower than baseline, and significantly lower than in the sham group (white star), indicative of an abolished general PIT in these subjects, in accordance with other studies reporting CeA requirement for general PIT expression (Blundell et al., 2001; Holland & Gallagher, 2003; Lingawi & Balleine, 2012).

However, it should be noted that the deficits observed by Corbit & Balleine (2005) following BLA lesions was quantitatively different to the deficit observed by Blundell and

colleagues. Indeed, Corbit and Balleine observed that, following BLA lesions, rats showed no increased responding in the *same* and *different* conditions when compared to baseline levels. Similarly, post-training lesions of the BLA have also been found to abolish the expression of specific PIT, with no excitatory influence of the CSs on responding (Ostlund & Balleine, 2008). More recently, chemogenetic inhibition of the BLA at test confirmed its requirement for the expression of specific PIT, with no preferential performance in the *same* condition as compared to the *different* condition in the full transfer paradigm (Derman et al., 2020). Inspection of the results suggest that there was also no excitatory influence of the CSs on performance.

That is, while Blundell and colleagues (2001) show that rats with BLA lesions appear able to show a general PIT effect and increase responding above baseline during both the *same* and *different* conditions, other studies have failed to observe this general invigoration of responding following BLA lesions (Corbit & Balleine, 2005; Derman et al., 2020; Ostlund & Balleine, 2008). This difference could be explained by the fact that general and specific PIT effects can only be dissociated when using the full transfer protocol (**Figure 10**). This suggests that the non-reinforcer specific PIT effect observed in the specific PIT procedure (Blundell et al., 2001) could be reflective of the isolated general PIT effect observed in the full transfer paradigm (Corbit & Balleine, 2005; Derman et al., 2020).

2.2. Cortical contributions to PIT

Studies investigating the cortical contribution to PIT have primarily focused on the medial prefrontal cortex (mPFC) and the orbitofrontal cortex (OFC). Using extracellular electrophysiological recordings, it was shown that the neural activity of mPFC (prelimbic

region) and lateral OFC neurons was selectively modulated during the presentation of the Pavlovian reward predicting-stimulus CS+ compared to the CS- and baseline periods during a general PIT test ([Homayoun & Moghaddam, 2009](#)). Moreover, there was an increased firing rate magnitude when subjects performed the instrumental action during the CS+, but not when the action was performed during the other test periods. It must be noted here that studies examining the *functional* involvement of cortical areas in general PIT remain sparse.

Attempts have also been made to delineate the role of distinct subregions of the mPFC and the OFC, particularly in specific PIT. Traditionally, the mPFC has been divided into four regions: the medial precentral (PrCm) or area Fr2, the ACC, the prelimbic area (PL), and the infralimbic cortex (IL). Bilateral lesions of the infralimbic cortex (IL) were found to abolish the expression of specific PIT, with no increase in responding during stimulus presentation in both the *same* and *different* conditions ([Keistler et al., 2015](#)). A disconnection procedure (contralateral lesions) revealed that the IL mediates this effect via its functional connectivity to the NAc shell ([Keistler et al., 2015](#)). In contrast, Corbit and Balleine showed that the adjacent prelimbic cortex was not required for the expression of specific PIT ([Corbit & Balleine, 2003](#)). Indeed, pretraining lesions of the PL spared the specific transfer effect, with no difference found in comparison to a sham group.

The OFC can also be divided into several areas, including the ventral (vOFC), lateral (lOFC), dorsolateral (dlOFC), and medial (mOFC) regions. Post-training lesions encompassing both vOFC and lOFC abolish specific PIT, evidenced by no facilitatory influence of the Pavlovian stimuli on action performance ([Ostlund & Balleine, 2007](#)). More recently, optogenetic stimulation of lOFC PV interneurons was shown to be sufficient in preventing Pavlovian stimuli to specifically bias instrumental responding towards their

shared outcome, confirming the role of IOFC in specific PIT (Leung et al., 2024). The mOFC also appears to play a role in specific PIT with mOFC lesions producing a non-reinforcer specific transfer, with equal responding during both same and different conditions (L. A. Bradfield et al., 2015). This result was later refined, revealing that the anterior but not the posterior part of the mOFC was required for specific PIT (L. A. Bradfield et al., 2018).

Altogether, these results reveal a dissociation within the mPFC in regards to specific PIT contribution, with the IL but not the PL being required, as well as in the OFC, with the anterior mOFC, the vOFC and the IOFC, but not the posterior mOFC, being necessary for specific PIT.

The interactions between the OFC and the BLA have also been studied in specific PIT. It was found that chemogenetic inhibition of IOFC terminals in the BLA at test did not alter the expression of specific PIT, while inactivation of BLA terminals in the OFC did, with no difference in responding between the baseline, the *same* and the *different* conditions (Lichtenberg et al., 2017). This suggests that BLA to OFC projections are required for rewarding stimuli to selectively bias responding in a specific manner, while OFC to BLA projections are not required at test for the expression of specific PIT (Lichtenberg et al., 2017). Sias and colleagues also showed that optogenetic inhibition of IOFC terminals in the BLA, as well as inhibition of the BLA only, during outcome delivery in the Pavlovian conditioning phase, abolishes the specific PIT effect at test, reflected by equal level of responding during both *same* and *different* conditions (Sias et al., 2021). This suggests that IOFC inputs to the BLA are required to encode S-O associations during Pavlovian conditioning. They also performed a serial disconnection of the IOFC→BLA pathway during Pavlovian conditioning, and the BLA→IOFC pathway during testing. To

do so, they optogenetically inhibited IOFC terminals in the BLA of one hemisphere during Pavlovian conditioning, and chemogenetically inhibited BLA terminals in the OFC in the contralateral hemisphere at test. This serial disconnection abolished the expression of the specific transfer effect, with subjects responding equally in *same* and *different* conditions.

This indicates that the lateral OFC and the BLA form a bidirectional circuit for the acquisition (IOFC→BLA) and retrieval (BLA→IOFC) of S-O associations to specifically bias action selection. That is, the BLA appears crucial for both the encoding of specific S-O appetitive memories, and their retrieval to specifically bias instrumental actions, notably through an IOFC-BLA-IOFC circuit.

2.3. *Striatal contributions to PIT*

There is much evidence that the dorsal striatum is critical for instrumental behavior (for a review see [Hart et al., 2014](#)) and, thus, it is again not surprising that several studies have also shown a role for this region in PIT. An initial study showed that inactivation of the dorsolateral part of the striatum (DLS) prevents Pavlovian stimuli to exert an excitatory influence on action performance, while inactivation of the dorsomedial striatum (DMS) leads to a non-reinforcer specific elevation of responding, above baseline but equally distributed between the *same* and the *different* conditions ([Corbit & Janak, 2007b](#)).

This dissociation in dorsal striatum was further characterized by inhibiting the DLS, the anterior part of the DMS (aDMS) or its posterior part (pDMS) via infusion of a baclofen/muscimol mixture ([Corbit & Janak, 2010](#)). Animals were first tested for their ability to encode A-O and S-O associations after inactivation of the targeted region during both instrumental and Pavlovian training phases, respectively. This first set of experiments revealed that both anterior DMS (aDMS) and posterior DMS (pDMS) are involved in the

acquisition of specific A-O associations. Moreover, it was also shown that the pDMS and the DLS are involved in the encoding of specific S-O associations. Following this, animals underwent a PIT test without inactivation. Subjects with inactivated DMS or DLS during training phases failed to show a specific PIT transfer effect. Although responding during CSs was significantly above baseline, it was equally distributed during both *same* and *different* conditions, indicating a non-reinforcer specific transfer effect.

These results show that both A-O acquisition, dependent on the DMS, and S-O encoding, relying on the pDMS and the DLS, are necessary for Pavlovian stimuli to bias performance towards the action sharing a common outcome. Thus, the involvement of the dorsal striatum in PIT is likely due to its role in S-O and A-O learning.

Many studies have also examined the involvement of ventral striatum in both general and specific PIT. Hall and colleagues first showed that pretraining lesions of the NAc core prevented a reward-predictive stimulus (CS+) to increase lever responding above baseline at test, while lesions of the NAc shell left the general transfer intact (Hall et al., 2001a). Additionally, findings by Corbit and Balleine, using the full transfer paradigm (cf. **Figure 5.C**), also found that the shell of the NAc is required for specific PIT, while general PIT requires the NAc core revealing a functional double dissociation within the accumbens, similar to the one observed in the amygdala (**Figure 11**). Pretraining lesions of the NAc shell prevented Pavlovian rewarding stimuli to specifically bias responding during the *same* and the *different* conditions at test (Corbit et al., 2001; Corbit & Balleine, 2011), and reversible inactivation of the shell using muscimol infusions prior to testing was similarly found to abolish specific, but not general, PIT (Corbit & Balleine, 2011). Moreover, the opposite pattern of results was observed when NAc core was reversibly inhibited using the same strategy, i.e. abolished general PIT but intact specific PIT.

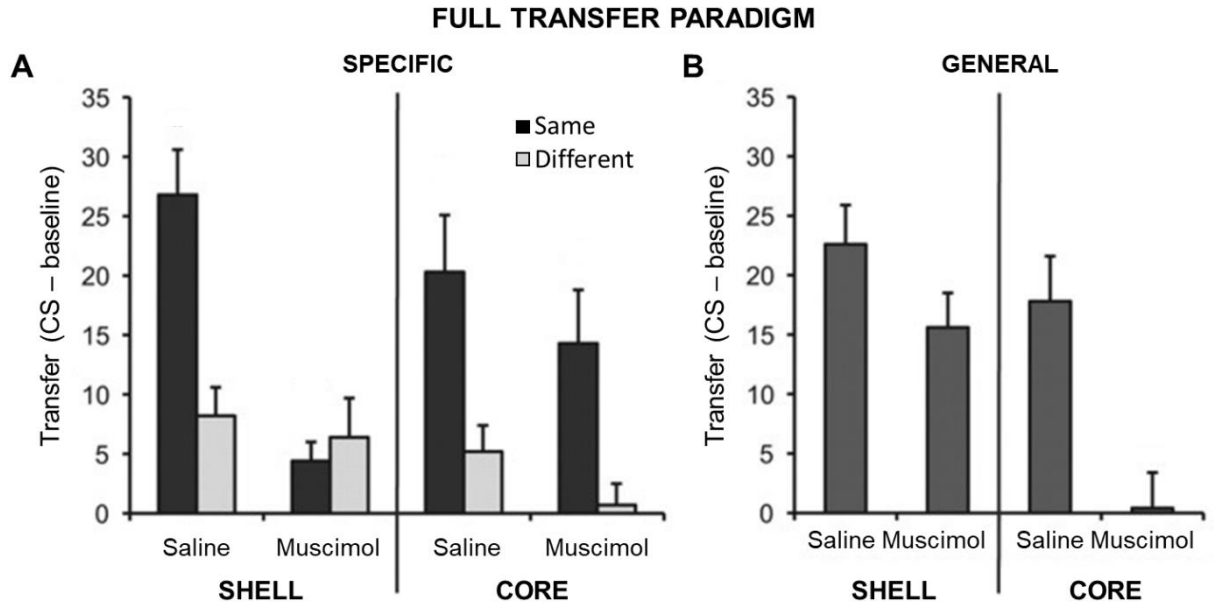


Figure 11. Double dissociation of NAc core and shell in general versus specific PIT, from Corbit & Balleine (2011). The effect of reversible inactivation of the NAc shell and core on the expression of the specific and general forms of PIT are presented separately but were assessed concurrently using the full transfer paradigm. The results are presented as the net increase of responding during stimuli presentations (\pm SEM), relative to baseline responding (absence of any stimulus). **(A)** Inhibition of the NAc shell via local infusion of muscimol at test abolished specific PIT as revealed by equal responding during same and different conditions, while control-treated subjects (saline infusion) showed intact specific PIT (significantly higher level of responding during the same condition compared to the different condition). Additionally, responding in the same condition was significantly decreased in muscimol-treated subjects compared to the vehicle group (black bars), further demonstrating the requirement of the NAc shell for specific PIT expression. Conversely, core-inactivated rats did not differ from controls, with a significant increase in responding in the same condition (black bars) compared to the different condition (grey bars). **(B)** The general PIT results revealed that the core but not the shell of the NAc was required for the expression of general PIT. Specifically, shell inactivation had no effect on general PIT, as revealed by similar performance in the saline and muscimol groups. Conversely, muscimol-induced inactivation of the core significantly decreased responding during stimulus presentation when compared to saline-treated subjects.

The contribution of amygdalar-striatal, striato-pallidal, and cortico-striatal pathways to specific PIT have also been explored. Disconnection of the BLA from the NAc shell, but not from the core, using contralateral lesions, was found to abolish the specific transfer suggesting a suppression of the excitatory influence of Pavlovian stimuli on action selection (Shiflett & Balleine, 2010). In line with previous studies, this reveals the central role of the BLA in specific PIT, and its communication with both NAc shell and IOFC, supporting the retrieval of S-O associations to specifically bias action selection. The medial ventral pallidum (VPm), which receives projections from the NAc shell, has also been found to be required for specific PIT, as well as the shell-VPm pathway (Leung & Balleine, 2013). Indeed, temporary inactivation by muscimol injections in the VPm during the PIT test, or in contralateral VPm and NAc shell, both prevented the previously rewarded stimuli to increase responding above baseline.

Together, these studies highlight the distinct contributions of striatal subregions and their pathways in the acquisition of A-O and S-O associations, and their role in memory retrieval to allow Pavlovian stimuli to exert excitatory and outcome-selective influences on action performance. A functional dissociation exists within the NAc, with the core required for the expression of general but not specific PIT, and the shell required for specific but not general PIT. Specific PIT also depends on amygdalar-striatal (BLA-shell but not BLA-core), striato-pallidal (shell-VPm), and cortico-striatal pathways (IL-shell). Within the dorsal striatum, it appears that both DMS and DLS are required for the expression of specific PIT. This is likely due to the involvement of these regions in the learning of action-outcome (DMS) and stimulus-outcome (DLS and posterior DMS) associations.

2.4. Thalamic contributions to PIT

The mammalian thalamus is composed of over 50 distinct nuclei, which are organized into seven major groups: anterior, medial, lateral, ventral, intralaminar, midline, and posterior (Roy et al., 2022). The mediodorsal thalamus (MD) is the largest nucleus within the medial group and includes subdivisions that are broadly homologous across rodents and primates (Wolff & Halassa, 2024). Notably, several studies have suggested a role of the MD in the acquisition of A-O associations (Alcaraz et al., 2016; L. A. Bradfield et al., 2013; Corbit et al., 2003; Fisher et al., 2020; Mitchell et al., 2007). Ostlund and Balleine investigated the contribution of the MD to instrumental action selection in situations where choice is guided either by the current reward value or by reward-predictive stimuli (Ostlund & Balleine, 2008). Results showed that while the MD was not required for value-guided actions (i.e. no deficit in outcome devaluation test), post-training lesions of the MD impaired specific PIT expression, as reflected by similar responding during baseline, *same* and *different* conditions.

The finding that the MD is required for stimulus-guided actions prompted further research to elucidate the neural circuitry involving this thalamic region. For example, Leung and Balleine (2015) investigated the effect of disconnecting the medial ventral pallidum (VPm) from the MD or the ventral tegmental area (VTA) during a specific PIT test. The results demonstrated that VPm projections to the MD, but not to the VTA, abolished specific transfer. While the excitatory influence of Pavlovian stimuli on action performance was preserved, such that responding during the CSs was significantly above baseline, the effect was non-reinforcer specific, with equal performance during both the *same* and *different* conditions (Leung & Balleine, 2015). Interestingly, VPm-VTA disconnection led to a higher rate of responding during the baseline period when

compared to the control groups, suggesting a role of the VTA in mediating the motivational component of specific PIT, in line with previous studies (Corbit et al., 2007).

More recently, it has been shown that the VPm exerts GABA_a-mediated inhibition of the MD, and inactivation of VPm terminals in the MD confirmed the requirement of the VPm→MD pathway for specific PIT (Leung et al., 2024). Conversely, chemogenetic stimulation of the VPm→MD pathway was found to enhance the specific PIT effect. The MD exerts feedforward inhibition over the lateral OFC (lOFC) via excitation of inhibitory PV neurons which in turn inhibit local pyramidal neurons. Chemogenetic stimulation, but not inhibition, of the MD-lOFC pathway was hence found to abolish the specific PIT effect. Finally, by combining the two previous chemogenetic treatments in the same subject in opposing hemispheres (i.e. inhibition of VPm→MD pathway and activation of MD→lOFC pathway), it was elegantly shown that expression of specific PIT requires a VPm→MD→lOFC circuit. Particularly, while subjects were sensitive to the excitatory influence of Pavlovian stimuli, stimulus-induced action selection was not reinforcer specific (Leung et al., 2024). These results highlight the involvement of a ventral pallidum-thalamocortical circuit in driving the biasing properties of predictive stimuli.

2.5. Contribution of ventral tegmental area to PIT

The functional role of the VTA, which is situated in the midbrain and serves as a primary source of dopaminergic projections, has been studied in the expression of both general and specific transfer. Murschall & Hauber (2006) transiently inactivated the VTA using baclofen/muscimol and found that general PIT was abolished such responding was not elevated during the CS+. Nonspecific drug effects on instrumental responding were ruled out as no significant differences in baseline or CS- responding were observed

among the different groups. Similarly, Corbit, Janak, and Balleine also inactivated the VTA with baclofen/muscimol before testing, using the full transfer paradigm (Corbit et al., 2007). They observed that performance in the general condition was similar to baseline, revealing an abolished general PIT effect. However, unlike Murschall and Hauber, they found that baseline responding was lower in the baclofen/muscimol group compared to the saline group, suggesting a more general role of the VTA in action initiation.

More recently, cre-dependent inhibitory DREADDs were injected in the VTA of TH-cre rats, to specifically express DREADDs in dopaminergic neurons (Halbout et al., 2019). Chemogenetic inhibition of VTA dopaminergic neurons during the general PIT test was shown to abolish the excitatory influence of the CS+ on action performance. Moreover, rats were implanted with cannulas either in the NAc core or in the mPFC to inhibit hM4Di-expressing dopaminergic terminals coming from the VTA, via local infusion of the DREADD ligand clozapine-n-oxide (CNO). Results revealed that general PIT was evident both when VTA→NAc core or VTA→mPFC were selectively inhibited, albeit a significantly attenuated transfer effect when the VTA→NAc core pathway was inhibited, in comparison to vehicle-infused controls. Collectively, these results suggest that dopaminergic innervation of the NAc core and the mPFC proves to be insufficient in driving the excitatory influence of Pavlovian stimuli on action performance.

In contrast to the dual requirement of both the CeA and the VTA in general PIT, disrupting their interaction has been shown to preserve general transfer, while disconnecting the CeA from the substantia nigra pars compacta (SNpc) significantly impairs the general transfer effect (El-Amamy & Holland, 2007). This may be explained by potential cross-hemispheric inhibitory connections between the two VTAs and CeA output to the SNpc (H. J. Lee et al., 2011).

Pharmacological inactivation of the VTA also abolishes the expression of specific PIT (Corbit et al., 2007), and interactions between the VTA and the BLA appear to mediate this effect. Indeed, Sias and colleagues recently demonstrated that optogenetic inhibition of the VTA-BLA pathway during Pavlovian conditioning abolishes the expression of specific PIT (Sias et al., 2024). TH-cre rats infected with a cre-dependent inhibitory opsin in the VTA, and an optical fiber placed in the BLA revealed that dopaminergic innervation of the BLA was required during Pavlovian learning to later bias action selection at test. While the general incentive properties of the Pavlovian stimuli on responding was preserved, action selection was not reinforcer specific when VTA dopaminergic terminals in the BLA were optogenetically inhibited.

2.6. Summary

The neural substrates of general and specific PIT have been extensively studied during the past two decades revealing notable dissociations within different brain structures (see **Table 2** and **Table 3** in **Appendix I** for a summary of results). Notably, a double dissociation has been identified within both the amygdala and the ventral striatum. Specifically, NAc core and CeA are required for general PIT, whereas NAc shell and BLA are essential for specific PIT (**Table 1**). Importantly, no structure has been shown to be functionally required for both general and specific PIT, except for the VTA (Corbit et al., 2007; Halbout et al., 2019; Murschall & Hauber, 2006).

Since the transfer effect is mediated by the integration of both Pavlovian S-O and instrumental A-O associations, brain regions involved in the acquisition or retrieval of such processes are also likely involved in PIT. As previously mentioned, the amygdala is crucial for processing S-O associations (Holland & Gallagher, 1999; Petrovich, 2011). This

suggests that the PIT deficits observed when inactivating amygdala subnuclei may result from an inability to process some aspects of Pavlovian contingencies. Similarly, A-O encoding requires the DMS (Balleine et al., 2009; Corbit & Janak, 2010) while S-O encoding requires both DMS and DLS (Corbit & Janak, 2010). Thus, the role of the dorsal striatum in PIT likely stems from its involvement in S-O and A-O learning (Corbit & Janak, 2007b, 2010). Interestingly, the NAc shell is not required for S-O or A-O learning (Bertran-Gonzalez & Laurent, 2018; Corbit et al., 2001) yet it is critical to show specific PIT, which suggests that it may be a key region in the *integration* of Pavlovian and instrumental associations and, hence, for stimulus-guided action.

Research on the cortical contributions to PIT has remained relatively scant, particularly for general PIT. Indeed, the ACC is the only cortical region that has been investigated in general PIT, and studies have shown that it is not required (Cardinal et al., 2003). By contrast, the IOFC, via its connections with the BLA, is required for specific PIT (Sias et al., 2021) and this is perhaps due to its role in tracking instrumental contingencies (Cerpa et al., 2023; Parkes et al., 2018) and in S-O encoding (Sias et al., 2021). The mOFC is also required for specific PIT (L. A. Bradfield et al., 2015). Beyond the OFC, two other prefrontal regions, the IL and the PL, have been assessed for their potential contribution to specific PIT. The PL has been shown to contribute to A-O acquisition in a relatively transient fashion, which may explain why pretraining PL lesions do not impair specific PIT (Corbit & Balleine, 2003). The IL, in contrast, is believed to contribute to habitual instrumental responding, and appears necessary for specific PIT expression (Keistler et al., 2015; Killcross & Coutureau, 2003). Additionally, specific PIT involves a cortico-amygdalo-cortical loop (IOFC→BLA→IOFC) as highlighted by Sias and

colleagues (2021), as well as a pallido-thalamo-cortical pathway (VPm→MD→IOFC), further emphasizing the central role of the IOFC in PIT (Leung et al., 2024).

Overall, the evidence reviewed in this section underscores the extensive network of brain regions involved in mediating the complex effects of reward-predicting stimuli on instrumental action (see **Table 1**). It is noted that the cortical contribution to PIT has garnered less attention than the contribution of subcortical regions, and general PIT has received less empirical attention than specific PIT. Notably, the role of the insular cortex (IC) in PIT has yet to be investigated. In the final section of this introductory chapter, we will explore evidence suggesting that the IC, particularly its gustatory region - the gustatory cortex - may be a key cortical structure in PIT.

Region	Subregion	General PIT	Specific PIT
Amygdala	BLA	×	✓
	CeA	✓	×
Cortex	ACC	×	?
	IL	?	✓
	IOFC	?	✓
	mOFC	?	✓
	PL	?	×
	VPm	?	✓
Striatum	DMS	?	✓
	DLS	?	✓
	NAc core	✓	×
	NAc shell	×	✓
Thalamus	MD	?	✓

Table 1. Summary of cortical and subcortical involvement in general and specific PIT. A green check mark (✓) to indicate regions required for PIT, a red cross (×) denotes non-essential regions, and a gray question mark (?) indicates that the region has not yet been investigated. **ACC:** anterior cingulate cortex; **BLA:** basolateral amygdala; **CeA:** central amygdala; **DMS:** dorsomedial striatum; **DLS:** dorsolateral striatum; **IL:** infralimbic; **IOFC:** lateral orbitofrontal cortex; **MD:** mediodorsal thalamus; **mOFC:** medial orbitofrontal cortex; **NAc core:** core of nucleus accumbens; **NAc shell:** shell of nucleus accumbens; **PL:** prefrontal cortex; **VPm:** medial ventral pallidum.

3. THE GUSTATORY CORTEX AND APPETITIVE BEHAVIOR

The rodent insular cortex (IC) is often described as a “*hub linking large-scale brain systems*” (for a review see [Gogolla, 2017](#)). The IC is intricately connected with an extensive network of cortical and subcortical regions involved in sensory, emotional, motivational, and cognitive functions. It receives sensory inputs from all modalities, processing information about both the external environment (auditory, somatosensory, olfactory, gustatory, and visual inputs) and internal bodily states (i.e., interoceptive information). This multimodal integration suggests that the IC is ideally positioned to monitor the environment as well as current emotional and physiological states, both of which are crucial for adaptive decision-making.

A particularly interesting region of the IC is its gustatory portion, the gustatory cortex (GC), which has been primarily studied for its role in taste perception but is also critical for food- and taste-related decision-making. As we will discuss further in this section, this region of IC plays a key role in making choices based on the current value of outcomes ([Balleine & Dickinson, 2000](#); [Parkes et al., 2015, 2018](#); [Parkes & Balleine, 2013](#)) and its activity is modulated by reward-predicting stimuli. Importantly, the IC is highly interconnected with the PIT neural network. Thus, we will explore compelling anatomical, physiological, and behavioral evidence that highlights the potential role of the IC, and in particular its gustatory region, in PIT.

3.1. Anatomical considerations

The rodent IC is a longitudinal strip occupying the dorsal bank of the rhinal sulcus, and is involved in various sensory and cognitive functions (Bermudez-Rattoni, 2014; Gogolla, 2017). Extending ventrally to the piriform cortex and dorsally to the somatosensory cortex, the IC spans from the lateral OFC to the perirhinal cortex along the antero-posterior axis as shown in **Figure 12.A-C** (Saper, 1982). Situated caudally to the lateral prefrontal cortex, the IC receives inputs notably from the gustatory (thalamic, amygdalar and prefrontal regions), visceral (parabrachial nucleus), somatosensory systems, processing polymodal information about the environment and bodily states (Cechetti, 1987; Fontanini, 2023; Gehrlach et al., 2019; Hanamori et al., 1998; Vincis & Fontanini, 2016). Along its dorsoventral axis, the IC consists of interconnected layers, including the granular, dysgranular, and agranular layers (**Figure 12.B**).

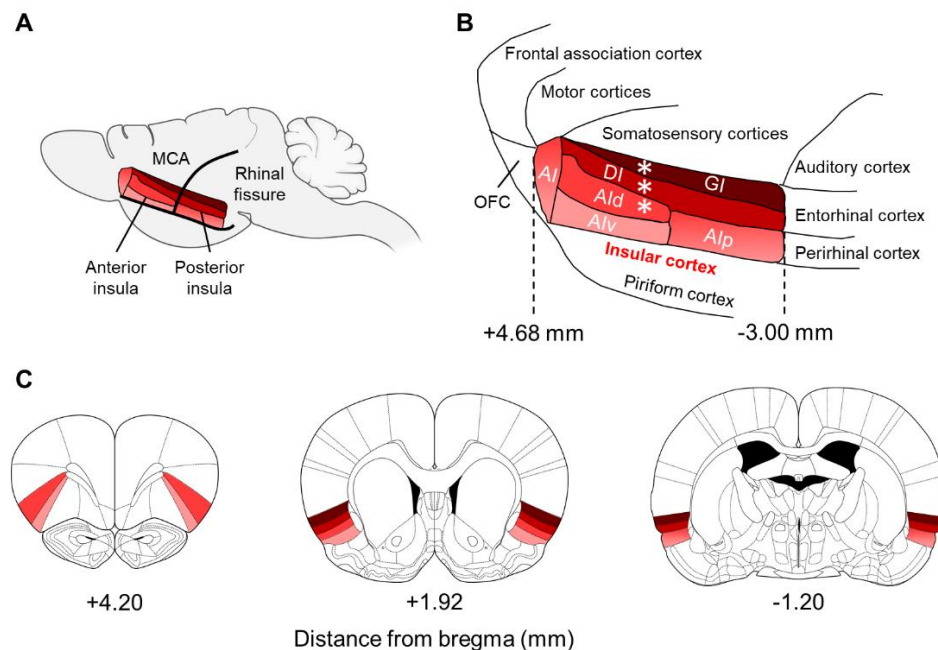


Figure 12. Location of the rodent insular cortex (IC). (A) IC is visible on the lateral surface of the brain, positioned above the rhinal fissure, with the medial cerebral artery (MCA) crossing over it. (B) Subdivisions include agranular IC (AI), with dorsal (Ald), ventral (Alv), and posterior (Alp) parts, dysgranular IC (DI), and granular IC (GI). White stars indicate regions that respond to gustatory stimuli (Ald, DI and GI). (C) Coronal cross-sections of the rat IC. Adapted from Gogolla (2017).

The IC is anatomically well placed within the neural circuitry known to mediate PIT (**Figure 13**). Notably, it sends inputs to the core and shell subregions of the NAc, and is reciprocally connected to the MD (G. J. Lee et al., 2022; Wright & Groenewegen, 1996), the BLA and the CeA (Gehrlach et al., 2020), the VTA (Gil-Lievana et al., 2020; Hernández-Ortiz et al., 2023) and to the OFC (Barreiros et al., 2021; Y. Chen et al., 2022; Mathiasen et al., 2023). Notably, MD, BLA and VTA are known to project to IC regions involved in gustatory-related information processing (Maffei et al., 2012). Thus, the IC appears to be ideally placed to contribute to both general and specific PIT.

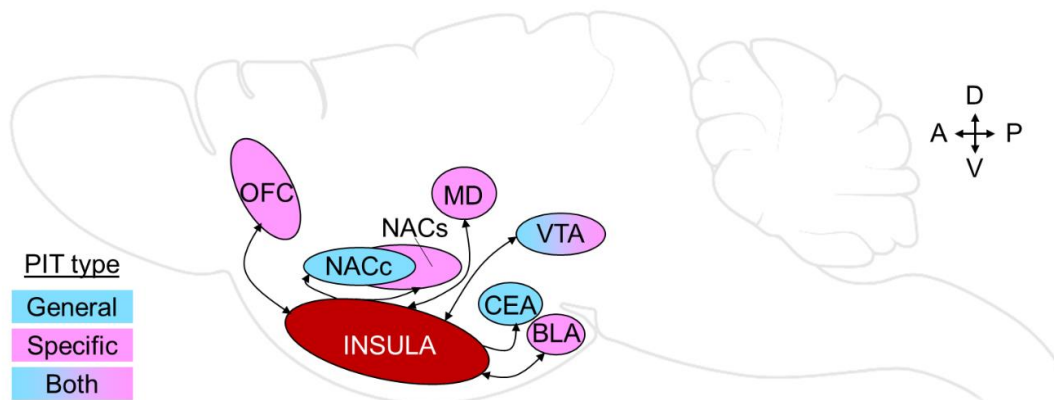


Figure 13. Pavlovian-to-instrumental transfer neural substrates and their connections with the insular cortex. The insular cortex (IC) or insula is anatomically connected to the nucleus accumbens core (NACc) and shell (NACs), central amygdala (CEA), basolateral amygdala (BLA), the orbitofrontal cortex (OFC), the mediodorsal thalamus (MD) and the VTA (ventral tegmental area). Hence, the IC is highly interconnected with brain structures playing a role in general PIT (blue), specific PIT (pink) or both (blue-pink gradient). *A*: anterior; *D*: dorsal; *P*: posterior; *V*: ventral.

Within the IC, the gustatory cortex holds particular significance, playing a crucial role in encoding taste-related information and occupying approximately 15% of the total IC area (Kosar et al., 1986; Yamamoto et al., 1980). It receives input from limbic regions, including the prefrontal cortex, which provides anticipatory and reward-related information as will be discussed later (Allen et al., 1991; Hoover & Vertes, 2011; Shi & Cassell, 1998). Somatosensory (texture, temperature), olfactory (retronasal), and visceral (hunger, thirst,

malaise) signals converging in the gustatory region of IC, might contribute to complex taste representations (Bouaichi et al., 2023; de Araujo & Simon, 2009).

How does taste-related, gustatory information reach the IC? Taste is first detected by taste buds on the tongue, from where gustatory information is relayed to 2nd-order neurons of the nucleus of the solitary tract (NST) in the medulla (**Figure 14**) (Frank & Barry, 2017). The sensory component of gustatory information is then sent to the thalamic gustatory relay, namely the parvicellular ventral posteromedial thalamic nucleus (VPMpc), also called gustatory thalamus, via the parabrachial nucleus (PBN) in rodents (Fontanini, 2023). Subsequently, VPMpc 3rd-order neurons project to the primary gustatory cortex. By definition, a primary sensory cortex is the cortical area that receives the most direct projections from the specialized sensory relay nucleus in the thalamus (Frank & Barry, 2017). Thus, the primary gustatory cortex must receive a considerable amount of direct projections from the VPMpc. The most extensive VPMpc projections are to the dorsal part of the IC, the so-called gustatory cortex, from where 4th-order neurons relay gustatory information to higher-order brain regions such as the prefrontal cortex, where they contribute to higher cognitive processes such as planning, anticipation and decision-making (Frank & Barry, 2017). From the PBN, the affective component of gustatory signals is conveyed to the amygdala (limbic pathway) before converging in the gustatory cortex (**Figure 14**).

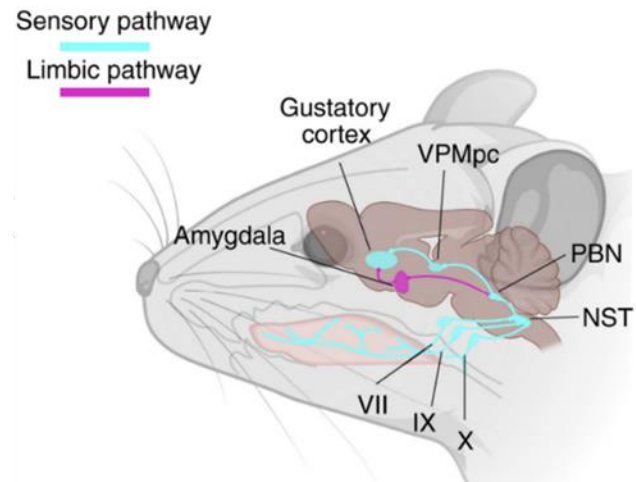


Figure 14. Simplified anatomical schematic of the gustatory system in rodents. Gustatory information is transmitted from the taste buds via three cranial nerves: the facial (VII), glossopharyngeal (IX), and vagus (X). These nerves innervate distinct regions of the tongue, palate, pharynx, and larynx. The information is first relayed to the nucleus of the solitary tract (NST) and then to the parabrachial nucleus (PBN) in rodents. From the brainstem, gustatory information bifurcates along two bottom-up pathways—a sensory and a limbic route—that converge in the gustatory cortex (GC). The sensory pathway (cyan) carries taste signals to the parvicellular portion of the ventral posteromedial nucleus of the thalamus (VPMpc), which then projects to the GC in the insular cortex. This area sends outputs to several frontal regions, including the medial prefrontal and orbitofrontal cortices, the contralateral GC, the olfactory cortex, the striatum and ventral striatum, and the amygdala. The limbic pathway (magenta) conveys the affective component of taste to the GC via the amygdala. Additional regions in the limbic system, such as the mediodorsal thalamus and lateral hypothalamus, also receive ascending gustatory inputs, bidirectional connections within the gustatory system and connections with other sensory systems. Adapted from [Fontanini \(2023\)](#).

Hence, the GC has been historically considered as the primary gustatory cortex, prompting extensive research on its role in taste perception. Early studies have primarily localized the GC within the dorsal agranular layer of the IC (**Figure 12.B**), using a combination of electrophysiological recordings, thalamocortical anatomical tracings and cytoarchitectural analysis ([Kosar et al., 1986](#)). However, more recent studies employing extracellular recordings of insular activity in anesthetized rodents revealed that taste-responsive neurons were, in addition to the agranular layer, also located in the granular and dysgranular layers (c. f. **Figure 12.B**), both known to receive projections from the gustatory thalamus ([Allen et al., 1991](#); [Maffei et al., 2012](#); [Nakashima et al., 2000](#)). Two-

photon calcium-imaging in alert mice further confirmed the notion that taste neurons are spatially distributed in the IC, with no evidence of a topographic (or gustatotopic) mapping (K. Chen et al., 2021). Moreover, taste-responsive neurons have been shown to respond to stimuli of various modalities, such as somatosensorial and visceral stimuli (Hanamori et al., 1998), olfactory (Blankenship et al., 2019), but also auditory and visual stimuli (Vincis & Fontanini, 2016), as well as aversive stimuli (Gehrlach et al., 2019). Due to the prevalence of polymodality and the absence of gustatotopic organization akin to that found in the visual or somatosensory cortex, Boughter and Fletcher challenged in a recent review the notion of a primary cortical area dedicated to taste perception or discrimination, arguing that the IC does not contain a specialized “gustatory cortex” (Boughter & Fletcher, 2021). Instead, they propose that taste inputs are among many sensory channels that converge within the IC, which functions more as an association cortex rather than a dedicated gustatory area.

Altogether, these anatomical considerations suggest that the IC, and particularly its “gustatory cortex” (GC), can encode multiple sensory, affective and cognitive signals associated with the experience of taste, ultimately controlling food intake (de Araujo & Simon, 2009). Here, we will explore research suggesting that the function of the gustatory region of IC extends beyond mere taste perception, integrating multiple types of information that potentially contribute to taste-related decision-making.

3.2. Taste memory hypothesis

In everyday life, animals and humans experience a limited set of taste qualities that each vary in perceptual intensity. Traditionally, tastes are categorized into four basic qualities: salty, sweet, sour, bitter. A fifth taste is sometimes considered umami (or savory). These tastes help individuals identify foods rich in essential nutrients (such as

sweet, salty, or umami) while avoiding potentially toxic substances (such as bitter and sour). Taste significantly influences food preferences and eating behaviors, encouraging the consumption of palatable foods. Thus, taste is crucial in helping individuals remember which foods are beneficial or harmful, aiding in future food choices. This is notably exemplified by the neophobia that rodents manifest when encountering an unknown taste, ingesting small portions to assess the safety of consuming a new food, or in conditioned taste aversion procedures, where a taste is paired with illness, leading to the devaluation of the associated food outcome. The resulting avoidance of a previously illness-paired food strongly relies on the subject's ability to encode and retrieve the association between a specific taste and the aversive event, as discussed later in this chapter (Braun et al., 1972; Kiefer et al., 1984), making taste critical for survival. Indeed, patients experiencing partial to complete loss of taste (hypoageusia or ageusia) may develop a reduced appetite due to the diminished ability to enjoy food (Rathee & Jain, 2024). This can result from difficulty in recognizing flavors, making eating a less pleasurable and sometimes frustrating experience. Consequently, these challenges may lead to weight loss or malnutrition. Additionally, the loss of taste can have significant psychological effects, potentially contributing to emotional distress, including depression or anxiety.

Research conducted during the 1950s and 1960s laid the groundwork for identifying the insular cortex as a pivotal component of the gustatory system (Braun et al., 1982). Early investigations primarily focused on understanding the IC's role in taste detection, taste learning, and taste memory. Notably, both sensory (taste quality) and limbic (affective taste-related signals) pathways converge in the IC (c.f. **Figure 14**), potentially contributing to complex taste representations, such as the pleasant sweetness of sucrose or the aversive bitterness of quinine. In the early 1970s, the first study on the involvement

of the IC in taste aversion learning emerged, revealing that rats with lesions of the gustatory region of IC (gustatory cortex, GC) rats failed to exhibit conditioned taste aversion (CTA) to saccharin (Braun et al., 1972). Water restricted rats with or without GC-lesions were presented with one of three aqueous drinking solutions, plain water, saccharin-flavored, or quinine-flavored water. Immediately following this first exposure to the gustatory stimuli, saccharin or quinine tastes were paired with illness. Subsequently, rats were subjected to the three drinking solutions to assess the CTA to the different gustatory stimuli. Results revealed that while non-lesioned rats drank less saccharin-flavored water when it was paired with illness, compared to plain water and vehicle-treated subjects, rats with GC-lesions did not show such an aversion to saccharin taste irrespective of treatment, drinking as much saccharin flavored water than plain water, similarly to vehicle-treated non-lesioned rats (**Figure 15**).

Moreover, vehicle-treated GC-lesioned rats showed a significant preference for saccharin over water, suggesting that the deficit observed is not sensory but rather associative. However, GC-lesioned rats for which quinine was paired with illness displayed normal rejection of the quinine-flavored water, arguing against a GC-lesion induced general inability of rats to associate gustatory stimuli with illness. The authors hypothesized that the learned rejection to quinine “*could represent simply an enhancement of an innate aversive tendency whereas rejection of the saccharin cue could be considered a reversal of an innate acceptance tendency, and one could argue that the latter performance would require a more elaborate mechanism than the former*” (Braun et al., 1972; p641), suggesting that the response to quinine could be innate. Nonetheless, it is clear from this study that GC-lesioned rats failed to utilize saccharin as a stimulus to guide ingestion or rejection responses.

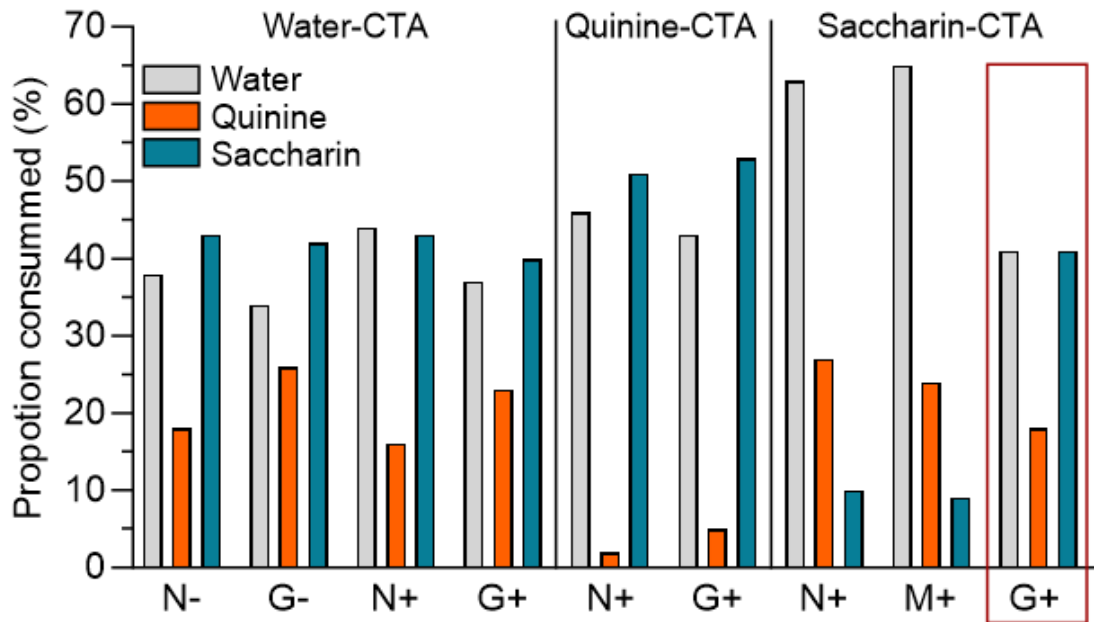


Figure 15. Ablation of the rat gustatory cortex abolishes conditioned taste aversion to saccharin but not to quinine. Rats were randomly assigned to one of three groups: the GC lesion group (G), the medial neocortex control lesion group (M), or the non-operated group (N). After the initial exposure to quinine (quinine-CTA), saccharin (saccharin-CTA), or water (water-CTA), the rats were injected with either a vehicle (saline, indicated by “-”) or the toxic agent cyclophosphamide to induce a taste-illness association (indicated by “+”). As expected, the CTA procedure did not forge an association between illness and water, as animals consumed less of the quinine-flavored solution compared to water and saccharin-flavored water, regardless of lesion or treatment status. Aversion to quinine was heightened after injection with cyclophosphamide in both non-lesioned and GC-lesioned rats, indicating no deficit in the acquisition of CTA to quinine. However, CTA to saccharin revealed different results. In cyclophosphamide-treated non-lesioned rats (N+) and rats with medial neocortex lesions (M+), consumption of saccharin-flavored water was markedly suppressed compared to plain water, and even quinine. In contrast, GC-lesioned rats (G+) failed to show such an aversion, drinking as much saccharin-flavored water as plain water, as highlighted by the red rectangle. These findings suggest that an intact GC is necessary to develop an aversion to a taste that is innately desirable. Adapted from [Braun et al., \(1972\)](#).

Nearly a decade later, Braun and colleagues conducted further experiments where rats were conditioned for taste aversion using either sucrose or saline, followed by GC lesions. Subsequent tests conducted a month later demonstrated that while control rats maintained specific aversions to each stimulus, GC-ablated rats exhibited a complete loss of aversions ([Braun et al., 1981](#)). Importantly, this taste agnosia was not attributed to

ageusia (i.e., a total loss of taste), as GC-lesioned rats displayed normal preferences for palatable solutions over aversive ones, and their taste thresholds for elementary tastes remained unaltered. Similarly, GC inactivation does not prevent rats to preferentially consume a non-devalued outcome (established via sensory specific satiety) over a devalued one, suggesting intact taste perception (Balleine & Dickinson, 2000; Parkes et al., 2015, 2018; Parkes & Balleine, 2013). Additionally, both control and GC-lesioned rats exhibit similar sensitivity to emetic treatments when trained to develop potentiated aversions to odors, suggesting that the observed agnosia was specific to gustation. Consequently, the “*taste memory*” hypothesis emerged. This hypothesis proposes that the GC enables animals to recall taste features over the delay between taste detection during consumption and detection of post-ingestive effects, thereby facilitating the association between taste and illness. This suggests some degree of dissociation between the primary perception of taste (detection and categorization), unaffected in GC-lesioned rats, and taste processing (learning and memory), which is impaired in GC-lesioned rats (Kiefer & Orr, 1992).

These findings emphasize the crucial role of the GC in retrieving associations between the specific sensory properties of a US, like taste, and its post-ingestive effects, such as illness. The inability of animals lacking a functional GC to infer the aversive consequences of consuming a taste previously paired with illness, as indicated by elevated consumption of the illness-paired solution, is further confirmed by assessment of conditioned orofacial rejection responses. When experiencing an aversive taste, rodents show typical behaviors of rejection reactions such as gapes, chin rubs and paw treads. GC-lesioned rats fail to show such rejection responses to illness-paired flavors, which are thought to rely on the retrieval of the sensory-specific aspects of outcomes (Kiefer & Orr, 1992). This result is in

accordance with the taste memory hypothesis, which stipulates that the GC facilitates the association between taste and illness, and highlights the GC's importance in informing digestive decision-making.

These findings highlight the critical role of the IC, and specifically the GC, in taste memory and the retrieval of specific sensory associations between taste and its post-ingestive consequences. Building on this understanding of the GC's involvement in associative learning, subsequent research has delved into the temporal dynamics of GC neural activity and how these patterns correlate with behavioral responses to different tastants. Several studies have identified distinct phases within the GC's response timeline, each corresponding to different aspects of taste processing. The initial epoch, lasting around 250 ms, encodes the taste's contact with the tongue; the second phase, from 250 ms to 1 second, captures the chemosensory properties of the tastant; and the third phase, after 1 second, represents the tastant's palatability, correlating with appetitive or aversive behaviors (N. Mukherjee et al., 2019). Essentially, over a 1.5-second period following taste's contact with the tongue (post-taste period), GC responses transition from encoding taste identity to predicting a rat's consumption decision, whether to ingest or reject the tastant (Katz et al., 2001; Maier & Katz, 2013; Sadacca et al., 2012). A brief optogenetic disruption of the GC during this transition delays the animal's decision to expel a bitter taste (N. Mukherjee et al., 2019). These findings suggest a specific epoch where the GC encodes choice-related information guiding consummatory decisions. This relies, in this case, on an innate, naturalistic behavior based on the aversiveness of bitter solutions.

The decision to ingest or reject a particular food is influenced not only by its taste but also by other modalities such as sensory, somatosensory, and visceral inputs, as well as emotional and motivational states. Taste-responsive neurons in the GC respond to a

variety of sensory modalities (Vincis & Fontanini, 2016), and play a crucial role in associative learning, as demonstrated in CTA experiments (Braun et al., 1972, 1981; Kiefer et al., 1984; Kiefer & Orr, 1992), which raises interesting questions regarding the GC's potential role in stimulus- and value-guided behaviors.

3.3. GC and stimulus-outcome learning

Imagine watching as the waiter serves the hot chocolate you ordered. The sight and sound of the beverage being poured, the steam rising from the mug, and the rich chocolate aroma floating in the air create a sensory anticipation—you can almost taste it. When you finally take your first sip, the flavor is no surprise; it is exactly as your eyes and nose had predicted. As developed in previous sections, the ability to anticipate the availability of desired outcomes relies on the ability to encode and retrieve stimulus-outcome (S-O) associations formed through Pavlovian learning. In this subsection, we will review evidence suggesting that the GC integrates information from various sensory modalities, and that associative learning shapes outcome representations in the GC.

Fontanini and colleagues recorded single-unit responses to auditory, visual, somatosensory, and olfactory stimuli in the GC of alert rats, both before and after these stimuli were paired with a sucrose solution (Vincis & Fontanini, 2016). In naïve rats (group untrained), 70% of taste-selective GC neurons (neurons showing significantly different responses to the four elementary gustatory stimuli) were exclusively activated by gustatory stimuli, while 25% and 5% were also activated by one or two cross-modal stimuli, respectively. Among cross-modal-responsive neurons (non-taste-selective), the majority (~84%) were activated exclusively by one of the four non-taste stimuli, while a minority responded to two or three cross-modal stimuli. This suggests that GC neurons

are modulated by taste *and* other sensory modalities. However, representations of stimuli from different sensory modalities overlap only minimally.

In a subsequent experiment, Fontanini and colleagues investigated the effectiveness of different cross-modal stimuli in facilitating the learning of stimulus-outcome (i.e., Pavlovian) associations. Rats were trained to associate each of four non-gustatory stimuli (auditory, visual, somatosensory, and olfactory) with the intraoral delivery of sucrose (group trained). Pavlovian conditioning was tracked by monitoring orofacial movements in response to the CS. After 14 days of conditioning, the rats demonstrated a significant increase in orofacial movements during the presentation of the stimuli, indicating anticipatory behavior in expectation of the sucrose reward. These findings suggest that each of the four non-gustatory stimuli can be successfully associated with taste.

Next, the authors investigated how Pavlovian learning shapes cross-modal representations in the GC. It was first observed that, in trained rats, the prevalence of neurons responding to non-gustatory stimuli specifically increased in comparison to untrained rats, while the proportion of taste-selective neurons remained unchanged. Moreover, trained rats showed a significantly higher number of GC neurons responding to both taste and non-gustatory stimuli (60%) compared to untrained rats (30%), indicating that Pavlovian conditioning induced an increase in stimulus-responsive neurons in the GC. Notably, there was a significant decrease in the proportion of neurons responding to only one non-taste stimulus in trained rats, and a significant increase in neurons responding to two non-taste stimuli.

Altogether, these findings suggest that S-O learning enhances the overlap between representations of multiple stimuli, and between representations of taste and non-gustatory stimuli, supporting the notion of an associative gustatory cortex. The greater

overlap in representations in GC neurons following stimulus-sucrose pairings could be assumed to reflect the encoding of a common property among these stimuli, potentially their predictive value or reward expectation. If this assumption is true, one could expect a decrease in conditioned, anticipatory, responses to reward-predicting stimuli when the GC is inactivated during presentation of these stimuli. Perhaps not surprisingly, during Pavlovian conditioning, neuronal activity in the mouse GC has been shown to be modulated throughout the different phases of stimulus-outcome pairings e.g. stimulus presentation (reward expectation), food delivery, and food consumption (M. P. H. Gardner & Fontanini, 2014; Kusumoto-Yoshida et al., 2015; Livneh et al., 2017; Samuelsen et al., 2012). More interestingly, pharmacological inactivation and optogenetic silencing of the GC during stimulus presentation significantly reduces conditioned food-port entries in rodents, suggesting that stimulus-evoked activity in the GC is required to drive food-directed behavior (Kusumoto-Yoshida et al., 2015).

Together, these data demonstrate that cross-modal stimulus-related activity is observed in the GC, and that associative learning profoundly shapes activity of GC neurons. This is illustrated by the convergence of taste and non-gustatory signals onto single GC neurons, possibly reflecting the encoding of a common reward-expectation across stimuli. These findings prompted further investigations regarding the temporality of taste perception and processing in the GC, in regards to food-directed choices.

As previously described, GC neurons are shaped by associative learning, during which representation of stimuli associated with a common reward converge onto single neurons, potentially reflecting reward-expectation (Vincis & Fontanini, 2016). It is however unclear at this point if, in addition to chemosensory information, the GC also encodes cognitive variables such as planning for a specific behavioral choice. This possibility was

also investigated by Vincis and colleagues (Vincis et al., 2020). In a perceptual discrimination task, head-restrained mice were trained to lick a central spout to receive one of four informative tastes during a sampling epoch as shown in **Figure 16.A** (Vincis et al., 2020). The mice then had to wait through a short delay epoch before deciding whether to lick left or right for a water reward. The taste cues were designed so that stimuli with similar perceptual features were associated to opposing actions: sweet sucrose or bitter quinine indicated that mice should perform a left lick for the water reward, while sweet maltose or bitter sucrose octaacetate indicated a right lick. Recording GC activity during this task revealed that, within the first 500 ms, GC neurons effectively discriminated between the four tastes (left panel **Figure 16.B**). However, during the subsequent epoch, population decoding of neural activity showed that GC neuron firing for tastants associated with the same action started to converge, indicating an impending decision (right panel, **Figure 16.B**). Importantly, while GC's inactivation during sampling of the tastant had no impact on performance (**Figure 16.C**), the same manipulation during the delay epoch significantly disrupted mice's choice behavior (decrease in correct choices) as shown in **Figure 16.D**. These findings suggest that the GC encodes signals that are essential for stimulus-guided choice, challenging the classical hierarchical view of primary sensory cortices, which proposes that such areas merely passively relay information to higher-order brain regions.

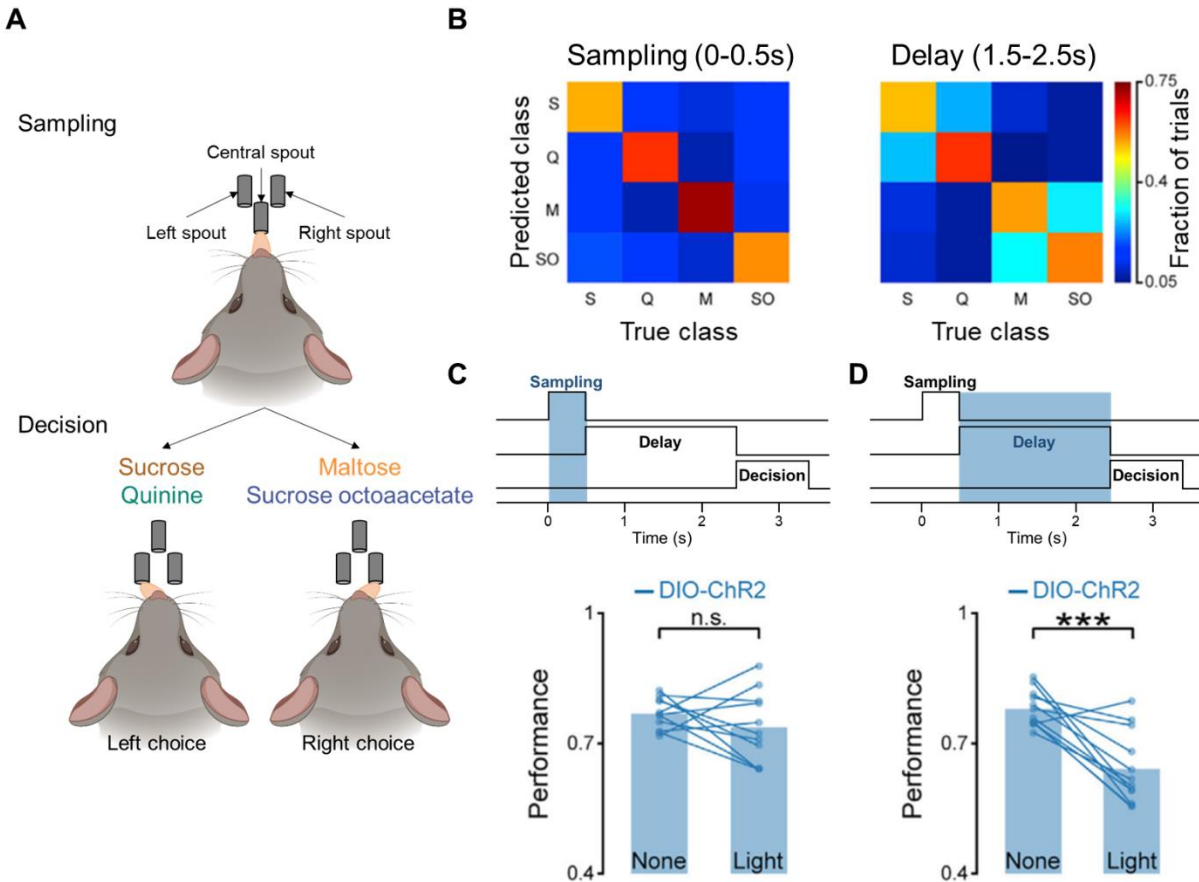


Figure 16. Transition from taste-representation to taste-guided choice in the rodent gustatory cortex supports the performance of stimulus-guided discrimination. (A) Water-deprived head-restricted mice sample one of four tastes from a central spout. Two of the tastants are sweet and palatable (sucrose: S, maltose: M) while the two others are bitter and non-palatable (quinine: Q, sucrose octoacetate: SO). Pairs of tastes were arranged such as each consisted of one sweet and one bitter taste. One pair indicated that the mouse had to lick the left spout (S or Q) while the other indicated a right lick (M or SO), to obtain a water reward. **(B)** Confusion matrices for population decoding of neural activity: the classifier was able to predict the true class of the experienced taste with a high accuracy during the first 500 ms of the trial, as revealed by the apparent diagonal. However, toward the second half of the delay period (1.5-2.5s post-stimulus), the classifier makes a considerable amount of incorrect predictions. Specifically, the classifier makes mistakes for pairs of tastes indicating the same licking direction, suggesting choice-related neural activity in the GC. **(C)** Top panel: schematic of trial structure and period of optostimulation (sampling epoch). Bottom panel: behavioral performance without and with light stimulation in PV-Cre mice injected in GC with excitatory opsin ChR2-EYFP reveals no deficit following stimulation during sampling period. **(D)** Top panel: schematic of trial structure and period of the optostimulation (covering the delay epoch). Bottom panel: behavioral performance in PV-Cre mice injected in GC with excitatory opsin ChR2-EYFP reveals a decrease in correct choices following stimulation of PV neurons during delay epoch. Bar plots represent the mean value of the performance. *** $p < 0.001$; n.s.: non-significant statistical difference. Adapted from Vincis et al., 2020.

3.4. GC and instrumental action

As described in the first section of this chapter, goal-directed actions are driven by knowledge of the relationship between a specific action and its outcome, as well as knowledge of the current value of the outcome. Such value-guided behaviors are critical for adaptive behavior as they permit organisms to pursue specific outcomes or goals that align with their current needs or desires. [Balleine & Dickinson \(2000\)](#) first investigated the role of GC in such value-guided actions using the outcome devaluation procedure. Rats received excitotoxic lesions of the GC and were then trained to perform two different actions to obtain two different food outcomes ([Balleine & Dickinson, 2000](#)). Acquisition of instrumental performance occurred similarly in sham- and GC-lesioned rats and it was shown that instrumental learning per se was intact in rats with GC lesions. Subsequently, one outcome was fed to satiety, inducing its sensory-specific devaluation. The effect of devaluation on action selection was then assessed in a non-rewarded test. Sham-operated rats showed a strong reduction in the performance of the action associated with the devalued outcome, preferentially performing the action associated with the still-valued outcome. However, such preference was not observed in GC-lesioned rats, for which performance was equally distributed over the two actions, reflective of impaired value-guided action selection.

Critically, a subsequent rewarded test (in which the outcomes were again available) showed that rats with GC lesions did indeed reject the devalued outcome, indicating no deficit in taste detection or assignment of incentive value to the taste. Hence, the deficit in action selection can't be attributed to an insensitivity to the devaluation treatment, nor to an inability to retrieve specific A-O associations. Rather, the deficit appeared to lie in the recall of the representation of the devalued outcome in its absence. Indeed, the results

indicated that GC specifically contributes to the retrieval of outcome value to guide action rather than the instrumental learning process itself (Balleine & Dickinson, 2000). Studies employing temporary perturbation of GC function have confirmed that the gustatory region of IC is required to retrieve outcome representations. Indeed, pharmacological (Parkes et al., 2015; Parkes & Balleine, 2013) or chemogenetic (Parkes et al., 2018) disruption of GC only during the test phase of the outcome devaluation paradigm renders rats unable to choose an action earning a valued outcome more than an action earning a devalued outcome. Moreover, intact communication between the GC and both the NAc core (Parkes et al., 2015) and the BLA (Parkes & Balleine, 2013) is also required. Collectively, these findings provide compelling evidence for the involvement of GC in the retrieval of outcome value to guide choice behavior.

These findings are consistent with the GC's taste memory hypothesis which posits that GC lesions would reduce the impact of sensory-specific satiety-induced outcome devaluation on action selection. Specifically, if subjects are unable to recall taste features, they would struggle to favor the non-devalued lever in the absence of sensory feedback. Consequently, any impairment in sensitivity to outcome devaluation due to GC lesions would likely result from an inability to recall the devalued taste, rather than issues with detecting the primary taste or assigning incentive value. The GC's taste memory hypothesis thus predicts that such deficits would be most evident in situations requiring the retrieval of mental representations of outcome values, as confirmed by Balleine and Dickinson (Balleine & Dickinson, 2000).

GC's contribution to value-guided choice was further strengthened by recent research employing recordings of GC neural activity during a task involving choices between immediate versus delayed rewards or between small and large rewards (Pribut

et al., 2021). Rats were trained to nose poke into a central port to sample one of three odor stimuli. These olfactory stimuli instructed rats to enter either a left or right well for sucrose reward (forced choice), while the third odor indicated that rats could earn a reward from either well (free choice). During the first block of the session, one well was designated to deliver a reward immediately (short delay, high value) while the other well delivered sucrose with delays increasing gradually (long delay, low value). These well contingencies were reversed in the second block. Results revealed that about a third of recorded GC neurons responded during odor sampling, indicating that GC's activity is modulated by olfactory stimuli (also see Vincis & Fontanini, 2016). Moreover, these odor-responsive neurons fired more frequently and strongly in response to stimuli predicting high-value rewards, suggesting stimulus-related value encoding in the GC. GC's activity was also modulated during the reward epoch (from 250 ms pre- to 1 s post-reward delivery), with an increased firing in about half of recorded GC neurons. Anticipatory activity was more strongly modulated by immediate than delayed rewards, with sustained firing from well entry (choice) to reward delivery in long delays, reflecting sustained reward-expectation and reward representations across delays. However, GC's activity at reward delivery was not modulated by reward size.

Thus, there is clear evidence from both electrophysiological recordings (neural correlates of value expectation) and functional inactivation studies that GC is required to use outcome value to guide choice.

3.5. Summary

Historically, the gustatory region of IC has been studied for its role in taste perception, prompting further research about the role of IC in learning. CTA studies revealed that not only does IC encode the identity of different tastes, but it also allows subjects to recall

tastes overtime (taste memory hypothesis), thereby facilitating associations between outcomes and consequences, such as paired-illness (Braun et al., 1972; Kiefer et al., 1984). Moreover, there is mounting evidence that IC is also required in stimulus-outcome learning processes as well as action selection. For example, IC neurons encode reward-anticipating signals (Vincis & Fontanini, 2016) and their activity is modulated throughout Pavlovian conditioning phases, demonstrating anticipatory neural activity in response to reward-predicting stimuli, which informs consummatory responses and correlates with planning and decision-making processes (M. P. H. Gardner & Fontanini, 2014; Kusumoto-Yoshida et al., 2015; Samuelsen et al., 2012). Recent findings also suggest that taste-representations transition to taste-guided choice in IC neurons, supporting performance in non-instrumental discrimination tasks (Vincis et al., 2020). Collectively, these results suggest that Pavlovian conditioning profoundly shapes IC's activity, which in conditioned subjects reflects the encoding of taste identity, cross-modal associations and upcoming stimulus-guided choice. Finally, while the IC is not required to learn A-O associations (Balleine & Dickinson, 2000; Parkes et al., 2015, 2018; Parkes & Balleine, 2013), in accordance with the taste memory hypothesis, the IC appears crucial to retrieve the current value of outcomes in their absence in order to guide action selection.

Collectively, these findings indicate that neurons within the gustatory cortex (GC) of IC are involved in perceptual processes, such as taste identification, hedonic coding, and recategorization, as well as cognitive functions including anticipatory activity, planning, and choice. This finding contrasts with the traditional view of primary sensory cortices, and argues in favor of an “*associative gustatory cortex*” (Boughter & Fletcher, 2021). Indeed, the GC not only processes the chemosensory features of tastants and regulates the timing of natural aversive reactions but also serves as a hub for taste-guided,

reward-related choices. Therefore, the GC appears crucial for forming and retrieving rich sensory representations of specific outcomes. Moreover, the GC's role extends beyond just gustatory stimuli, reflecting associations across various sensory modalities. This cross-modal activity is essential for driving food-directed behavior, emphasizing the GC's central role in integrating diverse sensory and decision-related variables to guide choice.

The evidence reviewed in this section provides compelling support for the IC's role in both anticipating and predicting events, as well as in exerting control over instrumental actions, particularly when those actions are guided by outcome value. This dual involvement in both major forms of associative learning – Pavlovian conditioning and instrumental action - raises an intriguing question: might the gustatory region of IC also be necessary when Pavlovian and instrumental processes are integrated to guide behavior, as is the case for general and specific PIT?

4. OBJECTIVES

This thesis examines how predictive stimuli guide decision-making processes. Indeed, much evidence indicates that our actions can be influenced by the presence of stimuli that we have learned signal particular outcomes (Colwill & Rescorla, 1988). The influence of predictive stimuli over instrumental action can be assessed using Pavlovian-to-instrumental transfer (PIT), which illustrates that Pavlovian predictive stimuli can energize (general transfer) or selectively guide instrumental actions (specific transfer). Theoretical accounts of PIT posit that general transfer results from the association between a stimulus and the motivational or affective qualities of the outcome, whereas specific transfer reflects Pavlovian associations between a stimulus and specific features of the outcome (c.f. Konorski, 1967). A representation of the appetitive outcome is thus essential for stimuli to exert their control over instrumental actions.

Several lines of evidence have revealed an important role for the gustatory region of the insular cortex (IC) in forming and retrieving rich sensory representations of appetitive outcomes and, therefore, we hypothesized that this region could represent a key cortical region in the influence of predictive stimuli over instrumental action. Moreover, despite considerable research efforts to understand the neural bases of PIT, the cortical contribution to both general and specific PIT is not particularly well understood. We thus used a chemogenetic approach to temporarily inactivate the IC during general and specific PIT (**Chapter II**). Preliminary work was also conducted to examine the broader neural circuitry, with a focus on the connections between IC and the mediodorsal thalamus (**Chapter III**).

Finally, it must be noted that we intended to include both male and female rats in this research. However, the results presented in empirical Chapters II and III were obtained exclusively from males. We encountered significant challenges in applying the PIT behavioural protocol across sexes. We began our experiments with male subjects which, in retrospect, may have been a limiting decision. When we attempted to replicate the protocol with female subjects, it did not produce consistent results, underscoring the need for sex-specific protocol optimization. Unfortunately, due to time constraints, we were unable to fully optimize the protocol for females, particularly for specific PIT. In **Appendix II**, we present our attempts to apply both general (Experiments 1-3) and specific (Experiment 4) PIT protocols to female rats.

CHAPTER II

ROLE OF INSULAR CORTEX IN PAVLOVIAN- TO-INSTRUMENTAL TRANSFER

Contribution of rat insular cortex to stimulus-guided action

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Running title: Rat insular cortex is required for PIT

ABSTRACT

Anticipating rewards in the environment is fundamental for decision-making. Animals often use cues to assess reward availability and to make predictions about future outcomes. The gustatory region of the insular cortex (IC), the so-called gustatory cortex, has a well-established role in the representation of predictive cues, such that IC neurons encode both a general form of outcome expectation as well as anticipatory outcome-specific knowledge. Here, we used Pavlovian-instrumental transfer (PIT) to assess if the IC is also required for predictive cues to exert both a general and specific influence over instrumental actions. Chemogenetic inhibition of IC abolished the ability of a reward-predictive stimulus to energize instrumental responding for reward. This deficit in general transfer was evident whether the same or different outcomes were used in the Pavlovian and instrumental conditioning phases. We observed a similar deficit in specific PIT, such that rats with IC inhibition failed to use a reward-predictive stimulus to guide choice toward actions that deliver the same food reward. These results are in accordance with a role for this IC region in the representation of appetitive outcomes and contribute to our understanding of the cortical bases of PIT.

Keywords (5 in alphabetical order): Decision-making, DREADD, Gustatory cortex, Pavlovian-instrumental transfer, Rodent

INTRODUCTION

The ability to accurately anticipate rewards in our environment is central to adaptive decision-making. Humans and animals frequently use cues to infer the availability of rewards and to make predictions about future outcomes. Our behaviour can thus be shaped by the presence of stimuli that we have learned signal particular outcomes ([Colwill and Rescorla, 1988](#)). This influence of predictive stimuli on decision-making processes is exemplified by Pavlovian-instrumental transfer ([Walker, 1942](#); [Estes, 1943](#); [Colwill and Rescorla, 1988](#); [Holmes et al., 2010](#)).

Pavlovian-instrumental transfer (PIT) refers to the capacity of Pavlovian outcome expectancies to affect instrumental action. Two forms of transfer have been observed. General transfer reveals that a reward-predictive stimulus elevates responding on an action that earns the same (or similar) rewarding outcome ([Estes, 1943, 1948](#); [Rescorla and Solomon, 1967](#)), due to the ability of the predictive stimulus to increase overall motivation via shared appetitive features of the outcomes ([Corbit and Balleine, 2005](#)). By contrast, in specific transfer, the predictive stimulus selectively facilitates performance on an action that delivers the same reward as the stimulus, but not other actions earning different rewards, on the basis of a shared outcome representation ([Kruse et al., 1983](#)). Thus, Pavlovian stimuli can energise (general PIT) or selectively guide (specific PIT) instrumental responding for rewards ([Rescorla and Solomon, 1967](#); [Corbit et al., 2001](#)).

The gustatory region of the insular cortex, the so-called gustatory cortex, has a well-documented role in the representation of predictive stimuli. In a particularly elegant series of studies over the past decade, Fontanini and colleagues (e.g., [Maffei et al., 2012](#); [Samuelsen et al., 2012, 2013](#); [Gardner and Fontanini, 2014](#); [Kusumoto-Yoshida et al., 2015](#); [Vincis and Fontanini, 2016](#); [Vincis et al., 2020](#)) have revealed that IC neurons show

robust responses not only to auditory stimuli predicting the general availability of taste ([Samuelsen et al., 2012, 2013](#)) but also to stimuli signaling specific taste outcomes ([Gardner and Fontanini, 2014](#); [Vincis et al., 2020](#); see also [Saddoris et al., 2009](#)). That is, neurons within the gustatory region of the IC appear to encode both a general form of outcome expectation as well as anticipatory knowledge regarding specific outcome identity. These anticipatory neural signals in IC are acquired with learning and extinguish, suggesting that such signals are tracking the predictive status of Pavlovian stimuli ([Gardner and Fontanini, 2014](#)). The functional role of these neural signals in reward prediction has also been established, as pharmacological or optogenetic inhibition of IC during presentation of an auditory stimulus predicting food pellet delivery decreases conditioned food approach responses ([Kusumoto-Yoshida et al., 2015](#)).

While the neural substrates of PIT have been extensively studied (for a review see [Cartoni et al., 2016](#)), the potential involvement of the IC in transfer effects remains unknown. This is particularly surprising given the clear evidence that the gustatory region of the IC encodes information about anticipated outcomes and that this outcome-related information may be used to guide instrumental action ([Saddoris et al., 2009](#)). Indeed, we and others have demonstrated that this same region of IC is specifically required for instrumental outcome devaluation in which the animal must recall changes in outcome value to inform action selection ([Balleine and Dickinson, 2000](#); [Parkes and Balleine, 2013](#); [Parkes et al., 2015, 2018](#)). The IC is also anatomically well-connected to the neural circuit known to mediate both general and specific PIT. Notably, it sends inputs to the nucleus accumbens ([Allen et al., 1991](#); [Lee et al., 2022](#)) and is reciprocally connected to the mediodorsal thalamus ([Wright and Groenewegen, 1996](#)), ventral tegmental area ([Ohara et al., 2003](#); [Naqvi and Bechara, 2009](#); [Hernández-Ortiz et al., 2023](#)), orbitofrontal cortex

([Barreiros et al., 2021](#); [Chen et al., 2022](#); [Mathiasen et al., 2023](#)), and the basolateral and central nucleus of the amygdala ([Sripanidkulchai et al., 1984](#); [Yamamoto et al., 1997](#); [Yamamoto, 2006](#); [Schiff et al., 2018](#); [Gehrlach et al., 2020](#); [Ponserre et al., 2020](#)). Thus, the IC appears ideally placed to contribute to Pavlovian-instrumental transfer effects.

Here, we examined the functional involvement of the gustatory region of the IC in general and specific Pavlovian-instrumental transfer. We first show that general transfer is abolished under chemogenetic inhibition when the same outcome is used during both Pavlovian and instrumental conditioning. However, while it is largely accepted that such experimental designs indeed elicit general transfer ([Hall et al., 2001](#); [Holland and Gallagher, 2003](#); [Corbit and Balleine, 2005, 2011](#)), it has been noted that these “single outcome” designs might allow for the possibility of mixed transfer effects (for a review see [Cartoni et al., 2016](#)). Therefore, to ensure that our results reflect deficits in general PIT and not specific PIT, we replicated these findings using two distinct outcomes for Pavlovian and instrumental conditioning and again found that IC inhibition impaired general PIT. Finally, we demonstrate that the IC is also required for specific PIT. These results are consistent with the known role for this IC region in representing both Pavlovian and instrumental outcomes and significantly contribute to a broader understanding of the cortical bases of PIT.

MATERIALS AND METHODS

Subjects

Subjects were 98 experimentally naïve, male Long-Evans rats, aged 3-4 months (Janvier, France). Rats were housed in pairs within plastic boxes in a climate-controlled room, maintained on a 12-hour light/dark cycle. All behavioural observations occurred during the light phase of this cycle. Rats were handled daily for five days prior to the behavioural procedures. Two days before behaviour, rats were food restricted to maintain their weight at approximately 90-95% of their ad libitum feeding weight. All experimental protocols adhered to French (Council Directive 2013-118, February 1, 2013) and European (Directive 2010-63, September 22, 2010, European Community) legislations and received approval from the local ethics committee.

Viral vectors

An adeno-associated viral vector carrying the inhibitory hM4Di designer receptor exclusively activated by designer drugs (DREADDs; Armbruster et al., 2007; Rogan and Roth, 2011) was obtained from Addgene (AAV8-CaMKIIa-hM4D(Gi)-mCherry; 1.46×10^{13} gc/ml; Addgene plasmid #50477; <http://n2t.net/addgene:50477>; RRID:Addgene_50477; gifted from Bryan Roth). A control vector lacking the hM4Di receptor was also used (AAV8-CaMKII-EGFP; 2.1×10^{13} gc/ml Addgene viral prep #50469-AAV8; <http://n2t.net/addgene:50469>; RRID:Addgene_50469; gifted from Bryan Roth). To activate the inhibitory receptor, the exogenous ligand deschloroclozapine (DCZ; MedChemExpress HY-42110) was dissolved in dimethyl sulfoxide (DMSO) to obtain a final concentration of 50 mg/mL, which was aliquoted and stored at -80°C (stock solution). This stock solution was further diluted in physiological saline to obtain a final concentration of 0.1 mg/mL, which was injected at 1 mL/kg (i.e., 0.1 mg/kg). DCZ was always handled

in dim light conditions and fresh solutions were prepared each day. Rats were injected with DCZ or vehicle (0.2% DMSO in physiological saline) intraperitoneally (i.p.) 30 minutes before behaviour. We, and others, have previously demonstrated the efficacy of this ligand (Cerpa et al., 2023; Nagai et al., 2020; Nentwig et al., 2022).

Surgery

Rats were anesthetized with isoflurane (5% induction, 1-2% maintenance) and placed on a stereotaxic apparatus (Kopf). The incision site was disinfected with betadine and subcutaneously injected with a local anesthetic mixture of lidocaine and ropivacaine. Vaseline was applied to the eyes and a heating pad was placed under the rat to maintain body temperature. Rats were rehydrated with subcutaneous injections of warm saline (0.9%, 10 ml/kg/hour) throughout the surgery. The viral vector was injected using a 10 μ l Hamilton syringe connected to a microinjector (UMP3 UltraMicroPump II with Micro4 Controller, World Precision Instruments). 1 μ l of AAV was injected at a rate of 0.2 μ l/min at two sites in each hemisphere of IC. The coordinates were (in mm from bregma): AP +0.3, ML \pm 5.5, DV -7.3, and AP +1.3, ML \pm 5.5, DV -7.3 (Paxinos & Watson, 2014). After surgery, rats were subcutaneously injected with a nonsteroidal anti-inflammatory drug (meloxicam, 2 mg/kg), and sutures were covered with aluminum spray. Rats were then individually housed in a warm cage with facilitated access to food and water, and continuously monitored for 1-2 hours. Finally, rats were returned to their collective home-cage for post-operative care and were allowed a minimum of 8 days to recover before behavioural procedures began. Injections of vehicle or DCZ occurred 4-5 weeks after surgery.

Behavioural Apparatus

Behavioural procedures were conducted in 8 operant cages (40 cm width × 30 cm depth × 35 cm height, Imetronic, France) that were individually enclosed in sound- and light-resistant shells. Each chamber was equipped with two pellet dispensers that delivered grain (45mg, BioServ) or sugar pellets (45 mg, Test Diet) into a food port. The cages also contained two retractable levers that could be inserted to the left and right of the food port, speakers that provided auditory stimuli (a 3kHz tone or a 10Hz clicker), and a house light.

Behavioural Procedures

Experiment 1: General Pavlovian-instrumental transfer – single outcome

Rats first received a habituation session to the two conditioned stimuli (CS+ and CS-, tone and clicker, counterbalanced). This session was identical to the Pavlovian conditioning sessions except no outcomes were delivered. Pavlovian conditioning began the following day. Rats received 8 Pavlovian training sessions, each lasting 1.5 h. During each session, rats were presented with 2 min presentations of the two CSs, one paired with grain pellets (CS+) and the other with no outcome (CS-). Grain pellets were delivered on a random time 30 s schedule throughout the CS+ (i.e., 4 pellets per CS+ presentation). During each session, the CS+ was presented 9 times and the CS- was presented 3 times in a pseudorandom order with a variable inter-trial interval (ITI) that averaged to 5 min (ranging from 3-7 min). Food port entries were recorded during CS+, CS-, and a 2 min interval before each CS presentation (preCS).

Following Pavlovian conditioning, rats underwent 12 sessions of instrumental conditioning (2 sessions per day). During each session, the left and right levers were available and responding on one lever (active lever) earned grain pellets (i.e., the same

outcome used for Pavlovian conditioning), whereas responding on the other lever (inactive lever) was unrewarded. Responding on the active lever was continuously reinforced on the first session and then shifted to a variable interval 30 s schedule of reinforcement for sessions 2-12. The identity of the active and inactive lever (left versus right) was counterbalanced. Rats could earn a maximum of 30 pellets per session and the maximum session duration was 30 min. The next day, all rats underwent instrumental extinction during which the two levers were extended for 30 min and no outcomes were delivered. Rats were then given a Pavlovian extinction session, which was identical to the CS habituation session previously described. The aim of these extinction sessions was to (1) reduce the baseline rate of instrumental responding, and (2) reduce competition between the Pavlovian and instrumental responses ([Holmes et al., 2010](#)).

Twenty-four hours after the extinction sessions, rats were injected i.p. with vehicle or DCZ and, 30 min later, they were given a PIT test. At the beginning of the test, the two CSs were each presented twice in the absence of the levers. Then, both levers were extended and instrumental responding was extinguished for 8 min (in the absence of the CSs) to reduce baseline instrumental responding. Subsequently, each CS was presented twice in a pseudorandom order while both levers were extended. Each CS lasted 2 min with a fixed 4 min ITI. Food port entries and lever pressing were recorded throughout the session and responses were separated into preCS, CS+, and CS- periods (2 min each). No outcomes were delivered during the PIT test.

Experiment 2: General Pavlovian-instrumental transfer – two outcomes

The procedure was identical to that described for Experiment 1 except that two distinct outcomes were used for Pavlovian and instrumental conditioning. That is, half of the rats were trained with sugar pellets during the Pavlovian phase and grain pellets during

the instrumental phase and vice versa for the remaining half of the rats. For all rats, CS+ was the clicker and CS- was the tone, and all rats were injected with DCZ prior to the general PIT test.

Experiment 3: Specific Pavlovian-instrumental transfer

Rats first received a single habituation session to the two CSs (tone and clicker). During this 90 min session, each CS was presented 6 times in a pseudo-random order, during which no outcomes were delivered. The duration of each CS was 2 min and the average ITI was 5 min (ranging from 3-7 min). Then, all rats received 8 sessions of Pavlovian conditioning (one session per day) during which each CS (tone and clicker) was associated with one of the food outcomes (grain or sugar pellets, counterbalanced). Each session consisted of four tone and four clicker presentations. During each 2 min CS, the associated reward was delivered on a 30 s random-time schedule, resulting in 4 pellet deliveries during each CS presentation. CSs were delivered pseudo-randomly with a variable 3-7 min ITI (average = 5 min). Food port entries were recorded and separated into CS period and a 2 min period before each CS presentation (preCS).

Rats then received instrumental training to perform two actions to earn two distinct food rewards (e.g., left lever earns a grain pellet and right lever earns a sugar pellet, or vice versa). These food rewards were the same outcomes that were used for Pavlovian conditioning. Rats were trained under continuous reinforcement (CRF) for the first 3 days, then under a random-ratio 5 schedule (RR5) on days 4-6 and, finally, an RR10 schedule on days 7-9. During these sessions, each lever was presented twice for a maximum of 10 min each or until 20 outcomes were earned. The ITI between lever presentations was 2.5 min. Hence, each subject could earn a maximum of 40 grain and 40 sugar pellets during each session.

The next day, rats were given an instrumental extinction session followed by a Pavlovian extinction session, as described above for the general PIT paradigm. Twenty-four hours later, all rats were injected with DCZ and were then given an outcome-specific PIT test 30 min later. The test procedure was identical to the general PIT test. Performance was evaluated under three conditions: lever pressing during the preCS period, pressing on the lever that shared the same outcome as the presented cue (same condition), and pressing on the lever that shared a different outcome with the presented cue (different condition). No outcomes were delivered during the PIT test.

Tissue Processing and Immunohistochemistry

After behavioural testing, rats received an i.p. injection of xylazine (20 mg/kg, i.p.) followed by an i.p. injection of pentobarbital (Euthasol; 200 mg/kg, diluted in saline). Rats were then perfused transcardially with 4% paraformaldehyde in 0.1M phosphate buffer. Subsequently, 40 µm coronal sections were cut using a VT1200S Vibratome (Leica Microsystems). Every fourth section was collected to form a series and immunoreactivity was performed for mCherry (hM4Di) but not for GFP as the endogenous fluorescence was sufficient to detect infected cells.

For hM4Di injected rats, free-floating sections were rinsed several times in 0.1M phosphate buffered saline (PBS), permeabilized with 0.3% Triton-X in 0.1M PBS (PBS-T), blocked for 1h (blocking solution: 4% normal goat serum in PBS-T) and placed in 1:1000 rabbit anti-RFP (CliniSciences, PM005) at room temperature for 24 h. Sections were then washed in PBS-T and incubated in 1:500 biotin goat-anti rabbit (Jackson ImmunoResearch, 111-065-144) diluted in PBS-T for 2 h at room temperature. Sections were then rinsed with PBS and incubated with 1:400 A594-Streptavidin (Jackson

ImmunoResearch, 016-580-084) in PBS. Finally, sections were rinsed in PB 0.1M then mounted and cover-slipped with Fluoroshield with DAPI (Invitrogen, 00-4959-52).

Slides were scanned using an upright fluorescent microscope system (Leica DM5500B) attached to a Jenoptik ProgRes MF Cool (#D-07739 Jena) that was equipped with a motorized stage (Märzhäuser #W215885) driven by the open source acquisition software Micro Manager (MMStudio V1.4.10). Acquired mosaics (10x objective) were stitched to reconstruct images, using the “Stitching - Grid/Collection” plugin of the Fiji freeware (Preibisch et al., 2009). Manual tracing of injection sites for each subject was performed using the mosaic images. These hand-traced injection sites were then superimposed onto corresponding atlas sections (Paxinos & Watson, 2014) and combined to visually convey the degree of overlap in virus expression across rats.

Data Analyses

All experiments used a mixed method design. In Experiment 1, separate statistical analyses were conducted on rats injected with the GFP control virus and rats injected with the inhibitory hM4Di virus. As such, the between-subject factor was treatment (vehicle versus DCZ) and within-subject factors were lever (active versus inactive), CS presentation (CS+ versus CS-), and session/time. In Experiment 2, the between-subject factor was group (GFP versus hM4Di) and within-subject factors were lever (active versus inactive), CS presentation (CS+ versus CS-), and session/time. All rats were injected with DCZ. For Experiment 3, the between-subject factor was again group (GFP versus hM4Di) and within-subject factors were CS presentation (same versus different) and session/time, and all rats were injected with DCZ.

The dependent variables were the rate of lever presses or food port entries (FPEs), which are presented as the response rate during the CS presentation minus the response

rate during the baseline preCS period (2 min period preceding each CS presentation). Data were analysed using a mixed-model ANOVA followed by simple effects analyses to establish the source of any significant interactions. Statistical significance was set at $p \leq 0.05$. Data are presented as mean \pm SEM and individual data points are supplied on histograms. Rats were excluded if they failed to properly learn the Pavlovian associations, defined as greater responding during CS- than during CS+ during the final two days of training for general PIT or greater responding during the preCS period versus the CS period during the final two training days for specific PIT. Four rats were excluded based on this criterion, two from general PIT and two from specific PIT.

RESULTS

Experiment 1: General transfer is impaired following IC inhibition.

We first assessed the impact of chemogenetic inhibition of insular cortex (IC) on the expression of general PIT using a single outcome in Pavlovian and instrumental training. Rats received bilateral IC injections of a virus carrying the inhibitory DREADD (hM4Di; N = 24) or a control virus (GFP; N = 30). Schematics of the viral expression in IC for each rat are illustrated in **Figure 1.A** along with representative images (**Figure 1.B**). One rat was excluded due to unilateral viral infection and two rats were excluded because they failed to properly discriminate between CS+ and CS- during Pavlovian conditioning. This yielded the following between-subject group sizes: GFP vehicle: n = 14, GFP DCZ: n = 15; hM4Di vehicle: n = 11, hM4Di DCZ n = 11.

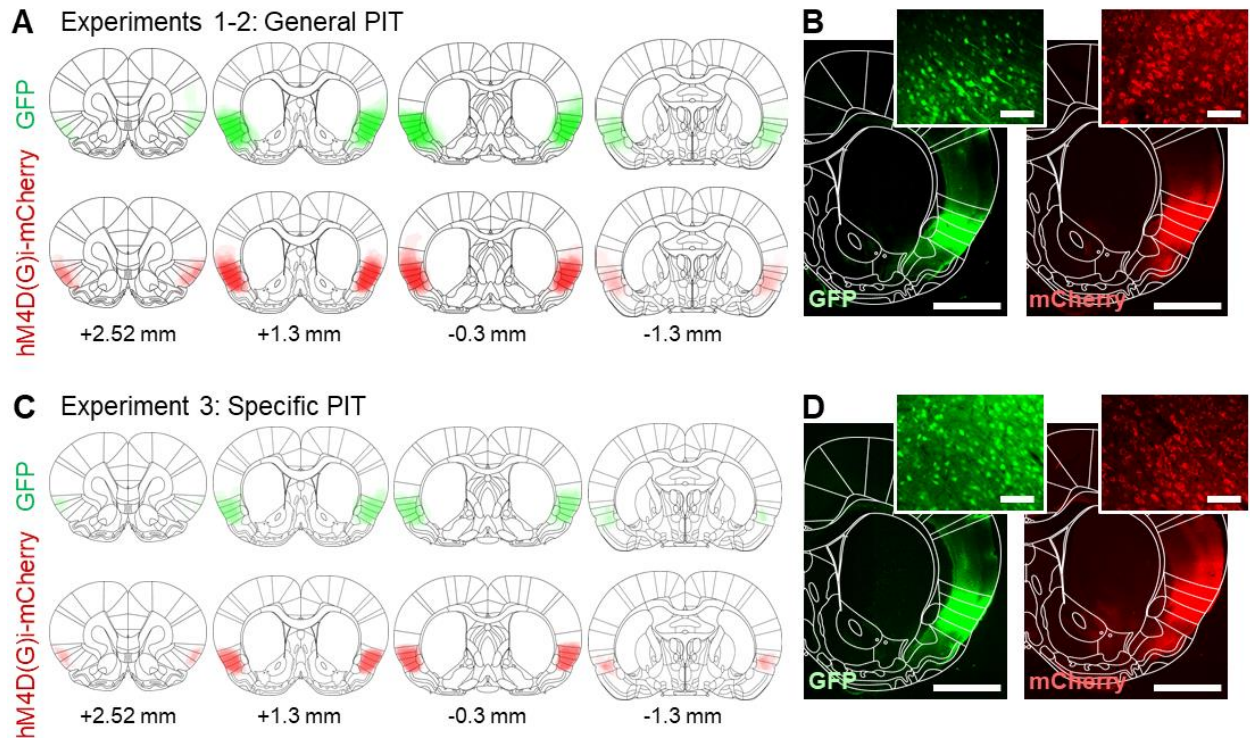


Figure 1. Schematics of the viral expression in IC (left; in mm from bregma) and representative images (right) for rats injected with the inhibitory DREADD (hM4Di) or control virus (GFP) in general PIT (**A, B**) and specific PIT (**C, D**) experiments. Scale bars represent 1 mm in the map image (10x) and 50 μm in the magnified image (20x, inset).

Following recovery, rats underwent the general PIT procedure with a single outcome, as shown in **Figure 2.A**. They first received Pavlovian conditioning during which CS+ was associated with the food reward and CS- was not. Rats were then given instrumental training whereby responding on the active lever earned the food reward while responding on the inactive lever was unrewarded. The food reward was the same for Pavlovian and instrumental training. Finally, all rats received a transfer test that assessed lever pressing in the presence of the two stimuli (CS+ and CS-). Rats were injected i.p. with vehicle or DCZ 30 min before the general PIT test.

Pavlovian conditioning proceeded smoothly for all groups. For GFP-injected rats (**Figure 2.B**), there was a main effect of CS ($F_{1,27} = 241.8$, $p < 0.01$), indicating that the rate of food port entries was higher in the presence of the rewarded stimulus (CS+) than

during the unrewarded stimulus (CS-). There was also a main effect of session ($F_{1,27} = 180.07$, $p < 0.01$), but no effect of treatment to be ($F_{1,27} = 0.02$, $p = 0.89$) or any significant interactions (largest $F_{1,27} = 1.32$, $p = 0.26$). A similar pattern of results was observed for rats injected with the hM4Di virus (**Figure 2.C**), with a main effect of CS ($F_{1,20} = 206.48$, $p < 0.01$) and session ($F_{1,20} = 33.15$, $p < 0.01$), but no effect of treatment to be or significant interactions (largest $F_{1,20} = 1.03$, $p = 0.32$).

Rats also successfully acquired instrumental responding with a main effect of session ($F_{1,27} = 157.87$, $p < 0.01$), lever ($F_{1,27} = 264.91$, $p < 0.01$), and a lever x session interaction ($F_{1,27} = 200.92$, $p < 0.01$) detected for GFP (**Figure 2.D**) and hM4Di groups (**Figure 2.E**; session: $F_{1,20} = 160.51$, $p < 0.01$; lever: $F_{1,20} = 291.50$, $p < 0.01$; session x lever interaction: $F_{1,20} = 148.94$, $p < 0.01$). Simple effect analyses conducted on the session x lever interactions indicated that lever pressing increased on the active (GFP: $F_{1,27} = 182.22$, $p < 0.01$; hM4Di: $F_{1,20} = 159.48$, $p < 0.01$) but not inactive levers (GFP: $F_{1,27} = 2.03$, $p = 0.17$; hM4Di: $F_{1,20} = 3.75$, $p = 0.07$). There were no significant differences in responding between rats that would receive vehicle or DCZ during the upcoming transfer test or any interactions between treatment and the other factors (GFP: largest $F_{1,27} = 2.56$, $p = 0.12$; hM4Di: largest $F_{1,20} = 0.87$, $p = 0.36$).

To reduce baseline instrumental responding and Pavlovian/instrumental response competition, all rats underwent a 30 min instrumental extinction session followed by a Pavlovian extinction session on the day preceding the transfer test (data not shown). For GFP rats, there was a significant main effect of lever ($F_{1,27} = 187.95$, $p < 0.01$), time ($F_{1,27} = 141.55$, $p < 0.01$), and a significant lever x time interaction ($F_{1,27} = 94.00$, $p < 0.01$) but no effect of treatment to be ($F_{1,27} = 0.34$, $p = 0.57$). Simple effects analyses indicated that responding on both the active ($F_{1,27} = 118.11$, $p < 0.01$) and inactive lever ($F_{1,27} = 18.58$,

$p < 0.01$) decreased across the session. Similar results were found for hM4Di rats (main effect of lever: $F_{1,20} = 243.24$, $p < 0.01$; time: $F_{1,20} = 93.74$, $p < 0.01$; lever x time interaction: $F_{1,20} = 76.58$, $p < 0.01$; no effect of treatment to be: $F_{1,20} = 0.58$, $p = 0.46$), and responding on both the active ($F_{1,20} = 98.20$, $p < 0.01$) and inactive lever ($F_{1,20} = 5.24$, $p < 0.01$) decreased across the session. The rate of food port entries across the Pavlovian extinction session did not differ between CS+ and CS- for GFP-injected rats ($F_{1,27} = 0.75$, $p = 0.39$), however hM4Di-injected rats responded more during CS+ than CS- ($F_{1,20} = 7.43$, $p = 0.01$). There were no main effects of time or treatment to be for GFP (largest $F_{1,27} = 0.66$, $p = 0.42$) or hM4Di rats (largest $F_{1,20} = 2.32$, $p = 0.14$), and no interactions (GFP: largest $F_{1,27} = 2.94$, $p = 0.10$; hM4Di: largest $F_{1,20} = 0.8$, $p = 0.38$).

Performance on the active lever during the general PIT test is shown in **Figure 2.F** for GFP- and **Figure 2.G** for hM4Di-injected rats. For GFP rats, lever pressing was higher during the CS+ than during the CS- ($F_{1,27} = 5.25$, $p = 0.03$), and there was no effect of treatment ($F_{1,27} = 0.09$, $p = 0.77$) or stimulus x treatment interaction ($F_{1,27} = 0.2$, $p = 0.66$), indicating successful general transfer.

A different pattern of results was observed for hM4Di rats. Statistical analyses revealed no main effect of treatment ($F_{1,20} = 0.01$, $p = 0.91$), but a main effect of CS ($F_{1,20} = 4.6$, $p = 0.04$) and a significant CS x treatment interaction ($F_{1,20} = 5.86$, $p = 0.03$). Simple effect analyses indicated that lever pressing was higher during CS+ than during CS- for rats that received vehicle ($F_{1,20} = 7.31$, $p = 0.01$) but not for rats that received DCZ ($F_{1,20} = 0.7$, $p = 0.41$).

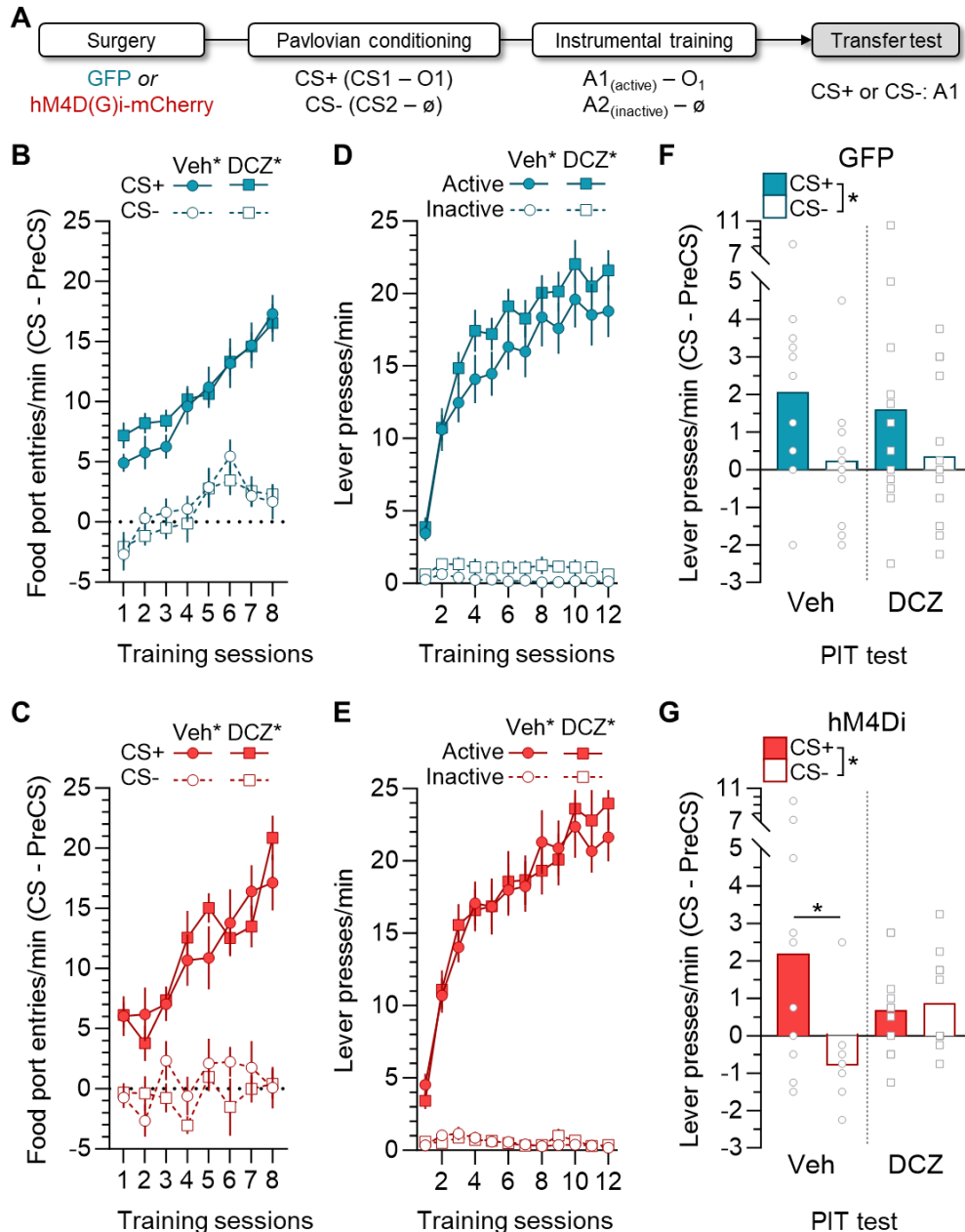


Figure 2. IC inhibition impairs general PIT when a single outcome is used. (A) Schematic of behavioural procedure, vehicle or DCZ was injected during the transfer test only. **(B, C)** Mean (\pm SEM) rate of food port entries (FPE) during CS+ (filled plot) and CS- (open plot) across Pavlovian conditioning for GFP (B) and hM4Di (C) rats. Calculated as rate of FPE during the 2 min CS minus the rate of FPE during the 2 min before CS onset (preCS). Vehicle (veh) groups are shown in circles and DCZ groups are in squares (veh*; DCZ* indicates treatment to be during the general PIT test). **(D, E)** Lever pressing rate (mean \pm SEM) across instrumental conditioning on active (filled plot) and inactive levers (open plot) for GFP (D) and hM4Di (E) rats. **F, G.** Performance on active lever during CS+ (filled bars) and CS- (open bars) in the general PIT test for GFP (F) and hM4Di (G) rats treated with vehicle or DCZ. Calculated as the pressing rate during the 2 min CS minus the pressing rate during the preCS. * statistical significance.

We also examined the rate of food port entries during the transfer test and found that all groups entered the food port more during CS+ than during CS- (**Supplemental Figure 1.A**). Moreover, vehicle and DCZ injected rats performed similarly in a progressive ratio task (**Supplemental Figure 2**), suggesting that the impairment in general PIT was not simply related to motor or motivational deficits.

Experiment 2: IC inhibition also impairs general transfer when two distinct outcomes are used.

The procedure was identical to that described for Experiment 1 except that half of the rats were trained with sugar pellets during the Pavlovian phase and grain pellets during the instrumental phase and vice versa for the remaining half of the rats (**Figure 3.A**). One rat was excluded for responding > 2 standard deviations during CS+ at the end of the Pavlovian extinction session (>20 entries per min during final two extinction trials). As in Experiment 1, rats received bilateral IC injections of the inhibitory hM4Di virus (n = 8) or the control GFP virus (n = 7; see **Figure 3.A**) and all rats were injected with DCZ 30 min prior to the general PIT test.

Across Pavlovian conditioning (**Figure 3.B**), food port entries were greater during CS+ than during CS- ($F_{1,13} = 39.38$, $p < 0.01$) and entries increased across training sessions ($F_{1,13} = 38.27$, $p < 0.01$) but this increase was greater for CS+ (CS x session interaction: $F_{1,13} = 9.83$, $p < 0.01$; simple effect of session for CS+: $F_{1,13} = 23.15$, $p < 0.01$; and CS-: $F_{1,13} = 7.87$, $p = 0.02$). GFP and hM4Di groups did not differ ($F_{1,13} = 0.29$, $p = 0.60$) and there were no interactions between group and any other factors (largest $F_{1,13} = 1.4$, $p = 0.26$).

Instrumental conditioning (**Figure 3.C**) was also successful with statistical analyses revealing a main effect of session ($F_{1,13} = 56.28, p < 0.01$), lever ($F_{1,13} = 165.10, p < 0.01$), and a lever x session interaction ($F_{1,13} = 66.88, p < 0.01$). Simple effects analyses revealed that responding increased on the active ($F_{1,13} = 62.33, p < 0.01$) but not inactive lever ($F_{1,13} = 0.98, p = 0.34$). There was no main effect of group ($F_{1,13} = 0.60, p = 0.45$) or any interactions between group and the other factors (largest $F_{1,13} = 2.18, p = 0.16$).

Lever pressing decreased across the instrumental extinction session (data not shown), with a main effect of session ($F_{1,13} = 117.67, p < 0.01$), lever ($F_{1,13} = 146.52, p < 0.01$), and a session x lever interaction ($F_{1,13} = 109.03, p < 0.01$). Simple effect analyses indicated that responding decreased across sessions on the active ($F_{1,13} = 125.31, p < 0.01$) but not the inactive lever ($F_{1,13} = 2.92, p = 0.11$). No main effect of group was detected ($F_{1,13} = 0.63, p = 0.44$) but the three-way interaction approached significance ($F_{1,13} = 4.29, p = 0.06$), suggesting that the decrease in responding on the active lever was perhaps greater in group GFP than group hM4Di. During Pavlovian extinction (data not shown), food port entries were greater during CS+ than during CS- ($F_{1,13} = 6.21, p = 0.03$), and there was no main effect of group ($F_{1,13} = 0.54, p = 0.48$), session ($F_{1,13} = 0.01, p = 0.92$) or any significant interactions (largest $F_{1,13} = 2.83, p = 0.12$).

Figure 3.D shows responding on the active lever during the general PIT test. Statistical analyses revealed a main effect of CS ($F_{1,13} = 6.62, p = 0.02$) and group ($F_{1,13} = 7.10, p = 0.02$), indicating that pressing was greater during the CS+ and that GFP rats pressed more than hM4Di rats. Importantly, there was also a significant CS x group interaction ($F_{1,13} = 4.82, p = 0.05$), and simple effects analyses indicated that group GFP responded more during CS+ than CS- ($F_{1,13} = 10.66, p < 0.01$) but this was not the case

for group hM4Di ($F_{1,13} = 0.08$, $p = 0.78$). Food port entries did not differ between groups (Supplemental Figure 1.B).

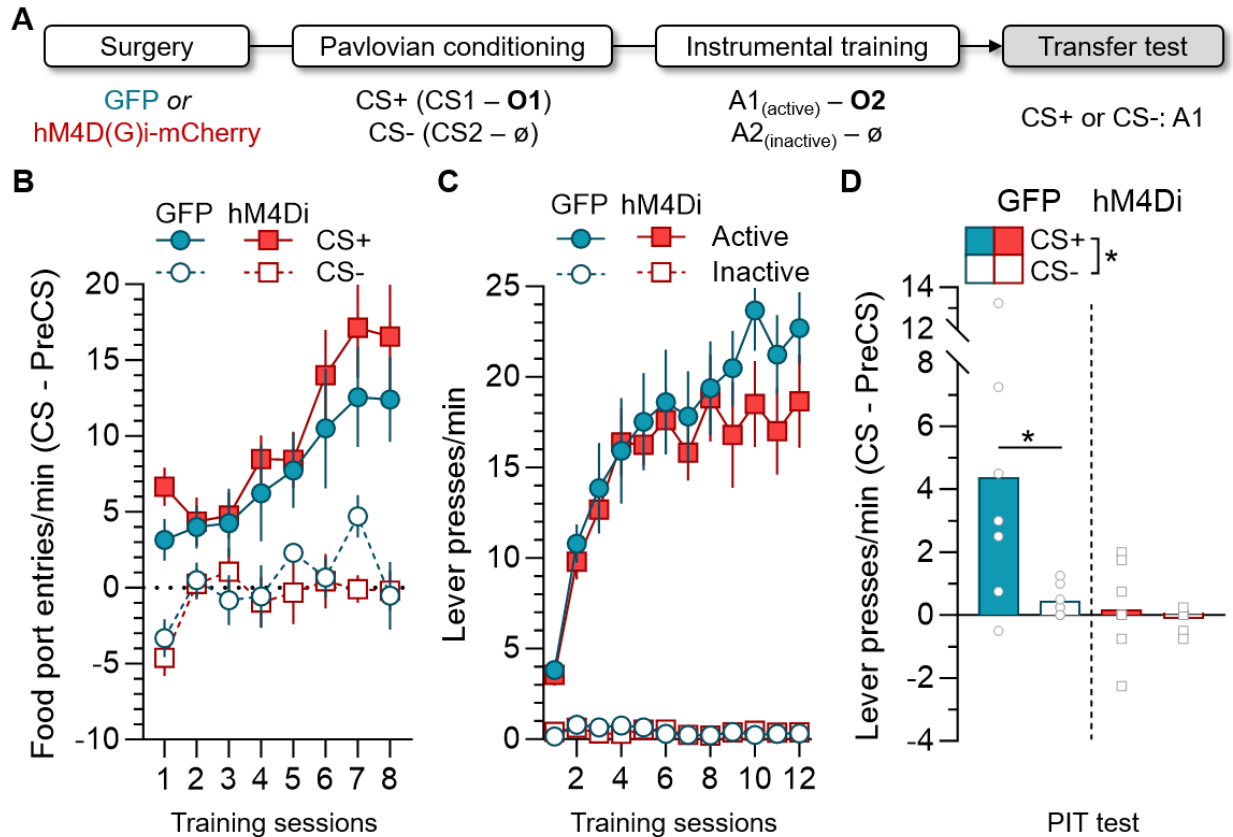


Figure 3. IC inhibition impairs general PIT when different Pavlovian and instrumental outcomes are used. (A) Schematic of the behavioural procedure, vehicle or DCZ was injected during the transfer test only. (B) Mean (\pm SEM) rate of food port entries (FPEs) during CS+ (filled plots) and CS- (open plots) across Pavlovian conditioning for GFP ($n = 7$) and hM4Di rats ($n = 8$). Calculated as the rate of entries during the 2 min CS minus the rate of entries during the 2 min before CS onset (preCS). (C) Mean (\pm SEM) lever pressing rate across instrumental conditioning on the active (filled plots) and inactive lever (open plots) for GFP and hM4Di rats. (D) Performance on the active lever during CS+ (filled bars) and CS- (open bars) in the general PIT test for GFP and hM4Di rats under DCZ injection. Performance is calculated as the rate of pressing during the 2 min CS minus the rate of pressing during the 2 min before CS onset (preCS). *statistical significance.

Experiment 3: Specific transfer is impaired following IC inhibition.

We next examined if IC was also required for specific PIT. Rats received bilateral IC injections of the inhibitory hM4Di virus ($n = 14$) or the control GFP virus ($n = 14$). Schematics of the viral expression in IC for each rat and representative images are shown in **Figure 1.C-D**. One rat was excluded due to misplaced viral expression and two rats were excluded because they failed to learn Pavlovian conditioning. This yielded the following between-subject group sizes: GFP: $n = 13$, hM4Di: $n = 12$. All rats were injected i.p. with DCZ during the specific PIT test.

Following recovery from surgery, rats underwent the specific PIT protocol (**Figure 4.A**). They first received Pavlovian conditioning during which two distinct stimuli (S1 and S2) were associated with two different food outcomes (O1 and O2). Rats were then trained to perform one action (e.g., left lever) to earn one outcome (e.g., O1) and another action (e.g., right lever) to earn a different outcome (e.g., O2). Finally, all rats were injected with DCZ and then received a transfer test that assessed responding on both levers during the presence of the two stimuli (S1 and S2).

As shown in **Figure 4.B**, Pavlovian conditioning was successful for both GFP and hM4Di groups with rats increasing their food port entries across sessions ($F_{1,23} = 23.69$, $p < 0.01$). There was no difference between GFP and hM4Di groups ($F_{1,23} = 0.01$, $p = 0.92$) and no significant interaction ($F_{1,23} = 0.47$, $p = 0.50$). Rats also acquired instrumental responding, with a main effect of session ($F_{1,23} = 248.23$, $p < 0.01$) but no effect of group or session x group interaction (**Figure 4.C**; largest $F_{1,23} = 0.9$, $p = 0.35$).

All rats were then given an instrumental extinction session followed by a Pavlovian extinction session on the day preceding the transfer test (data not shown). Instrumental responding decreased across the extinction session ($F_{1,23} = 133.42$, $p < 0.01$), and there

was no significant difference between groups or a significant group x session interaction (largest $F_{1,23} = 2.63$, $p = 0.12$). Food port entries across the Pavlovian extinction session did not differ between groups ($F_{1,23} = 0.66$, $p = 0.43$) and there was no effect of time or group x time interaction (largest $F_{1,23} = 0.85$, $p = 0.37$).

The results from the specific PIT test are shown in **Figure 4.D**. Data are plotted as the rate of lever presses (collapsed across A1 and A2) during the stimulus (S1 or S2) that shared a common outcome (same condition) or a different outcome (different condition). Statistical analyses revealed no main effect of group ($F_{1,23} = 0.03$, $p = 0.86$) or lever ($F_{1,23} = 3.96$, $p = 0.06$) but a significant group x lever interaction ($F_{1,23} = 4.5$, $p = 0.045$). Simple effects conducted on the interaction indicated that GFP rats responded significantly more on the same lever than on the different lever ($F_{1,23} = 8.81$, $p = 0.007$) however, hM4Di rats did not ($F_{1,23} = 0.008$, $p = 0.93$). Food port entries were greater during the CSs than during the preCS period, with no differences between groups (**Supplemental Figure 1.C**).

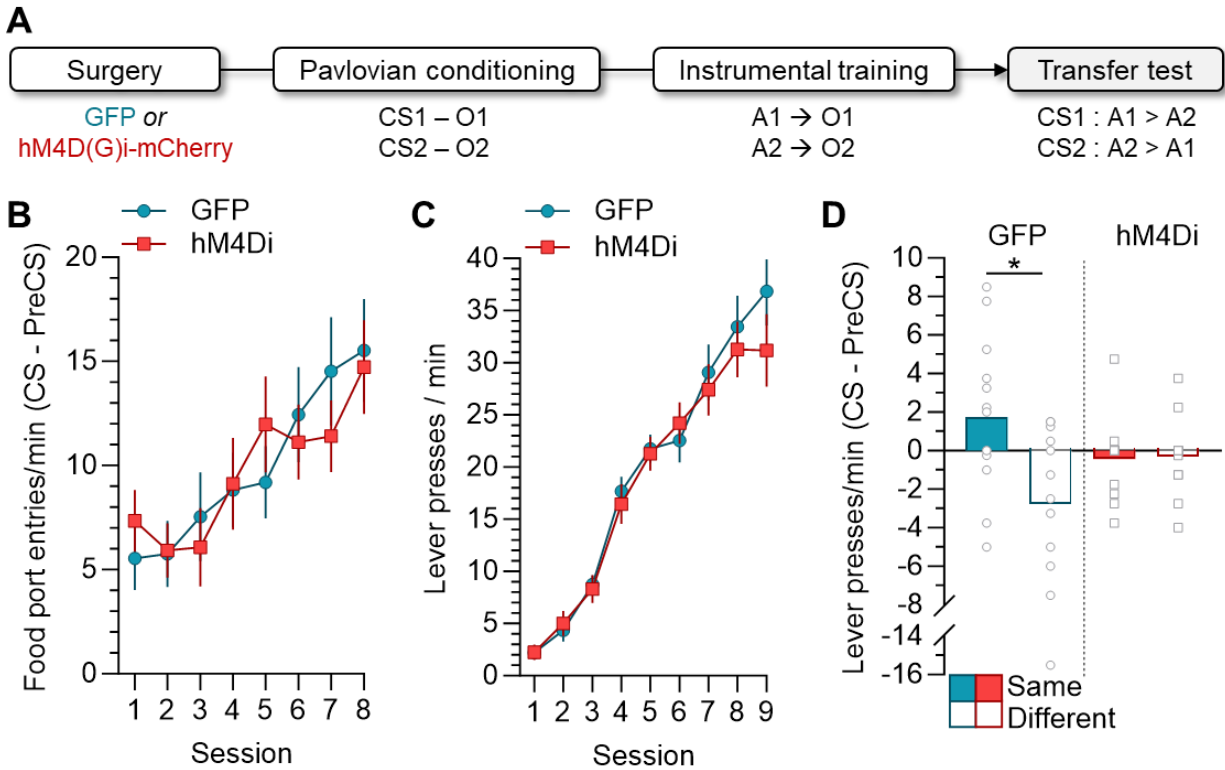


Figure 4. IC inhibition abolishes specific PIT. (A) Schematic of the behavioural procedure. **(B)** Mean (\pm SEM) rate of food port entries (FPEs) during CS presentations (clicker and tone averaged) across Pavlovian conditioning for GFP (circles) and hM4Di rats (squares). Calculated as the rate of entries during the 2 min CS minus the rate of entries during the 2 min before CS onset (preCS). **(C)** Mean (\pm SEM) lever pressing rate across instrumental conditioning (left and right lever averaged) for GFP (circles) and hM4Di rats (squares). **(D)** Lever pressing during the CS that shared the same outcome (Same; filled bars) and the different outcome (Different; open bars) in the specific PIT test for GFP and hM4Di rats under DCZ injection. Performance is calculated as the rate of pressing during the 2 min CS minus the rate of pressing during the 2 min before CS onset (preCS). . * indicates statistical significance.

DISCUSSION

Here, we examined the role of the gustatory region of the insular cortex (IC) in the general and specific forms of Pavlovian-instrumental transfer (PIT). Our findings revealed that chemogenetic inhibition of the IC during the transfer test abolished both general and specific PIT, indicating that IC is required for predictive cues to both energize instrumental actions and to selectively guide choice towards actions associated with specific outcomes.

We first observed that inhibition of IC abolished general transfer such that instrumental responding was similar both in the presence and absence of the reward-predictive stimulus. We then replicated this effect using a modified general PIT design in which two distinct outcomes were used in the Pavlovian and instrumental conditioning phases. As expected, this design also produced a general transfer effect in control rats, which confirms that PIT protocols utilizing a single action-outcome (A-O) association can produce general transfer regardless of the reinforcers used, provided they are equally desirable and of the same nature ([Baxter and Zamble, 1982](#)). IC inactivation again abolished this general transfer effect, with instrumental actions remaining essentially unaffected by the Pavlovian stimuli. Importantly, there appeared to be no deficit in the retrieval or expression of stimulus-outcome (S-O) associations as IC-inactivated rats visited the food port more during the rewarded stimulus than during the unrewarded stimulus. Moreover, IC inhibition did not diminish the rats' motivation to perform an instrumental action, nor did it affect their ability to recall which action was rewarded and which was not.

Finally, we demonstrated that IC inhibition also impaired specific PIT and the pattern of results was similar to what was observed in general PIT. That is, instrumental

performance appeared insensitive to the presence of reward-predictive stimuli, as evidenced by similar rates of lever pressing across different test periods (*same, different, baseline*). We again observed that the Pavlovian associations appeared to be intact following IC inhibition as rats visited the food port more frequently during the reward-predictive stimuli than during the baseline period. However, it is difficult to know whether this behaviour reflects an outcome-specific expectancy, as only a single food port was used for delivering the outcomes predicted by both stimuli. Therefore, the Pavlovian conditioned responses observed in the specific PIT experiment could result from either a general or specific reward expectancy. Similarly, rats with IC inhibition performed the instrumental actions in a similar manner to controls, but we cannot be sure if the rats were able to recall the specific A-O associations (i.e., A1-O1 and A2-O2) or if they simply retrieved a general A-reward association. However, several lines of evidence show that IC inhibition does not affect the ability learn or recall specific A-O associations ([Balleine and Dickinson, 2000](#); [Parkes and Balleine, 2013](#); [Parkes et al., 2015, 2018](#)).

Here, our aim was to explicitly test the involvement of IC in transfer effects, not in the acquisition of S-O or A-O associations. As such, we restricted the IC inhibition to the test phase only. The available evidence indicates that IC is not involved in the acquisition or expression of A-O ([Balleine and Dickinson, 2000](#); [Parkes and Balleine, 2013](#); [Parkes et al., 2015, 2018](#)), and our current results also indicate that the expression of both A-O and S-O associations is intact in rats with IC inhibition. However, we did not test if the gustatory region of IC is necessary for the acquisition of S-O associations and the potential role of IC in appetitive Pavlovian learning remains to be clarified.

Altogether, these results show that IC inhibition renders instrumental actions insensitive to reward-predictive stimuli, even though both Pavlovian conditioned

responses and instrumental performance appear to be preserved. The gustatory region of IC is thus necessary for Pavlovian stimuli to exert both general excitatory and specific influences on instrumental actions. The involvement of IC in *both* general and specific transfer is consistent with evidence that IC neurons respond to Pavlovian cues predicting the availability of multiple tastants ([Samuelson et al., 2012, 2013](#)) and also show selective responses to auditory cues predicting distinct outcomes ([Gardner and Fontanini, 2014](#); [Vincis and Fontanini, 2016](#); [Vincis et al., 2020](#)). Immediate early gene activation is also increased in IC following presentation of a Pavlovian reward-predictive cue ([Dardou et al., 2006, 2007](#); [Kerfoot et al., 2007](#); [Saddoris et al., 2009](#)) and this activation appears to be outcome-specific ([Saddoris et al., 2009](#)).

Moreover, this anticipatory activity in IC is functionally relevant. [Vincis et al \(2020\)](#) used a perceptual discrimination task in which head-restrained mice were trained to lick a central spout to receive one of four taste cues during a sampling epoch. The mice then had to wait through a short delay before deciding whether to lick left or right for a water reward. The taste cues were designed so that stimuli with similar perceptual features signaled that the left or right lick would be rewarded: sweet sucrose or bitter quinine indicated that mice should perform a left lick for the water reward, while sweet maltose or bitter sucrose octaacetate indicated a right lick. Electrophysiological recordings demonstrated that, within the first 500 ms, IC neurons effectively discriminated between the four tastes. However, during the subsequent delay epoch, population decoding of neural activity showed that firing for tastants associated with the same action started to converge. Importantly, while optogenetic inactivation of IC during sampling of the tastant had no impact on performance, the same manipulation during the delay epoch decreased the number of correct choices ([Vincis et al., 2020](#)).

Our findings are also in accordance with studies implicating the gustatory region of IC in the retrieval of outcome value in situations where animals must choose between actions ([Balleine and Dickinson, 2000](#); [Parkes and Balleine, 2013](#); [Parkes et al., 2015, 2018](#)). In these experiments, rats learn to perform two actions to earn two distinct rewards and then one of the rewards is devalued before a choice test between the two actions. Typically, rats choose the action associated with the non-devalued outcome more than the action associated with the devalued outcome, indicating that they have both encoded the new value of the outcome and are able to retrieve this outcome representation to inform their instrumental responding. However, lesions or inactivation of NMDA receptors in the gustatory region of IC renders rats unable to show a preference for the action earning the non-devalued outcome. Specifically, the evidence indicates that the IC is necessary for retrieving the outcome representation (here, the outcome value) ([Balleine and Dickinson, 2000](#); [Parkes and Balleine, 2013](#); [Parkes et al., 2015, 2018](#)).

Taken together, these studies demonstrate that the IC is not required to learn about predictive cues or instrumental responses per se but rather to use outcome representations to guide responding ([Saddoris et al., 2009](#)). That is, the IC seems to be involved in using associative representations of (taste) outcomes. The associative cybernetic model of instrumental conditioning is currently the most comprehensive theoretical account for transfer effects ([Dickinson and Balleine, 1994](#); [Balleine and Ostlund, 2007](#); [Cartoni et al., 2013](#)). A key feature of this model is that transfer is mediated by a stimulus-induced outcome representation, which serves as a stimulus to trigger the representation, and subsequent performance, of the associated instrumental action. The emergence of this S^O -A chain results notably from the ability to use the backward A-O association (i.e., the O-A association), where the representation of the outcome (S^O) leads

to a mental representation of the corresponding action, resulting in the activation of motor responses ($S1^{O1}$ -A1; $S2^{O2}$ -A2), thus generating specific PIT. In addition, the model proposes that reward predictive stimuli can prompt a general expectancy for reward, which in turn excite motor responses through a S^{reward} -A chain.

Thus, the model proposes that both forms of PIT rely on the use of a S-O-A chain through an O-A association (Balleine & Ostlund, 2007). Based on this model, the lack of transfer effects observed in rats with IC inhibition may result from either a failure to represent the outcome as a stimulus (S^O), which is required to activate the representation of the associated action (A), or an inability to use the triggered A-O representation in a backward manner. In both cases, there appears to be a failure to use the S^O -A associative chain within S-A memory, reflecting an inability of stimulus-induced outcome expectations to influence instrumental actions. It is currently unknown if the IC is indeed required to use backward A-O associations but this could be assessed using outcome reinstatement, whereby a previously extinguished instrumental action is reinstated by “free” delivery of its associated outcome, where the outcome serves as a stimulus signaling its availability, stimulating the performance of the associated action.

While we have shown that the IC contributes to the expression of both forms of PIT, distinct insular pathways are likely at play. The IC is anatomically connected to the basolateral amygdala (BLA) (Sripanidkulchai et al., 1984; Yamamoto et al., 1997; Yamamoto, 2006; Schiff et al., 2018; Gehrlach et al., 2020; Ponserre et al., 2020), the lateral OFC (Barreiros et al., 2021; Chen et al., 2022; Mathiasen et al., 2023), and the nucleus accumbens shell (Allen et al., 1991; Lee et al., 2022), which have all been exclusively implicated in specific PIT. One might therefore hypothesize that the IC-BLA, IC-shell and IC-lateral OFC pathways could support specific, but not general, PIT.

Consistent with this suggestion, previous studies have shown that the IC-BLA may be particularly important. For example, pharmacological inactivation of the BLA reduces the neural excitatory responses to predictive stimuli in the IC ([Samuelsen et al., 2012](#)) and evidence from outcome devaluation studies proposes that the BLA updates and encodes information about the value of the instrumental outcome and then sends this information to IC, where it is retrieved to guide behaviour ([Parkes & Balleine, 2013](#)).

By contrast, general PIT may require interaction between the IC and the central amygdala ([Gehrlach et al., 2020](#)) and/or NAc core ([Wright and Groenewegen, 1996](#); [Reynolds and Zahm, 2005](#)), as these regions are involved in mediating the general excitatory influences of reward-predictive stimuli on instrumental action ([Hall et al., 2001](#); [Holland and Gallagher, 2003](#); [Corbit and Balleine, 2005, 2011](#); [Lex and Hauber, 2008](#); [Lingawi and Balleine, 2012](#)). This is supported by the crucial role of the CeA in generating stimulus-induced general motivation for natural rewards, as reviewed by [Warlow and Berridge \(2021\)](#), and evidence showing the IC-CeA pathway contributes to taste-related choice behaviour ([Schiff et al., 2018](#)). Again, whether IC inputs to the central amygdala (CeA) or NAc core are required for reward-predictive stimuli to invigorate instrumental actions warrants further investigation. Nevertheless, we propose that a double dissociation may exist at the cortico-striatal and cortico-amygdalar level, such that IC-core and IC-CeA may support general (but not specific) transfer, and IC-shell and IC-BLA potentially mediate specific (but not general) PIT.

CONCLUSION

Extensive evidence indicates that Pavlovian expectancies can invigorate or bias instrumental action. Such effects are pervasive and have so far been demonstrated in rodents, monkeys, and humans (Holmes et al., 2010; Colagiuri and Lovibond, 2015; Cartoni et al., 2016), as well as in equines (Lansade et al., 2013), pigeons (Herrnstein and Morse, 1957; Foree and LoLordo, 1973; Overmier et al., 1983) and rabbits (Lovibond, 1983). Our results provide the first demonstration that the gustatory region of the insular cortex mediates the ability of predictive cues to exert both a general and specific influence over instrumental responding, likely via the retrieval of outcome representations to guide behaviour. These findings significantly contribute to our understanding of the cortical bases of the general and specific forms of Pavlovian-instrumental transfer and add to the growing body of literature investigating the broad role of the IC in decision-making.

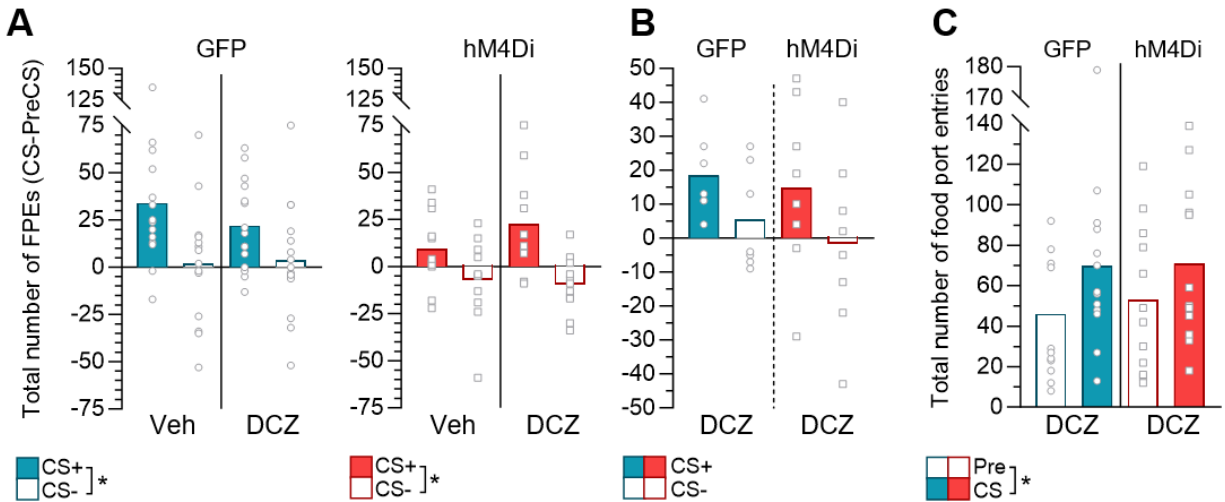
FUNDING

This work was supported by the French National Agency for Scientific Research (CoCoChoice ANR-19-CE37-0004-07) and the French government in the framework of the University of Bordeaux's IdEx "Investments for the Future" program/GPR BRAIN_2030.

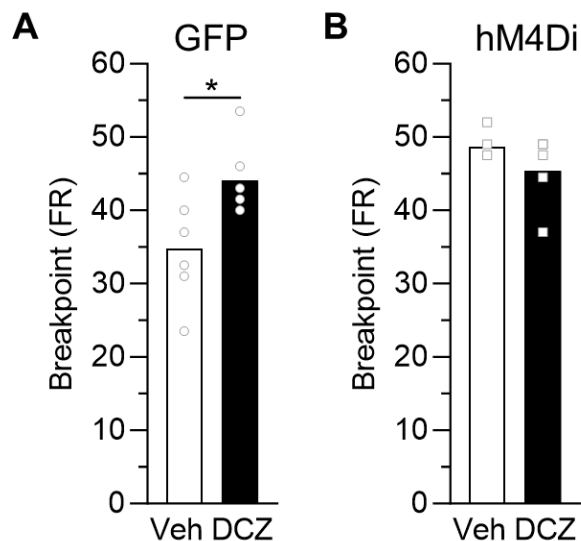
ACKNOWLEDGEMENTS

We thank Y. Salafranque for the care provided to the rats during experiments as well as Gilles Courtand and Angélique Faugère for their technical assistance. We also thank Dr. Etienne Coutureau for comments on an earlier version of this manuscript.

SUPPLEMENTAL FIGURES



Supplemental Figure 1. Food port entries during the transfer tests. Due to potential response competition between food port entries (FPE) and lever presses during the transfer test, we examined total FPE during the four CS alone presentations prior to lever extension and the four CS presentations while the levers were present. **(A)** In Experiment 1, both GFP ($F_{1,27} = 8.30$, $p < 0.01$) and hM4Di rats ($F_{1,20} = 10.64$, $p < 0.36$) entered the food port more during CS+ than CS-, and there was no effect of treatment (GFP: $F_{1,27} = 0.47$, $p = 0.50$; hM4Di: $F_{1,20} = 0.97$, $p = 0.34$) or CS x treatment interaction (GFP: $F_{1,27} = 0.64$, $p = 0.43$; hM4Di: $F_{1,20} = 1.10$, $p = 0.33$). **(B)** In Experiment 2, there was no main effect of CS ($F_{1,13} = 2.48$, $p = 0.14$), group ($F_{1,13} = 1.05$, $p = 0.32$), or CS x group interaction ($F_{1,13} = 0.04$, $p = 0.85$). **(C)** In Experiment 3, rats entered the food port more during the CSs than during the preCS period ($F_{1,23} = 11.06$, $p < 0.01$) and there was no effect of group ($F_{1,23} = 0.09$, $p = 0.77$) or CS period x group interaction ($F_{1,23} = 0.21$, $p = 0.65$). * indicates statistical significance.



Supplemental Figure 2. Performance in a progressive ratio task. A subset of rats from Experiment 1 also underwent a progressive ratio (PR) task to assess general locomotion and motivation to lever press under IC inhibition (GFP vehicle $n = 6$; GFP DCZ $n = 6$; hM4Di vehicle $n = 5$; hM4Di DCZ $n = 5$). Rats were injected with either vehicle or DCZ 30 minutes prior to PR. During the PR, rats were required to press the lever an increasing number of times on a fixed ratio (FR) 3 to obtain successive food pellet rewards (+3 increments, e.g., 1 press for the first pellet, 4 presses for the second pellet, 7 presses for the third pellet, etc.). The breakpoint, defined as the last FR completed (number of lever presses to obtain the final pellet), was recorded. **(A)** The breakpoint was significantly lower for GFP rats injected with vehicle compared to GFP rats injected with DCZ ($F_{1,10} = 6.31$, $p = 0.03$; indicated by *). **(B)** The breakpoint did not differ for hM4Di rats injected with vehicle versus those injected with DCZ ($F_{1,8} = 1.86$, $p = 0.21$). * indicates statistical significance.

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CHAPTER III

PRELIMINARY DATA: ROLE OF INSULAR CORTEX INPUT TO MEDIODORSAL THALAMUS IN PIT

1. PREFACE

Several lines of literature have shown that general and specific PIT are often dissociated at the neural level. In **Chapter II**, however, we provide evidence that the insular cortex, namely its gustatory region, is required for both general and specific PIT. We hypothesized that while the IC may be involved in both forms of transfer, it is likely that distinct IC pathways mediate general versus specific PIT. We thus next aimed at investigating the broader circuitry in which IC contributes to transfer effects.

There are several IC circuits that could be of interest, including cortico-amygdala, cortico-striatal, and cortico-thalamic pathways. For example, general PIT could rely on IC-NAc core and IC-CeA pathways, while specific PIT could rely on IC-NAc Shell, IC-BLA and IC-MD (see **Neural bases of PIT** in Chapter I). Another interesting candidate that has been less well studied in PIT is the mediodorsal thalamus (MD). The IC and MD are anatomical partners ([Wright & Groenewegen, 1996](#)) and there is increasing evidence that the MD also plays crucial roles in value- and stimulus-guided choice. Hence, based on anatomical and behavioral evidence, we explored the contribution of the IC-MD pathway to both forms of PIT.

The MD is the largest nucleus within the medial thalamic nuclei and includes subdivisions that are broadly homologous across rodents and primates ([Wolff & Halassa, 2024](#)). The MD comprises three main subdivisions: the medial (MDm), central (MDc), and lateral (MDl) divisions (**Figure 17**). This nucleus is primarily composed of excitatory neurons, playing a crucial role in integrating and relaying information to various cortical

and subcortical regions. Importantly, IC and MD are reciprocally connected (Odegaard et al., 2024; Wright & Groenewegen, 1996).

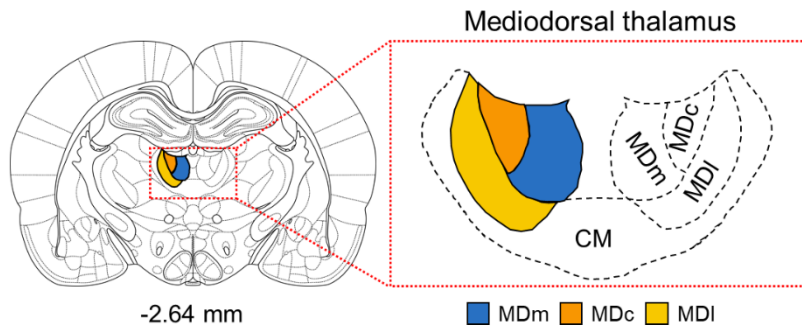


Figure 17. Anatomy of the rat mediodorsal thalamus. The mediodorsal thalamus is composed of three subdivisions, the medial (MDm), central (MDc), and lateral (MDI) parts, lying dorsally to the central medial thalamic nucleus (CM).

As previously discussed (see **Chapter I**), the MD has been implicated in specific PIT, although its role in general PIT has not yet been investigated. During a specific PIT test, rats with post-training lesions of the MD exhibit a marked insensitivity to Pavlovian stimuli, and press similarly across baseline, *same*, and *different* conditions, indicating a failure to differentiate between the stimuli based on their associated outcomes (Ostlund & Balleine, 2008). Furthermore, when MD-lesioned rats were subjected to a Pavlovian contingency degradation procedure - where one of the two S-O associations was degraded by delivering the reward independently of its CS - they displayed an atypical pattern of behavior (Morceau et al., 2022; Ostlund & Balleine, 2008). Normally, such degradation results in a reduced conditioned response (e.g., fewer food port entries) to the stimulus that no longer reliably predicts its outcome. However, MD-lesioned rats showed a non-specific reduction in responding to both stimuli, regardless of their predictive validity. This suggests that the MD may be critical for generating outcome-specific Pavlovian expectancies, and its disruption leads to a generalized impairment in processing stimulus-outcome contingencies.

Furthermore, a VP-MD-OFC pathway has been demonstrated to support the specific transfer effect (Leung et al., 2024; Leung & Balleine, 2015). Disconnecting VP and MD using contralateral lesions produced deficits in specific PIT such that subjects responded significantly above baseline in both *same* and *different* conditions in a similar manner, suggesting that this pathway is required for predictive stimuli to specifically bias instrumental responses but perhaps not for the general motivating effect of predictive stimuli on lever pressing (Leung & Balleine, 2015; Ostlund & Balleine, 2008). More recently, using chemogenetics and optogenetics, it was again shown that inhibiting VP-MD and/or MD-OFC pathways lead to a non-reinforcer specific increase in instrumental responding to reward-predictive stimuli (Leung et al., 2024). That is, when these MD pathways were inhibited, rats responded similarly on the same and different levers and responding was greater than during baseline. These findings suggest a critical involvement of the MD in the neural circuitry underlying specific PIT. Moreover, the pattern of deficits observed suggest that while MD may be required to bias action selection toward the appropriate response, it may not be required for the general energizing effect that predictive stimuli can exert over instrumental actions. That is, while MD is required for specific PIT it is unlikely to be required for general PIT.

Here, we investigated the potential involvement of the IC-MD pathway in general and specific PIT using a chemogenetic approach that allowed us to inhibit MD neurons receiving IC input during the PIT test. To achieve this, we used a double viral strategy involving the injection of a transsynaptic anterograde viral vector carrying Cre recombinase (AAV1-Cre) into the IC and a cre-dependent virus carrying the hM4Di inhibitory DREADDs into the MD (Armbruster et al., 2007; Castle et al., 2014; Rogan & Roth, 2011; Zingg et

al., 2017). This results in the expression of hM4Di receptors in MD neurons receiving IC inputs, allowing for the inhibition of this thalamic neural population during the PIT test.

2. METHODS

2.1. *Subjects*

Subjects were 28 experimentally naïve, male Long-Evans rats, aged 3-4 months (Janvier, France). Housing conditions, handling, and food restriction were exactly as described in Chapter II, and complied with French and European legislations and the local Ethics Committee (CE50).

2.2. *Viral vectors*

An adeno-associated viral vector was used to carry a cre-dependent inhibitory hM4Di designer receptor exclusively activated by designer drugs [DREADDs; (Armbruster et al., 2007; Rogan & Roth, 2011)]. This vector was obtained from Addgene (viral prep #44362-AAV8, AAV8-hSyn-DIO-hM4Di(Gi)-mCherry, plasmid gifted from Bryan Roth) as well as a control virus (viral prep #50459-AAV8 AAV8-hSyn-DIO-mCherry, plasmid gifted from Bryan Roth). The transynaptic anterograde virus was also obtained from Addgene (viral prep #105553-AAV1, AAV1-hSyn-Cre-WPRE-hGH) to induce the recombination of the mCherry-conjugated floxed transgenes.

2.3. *Surgeries*

Surgeries were performed exactly as described in **Chapter II**. A total of 12 rats were infused with 0.5 μ L/site of the floxed inhibitory DREADD (1.2×10^{12} gc/ml) in the MD at the following coordinates using a Hamilton syringe, at a rate of 0.2 μ L/min: AP: -2.3, ML: \pm 0.7, DV: -5.3 and AP: -2.7, ML: \pm 0.7, DV: -5.3, in mm from the dura. The

transynaptic anterograde AAV1-Cre virus (2.2×10^{13} gc/ml) was infused in the IC, at a volume of 1 μ L/site and a rate of 0.2 μ L/min as well, at the following coordinates (in mm from bregma): AP: +1.3, ML: \pm 5.5, DV: -7.3 and AP: +0.3, ML: \pm 5.5, DV: -7.3. Controls animals (n = 16) were similarly operated, at the difference that they were infused with a floxed virus lacking the inhibitory DREADD (4.4×10^{12} gc/ml).

2.4. Apparatus

Same as previously described (see **Chapter II**).

2.5. Behavioral procedures

Following surgery and recovery, rats were subjected either to a general (single outcome) or a specific PIT protocol, followed by a progressive ratio task, as described in.

2.6. Histology

At the end of the behavioral experiments, the animals were transcardially perfused, and the tissues were processed exactly as described in **Chapter II**.

2.7. Data analysis

Both experiments used a mixed method design with between- and within-subject factors. The between-subject factor was group (mCherry versus hM4Di) and the within-subject factor was CS presentation (CS+ versus CS- or same versus different). All rats were injected with DCZ. The dependent variables were the rate of lever presses or food port entries (FPEs). All lever presses and FPEs are presented as the rate of responding during the CS presentation minus the rate of responding during the preCS (2 min period preceding each CS presentation). Data were analysed using a mixed-model ANOVA followed by simple effects analyses to establish the source of any significant interactions.

Statistical significance was set at $p \leq 0.05$. All data are presented as the mean plus individual data points.

3. RESULTS

As illustrated in **Figure 18.A**, we injected a transsynaptic anterograde AAV1-hSyn-Cre-WPRE-hGH vector into the IC and a cre-dependent inhibitory AAV8-hSyn-DIO-hM4Di(Gi)-mCherry DREADDs (or mCherry-DIO for controls) into the MD. The viral spread for rats injected with the hM4Di virus and a representative coronal section is shown in **Figure 18.B** and **C**, respectively.

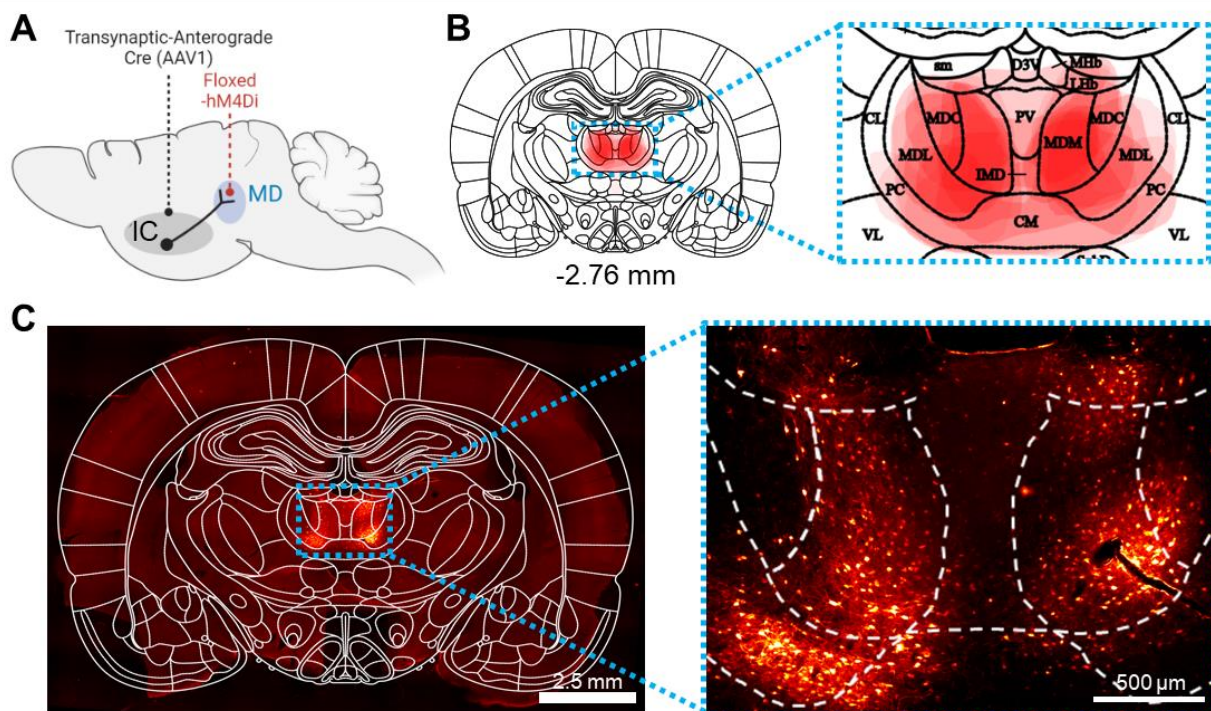


Figure 18. Strategy to target projection-defined thalamic neurons. **(A)** Rats were injected with a transsynaptic anterograde AAV1-hSyn-Cre-WPRE-hGH vector in the IC and AAV8-hSyn-DIO-hM4Di(Gi)-mCherry DREADDs (or mCherry-DIO control virus) in the MD. **(B)** Shading reflects the viral expression, with each rat that received the hM4Di virus represented as a separate, stacked layer (N = 12). mCherry expression was observed in the MD, with labeling also observed in the central medial (CM) thalamus and paracentral thalamic nuclei (PC). **(C)** A representative coronal section illustrates mCherry+ neurons in the MD using a red-hot lookup table. Scale bar: 2.5 mm. Right panel shows a magnified image of the coronal section (scale bar: 500 μ m). **CL**: centrolateral thalamic nucleus; **D3V**: dorsal 3rd ventricle; **IMD**: intermediodorsal thalamic nucleus; **LHb**: lateral habenular nucleus; **MDC**: mediodorsal thalamic nucleus, central part; **MDI**: mediodorsal thalamic nucleus, lateral part; **MDm**: mediodorsal thalamic nucleus, medial part; **MHb**: medial habenular nucleus; **PV**: paraventricular thalamic nucleus; **sm**: stria medullaris; **VL**: ventrolateral thalamus.

Following recovery from surgery, one group of rats underwent general PIT (**Figure 19.A**; hM4Di n = 5; mCherry = 9) and another group underwent specific PIT (**Figure 19.B**; hM4Di n = 7; mCherry = 7). All rats were injected with DCZ 30 min prior to the PIT test.

Responding on the active lever during the general PIT test is shown in **Figure 19.C**. Statistical analyses revealed a significant main effect of CS ($F_{1,12} = 10.88$, $p = 0.006$) but no main effect of group ($F_{1,12} = 2.95$, $p = 0.11$) or interaction between these factors ($F_{1,12} = 1.82$, $p = 0.2$). This suggests that the IC-MD pathway is not required for general PIT, as both hM4Di and mCherry groups exhibited a significantly higher rate of lever pressing during CS+ presentations than during CS-, relative to baseline. Similarly, the rate of food port entries during the general PIT test (**Figure 19.D**) was significantly higher during CS+ compared to CS- ($F_{1,12} = 14.88$, $p = 0.002$) and there was no effect of group ($F_{1,12} = 0.001$, $p = 0.98$) or CS x group interaction ($F_{1,12} = 0.85$, $p = 0.38$).

The results from the specific PIT test are shown in **Figure 19.E**. Statistical analyses revealed no main effect of group ($F_{1,12} = 3.03$, $p = 0.1$) or CS period (same vs. different: $F_{1,12} = 0.73$, $p = 0.4$) however, a significant group x CS period interaction was observed ($F_{1,12} = 5.77$, $p = 0.033$). Simple effects analyses showed that the specific transfer effect was significant in mCherry control subjects, as indicated by a significantly higher rate of responding in the *same* condition compared to the *different* condition ($F_{1,12} = 5.31$, $p = 0.04$). By contrast, rats in group hM4Di did not show the specific transfer effect ($F_{1,12} = 1.2$, $p = 0.3$). The rate of food port entries during the specific PIT test (**Figure 19.F**) also did not significantly differ between mCherry and hM4Di groups ($F_{1,12} = 1.61$, $p = 0.23$). Overall, these results indicate that inhibition of MD cells receiving IC input impairs specific PIT.

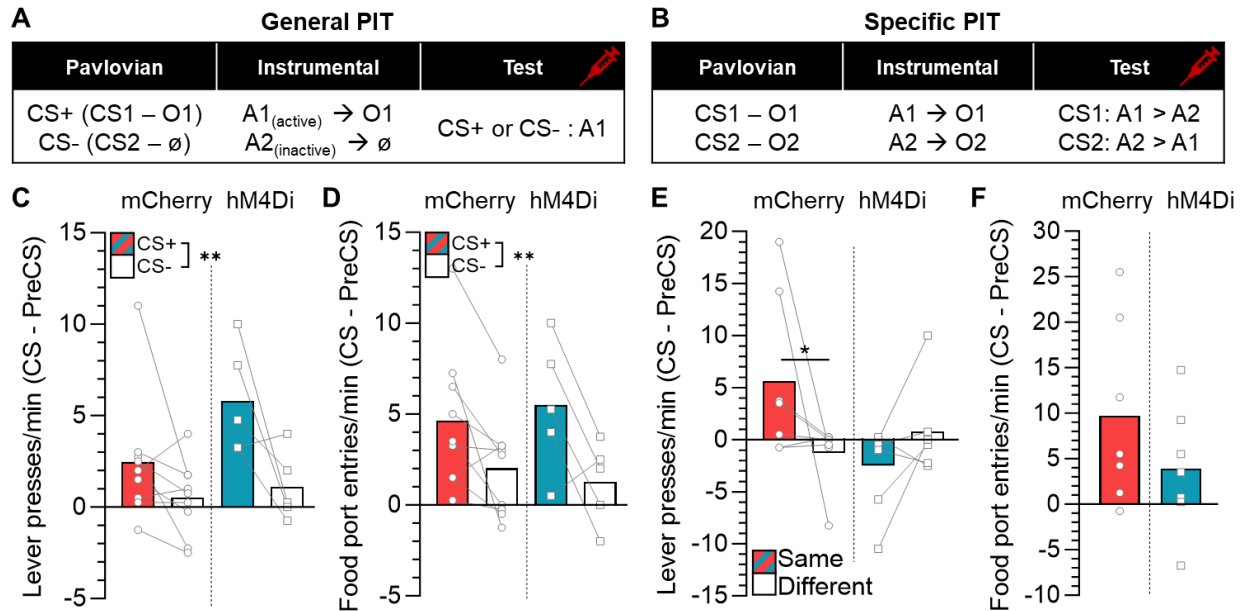


Figure 19. Inactivation of MD neurons receiving GC inputs impairs specific but not general PIT. Schematic representation of the behavioural procedure used for general **(A)** and specific PIT **(B)**. Rats were injected with DCZ 0.1M 30 min prior to testing. **(C)** Rate of responding on the active lever during the general PIT test for mCherry ($n = 9$) and hM4Di ($n = 5$) groups during stimulus presentations. Responding is calculated as lever pressing during the CS period (CS+ or CS-) minus the rate of pressing during the preceding baseline period (CS - PreCS). **(D)** Rate of food port entries (FPEs) during the general PIT test. FPEs are calculated as the rate of FPEs during the CS presentation minus the rate of FPE during the preCS. **(E)** Rate of responding on the active lever during the specific PIT test for mCherry ($n = 7$) and hM4Di ($n = 7$) groups during stimulus presentations. Responding is calculated as the rate of lever pressing during the CS period (same or different) minus the rate of pressing during the preceding baseline period (CS - PreCS). **(F)** Rate of food port entries (FPEs) during the specific PIT test. FPEs are calculated as the rate of FPEs during the CS presentation minus the rate of FPEs during the preCS.

Following completion of PIT protocols, animals from both experiments underwent a progressive ratio task (**Figure 20**). In this task, rats were required to press the lever an increasing number of times to obtain a single reward. Specifically, the fixed ratio (FR) schedule increased by 3 after each reward, meaning the first reward was delivered after one press, the second after four presses, the third after seven presses, and so on. The breakpoint, defined as the highest FR completed before the animal ceased responding, was used as a measure of motivation. The general motivation to lever press was found to be similar between control mCherry rats and hM4Di rats in both the general ($F_{1,12} = 0.53$, $p = 0.48$; **Figure 20.A**) and specific PIT experiments ($F_{1,12} = 0.18$, $p = 0.68$; **Figure 20.B**).

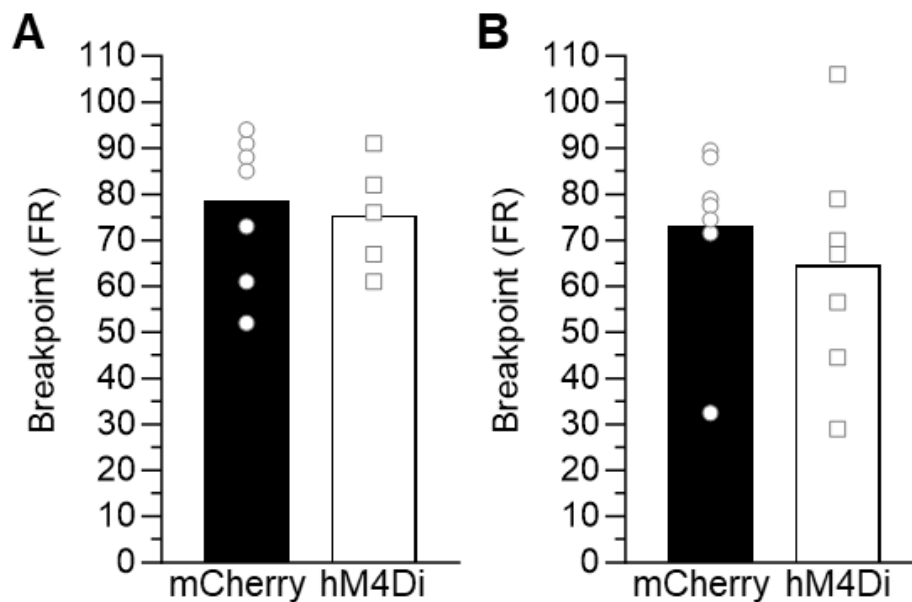


Figure 20. Inactivation of MD neurons receiving IC input does not impair motivation to lever press. Following completion of the general (**A**) or specific PIT protocol (**B**), animals were briefly retrained to lever press and were then subjected to a progressive ratio task. The breakpoint, defined as the highest fixed ratio (FR) completed before the animal ceased responding, was used as a measure of motivation.

Collectively, these results suggest that the IC-MD pathway is crucial for the expression of specific but not general PIT. Moreover, the deficit observed in specific PIT cannot be attributed to a lack of motivation to lever press. Rather the deficit observed reflects an inability to use specific S-O associations to bias instrumental action selection.

4. DISCUSSION

In the present preliminary work, we used a double viral approach utilizing a transynaptic anterograde vector carrying Cre (injected in IC) and cre-dependent inhibitory DREADDs (injected in MD). This method was intended to restrict DREADDs expression to MD neurons receiving inputs from the gustatory region of the IC. However, AAV1 has some limitations, as it can be transported in a retrograde direction in addition to its primary anterograde properties ([Kitanishi et al., 2022](#); [Rothermel et al., 2013](#); [Zingg et al., 2017](#)). Given the reciprocal connections between the IC and MD ([Odegaard et al., 2024](#); [Wright & Groenewegen, 1996](#)), there is a possibility that AAV1 could be transported both anterogradely and retrogradely between these regions, potentially targeting both MD neurons receiving inputs from the IC and MD neurons projecting back to the IC. Consequently, any deficits observed from manipulating the IC-MD pathway using this chemogenetic approach could result from effects in either direction.

Therefore, our viral approach does not allow to unambiguously determine the direction of communication (cortico-thalamic and/or thalamo-cortical) due to the minor retrograde properties of the anterograde Cre-carrying vector and the reciprocal connections between the IC and MD. Nevertheless, these preliminary results suggest a dissociation in the role of IC-MD pathways in specific versus general PIT, with interaction between these structures being required for the former but not the latter. It is also possible that our viral method infected MD neurons projecting to the IC, and if these neurons synapse onto insular interneurons ([Ohara et al., 2003](#); [Ramos-Prats et al., 2022](#); [Sun et al., 2020](#)), this could have led to IC inhibition alongside the inhibition of MD neurons receiving IC inputs. We think this is unlikely as we showed in the previous chapter that IC inactivation at test

abolishes general PIT, an effect we did not observe when manipulating the IC-MD pathway. Thus, the observed effects likely stem from the cortico-thalamic direction rather than thalamo-cortical, although we cannot entirely rule out the latter in this setup.

Consistently with a role of the IC-MD pathway in associative learning and stimulus-guided choice, it has recently been shown that the MD is integral to the gustatory pathway, encoding taste-related signals and processing associative information essential for ingestive related behaviors (Odegaard et al., 2024). Moreover, the MD encodes taste information more slowly than the IC but faster than the prefrontal cortex (PFC), raising the possibility that MD relays outcome-related information relevant to consumption decisions from the IC to the PFC (Odegaard et al., 2024). While the MD seem to not be required for S-O learning *per se*, as MD-lesioned rats are able to develop conditioned responses to Pavlovian stimuli, there is evidence of its critical involvement in generating outcome-specific Pavlovian expectancies, as demonstrated by the inability of MD-lesioned rats to adapt to changes in stimulus-outcome contingencies (Morceau et al., 2022; Ostlund & Balleine, 2008). These results are consistent with recent findings suggesting that the MD plays a critical role in attending to specific stimuli to choose the most appropriate response strategy (A. Mukherjee et al., 2021; Schmitt et al., 2017; Wolff & Halassa, 2024). Collectively, these data suggest that the MD plays a critical role in tracking relevant environmental changes to generate outcome-specific expectancies, crucial for Pavlovian stimuli to specifically bias instrumental responding.

In contrast to S-O learning, the MD has been found to be critical for A-O learning, but not for the retrieval of these A-O associations (Alcaraz et al., 2018; L. Bradfield et al., 2013; Corbit et al., 2003; Fisher et al., 2020; Mitchell et al., 2007; Ostlund & Balleine,

2008; Pickens, 2008; Wicker et al., 2018). However, this requirement seems to be confined to scenarios where multiple responses have been trained to earn reinforcers, that is, in situations where outcome-specific expectancies develop, rather than situations where only a single response has been learned, leading to a general reward expectancy (Pickens, 2008).

Our results align with previous studies that have demonstrated a central role of the MD in the expression of specific PIT (Leung et al., 2024; Leung & Balleine, 2015; Ostlund & Balleine, 2008). However, the observed patterns of results vary depending on the experimental conditions. Consistent with our findings, Ostlund & Balleine (2008) reported that post-training lesions to the MD rendered rats insensitive to Pavlovian stimuli, resulting in similar rates of instrumental responding across test periods (baseline, *same*, *different*). In contrast, research by Leung and colleagues showed that while the excitatory influence of Pavlovian stimuli on instrumental actions was preserved, it became non-reinforcer-specific following inactivation of the VPm-MD, MD-IOFC, and VPm-MD-IOFC pathways using a combination of pharmacological, chemogenetic, and optogenetic approaches (Leung et al., 2024; Leung & Balleine, 2015). In the study from Ostlund & Balleine (2008), the animals underwent an outcome devaluation procedure prior to PIT test, which involved repeated instrumental testing under extinction conditions that likely reduced baseline levels of responding—similar to the extinction treatment administered to our rats before PIT testing. Additionally, they employed a lesion approach, with some lesions extending into surrounding thalamic nuclei. In contrast, Leung's research utilized a protocol similar to ours but without an instrumental extinction session prior to testing (Leung et al., 2024; Leung & Balleine, 2015). While our preliminary results suggest that the GC-MD pathway

is not required to mediate the general excitatory influence of a reward-predicting stimulus, MD inactivation during a general PIT test has not been previously investigated. Leung's research suggests that this excitatory influence is preserved, and our failure to observe this effect may be attributable to the instrumental extinction session included in our protocol.

In summary, the role of the MD in instrumental behavior seems to be limited to the acquisition of A-O associations, and while MD is not required for the acquisition of S-O associations, it is critical to adapt to new Pavlovian contingencies, by generating outcome-specific expectancies. This suggests that the deficit observed in specific PIT following MD inactivation does not result from an inability to retrieve A-O associations, but could stem from an inability to use outcome-specific predictions based on stimulus presentations, reflecting an impairment in the processing of Pavlovian stimuli in new situations. This is further supported by the ability of Pavlovian stimuli to exert a general excitatory influence on instrumental actions in MD-inactivated subjects, as demonstrated by the significant general PIT effect observed following MD inhibition. That is, in a situation where the subject does not need to retrieve complex outcome-specific representations, the MD is not required for reward-predictive stimuli to increase instrumental responding. This aligns with the proposed role of the MD in forming outcome-specific expectancies, rather than mediating the general excitatory effects of Pavlovian stimuli on instrumental actions. Moreover, motivation for lever pressing is not impaired by MD inactivation, as demonstrated by performance in the progressive ratio task, which was similar to controls. Altogether, these preliminary data suggest that functional communication between the IC and the MD mediates the performance of outcome-specific stimulus-guided actions.

CHAPTER IV

GENERAL DISCUSSION

Stimulus-guided action is an adaptive process that enables organisms to use environmental stimuli to pursue favourable outcomes or goals. This process involves the independent acquisition, storage, and retrieval of stimulus-outcome (S-O; Pavlovian) and action-outcome (A-O; instrumental) associations, and can be empirically studied using Pavlovian-to-instrumental transfer (PIT). In the present research, we aimed to elucidate the role of the gustatory region of the insular cortex (IC) in both the general and specific forms of PIT. The IC is not only highly connected within the PIT circuitry, but has also been found to represent stimuli predicting both general and specific food-related events (M. P. H. Gardner & Fontanini, 2014; Samuelsen et al., 2012; Vincis et al., 2020; Vincis & Fontanini, 2016). We thus hypothesized that the IC is a key region in mediating the transfer of Pavlovian predictions to the control of instrumental actions. To test this hypothesis, we employed a chemogenetic approach to specifically inhibit the IC during the transfer test. Our findings indicate that IC is required for Pavlovian stimuli to exert both general excitatory and specific influences on instrumental actions. Preliminary results also suggest that the latter may particularly depend on an intact IC-MD pathway.

1. Insular cortex is required for general and specific transfer

The PIT effect manifests in two forms: general PIT, where invigoration of instrumental responding is driven by the general motivational and appetitive properties of Pavlovian stimuli, and specific PIT, which relies on the specific sensory features of the outcomes to selectively bias choice toward instrumental actions associated with the same, but not different, outcome. We found that temporarily inactivating the IC specifically during the test phase abolished both forms of PIT.

In the first experiment, rats injected with a control GFP virus in IC exhibited a general transfer effect, pressing the active lever during the reward-predictive stimulus (CS+) at a

significantly higher rate than during CS- presentations (relative to baseline). Importantly, this transfer effect was evident in rats treated with vehicle and in rats treated with the DREADD ligand, DCZ. Thus, our results indicate that the DCZ treatment itself does not affect the transfer effect. Moreover, rats expressing the inhibitory hM4Di receptor in the IC also showed general transfer if they were injected with vehicle. However, critically, rats expressing the hM4Di receptor who were injected with DCZ during the PIT test failed to show general PIT. Specifically, their rates of instrumental responding were similar across all periods (CS+, CS-, and baseline), suggesting that IC inactivation rendered instrumental actions virtually insensitive to reward-predictive stimuli.

In this initial experiment, we used a single outcome protocol in which both the Pavlovian stimulus and instrumental actions were reinforced with the same outcome. While this protocol is commonly employed in the literature, and it is generally accepted that single A-O protocols produce general transfer effects ([Cartoni et al., 2016](#)), we were concerned that the observed deficits could be due to an insensitivity of the subjects' instrumental actions to the sensory-specific predictive properties of the Pavlovian stimulus rather than its general excitatory influence. To rule out this possibility, we conducted a second general PIT experiment using a dual outcome paradigm, where Pavlovian conditioning and instrumental training were conducted with different reinforcers (i.e. grain pellets for Pavlovian conditioning and sugar pellets for instrumental conditioning or vice versa). As expected, this design also produced a general transfer effect, as evidenced by the significant invigoration of instrumental responding during CS+ in control rats. This finding confirms that PIT protocols utilizing a single A-O association can produce general transfer effects regardless of the reinforcers used, provided that they are equally desirable and of the same nature ([Baxter &](#)

Zamble, 1982). However, IC inactivation again abolished this transfer effect, with instrumental actions remaining essentially unaffected by the Pavlovian stimuli.

We then assessed the IC's contribution to specific PIT. We found that IC inactivation also abolished this form of PIT and the pattern of results was similar to what was observed in the general PIT experiments. That is, instrumental actions in IC-inactivated rats appeared insensitive to the presence of reward-predictive stimuli, as evidenced by similar rates of lever pressing across different test periods (*same, different, baseline*).

Together, these findings indicate that IC plays a role in the expression of both general and specific PIT. Importantly, supplementary analyses revealed that, when considered independently, the Pavlovian and instrumental responses of control rats and those with IC inhibition were virtually indistinguishable. Indeed, Pavlovian conditioned responses remained intact with IC inactivation, indicating no deficit in the retrieval or expression of S-O associations. For instance, in the general PIT experiments, IC-inactivated rats visited the food port significantly more during CS+ than during CS-, relative to baseline, not only suggesting sensitivity to the general rewarding properties of the CS+, but also discrimination between the two auditory stimuli. This indicates that while general PIT was abolished, the rats were able to recall which CS was rewarded and which was unrewarded. In the specific PIT experiment, IC-inactivated rats also visited the food port more frequently during the reward-predictive stimuli than during the baseline period, further demonstrating their intact sensitivity to the general rewarding properties of the Pavlovian stimuli. However, it is not possible to determine whether this behavior reflects an outcome-specific expectancy, as the same food port was used for delivering the outcomes predicted by both stimuli. Therefore, the Pavlovian conditioned responses observed in the specific PIT experiment could result from either a general or a specific reward expectancy.

Instrumental responding per se also appeared unaffected following IC inhibition. For instance, IC inhibition did not diminish the rats' motivation to perform an instrumental action, even when the effort required to obtain the reward was progressively increased, and the total number of instrumental actions performed by IC-inactivated rats was comparable to that of the control rats across all three experiments. Furthermore, in the general PIT experiments, IC-inactivated rats performed the rewarded action (active lever) significantly more than the action that was never reinforced (inactive lever), suggesting that IC inactivation did not impair the retrieval of the action-reward association. In the specific PIT experiment, while rats performed the instrumental responses in a similar manner to controls, we cannot be sure if the rats were able to recall the specific A-O associations (i.e., A1-O1 and A2-O2) or if they simply retrieved a general A-reward association (i.e., A1-O and A2-O). However, importantly, several other studies using very similar procedures have shown that rats with IC inhibition are perfectly able to recall specific A-O associations, in addition to general A-reward associations (Balleine & Dickinson, 2000; Parkes et al., 2015, 2018; Parkes & Balleine, 2013). It thus seems that the IC is not required to recall or express instrumental associations.

Given the similar pattern of results across experiments, it is tempting to suggest that the nature of the impairment is the same in both general and specific PIT. That is, if IC inhibition renders rats insensitive to general transfer, perhaps it is not surprising that they also don't show specific transfer. However, it must be noted that an inability to express general PIT does not systematically reflect a deficit in specific PIT expression. For instance, rats lacking a functional CeA or NAc core show evidence of specific PIT despite abolished general PIT (Blundell et al., 2001; Corbit et al., 2001; Corbit & Balleine, 2005, 2011a; Derman et al., 2020; Keistler et al., 2015; Leung & Balleine, 2013; Ostlund & Balleine, 2008; Shiflett & Balleine, 2010; Sias et al., 2021, 2024), while BLA or NAc shell inactivations spare general but not

specific PIT (Corbit & Balleine, 2005, 2011a; Hall et al., 2001; Holland & Gallagher, 2003; Lex & Hauber, 2008; Lingawi & Balleine, 2012).

Collectively, our results suggest that IC inhibition renders instrumental responding insensitive to reward-predictive stimuli, even though both Pavlovian conditioned responses and instrumental performance appear to be preserved. These results constitute the first demonstration that IC is required for transfer effects and significantly contribute to our understanding of the cortical bases of PIT.

2. A role for insular cortex in outcome representation

The involvement of the gustatory region of IC in *both* general and specific transfer is highly consistent with evidence showing that IC neurons exhibit a strong and consistent response to Pavlovian cues predicting the general availability of taste (Samuelsen et al., 2012; 2013) as well as selective neural responses to cues predicting distinct outcomes (Gardner & Fontanini, 2014). That is, the IC appears to encode both a general anticipatory signal as well as outcome-specific expectations (M. P. H. Gardner & Fontanini, 2014; Saddoris et al., 2009; Samuelsen et al., 2012; Vincis et al., 2020; Vincis & Fontanini, 2016).

Indeed, in line with IC's role in general PIT, Samuelsen and colleagues demonstrated that stimulus-induced general reward expectation modulates the activity of IC taste responsive neurons, an anticipatory response that subsequently promotes the faster encoding of taste (Samuelsen et al., 2012). Follow-up studies demonstrated that IC's neural activity was also modulated by specific reward expectation, in addition to general reward expectation, consistent with our finding that IC is also crucial for the expression of specific PIT (M. P. H. Gardner & Fontanini, 2014; Vincis et al., 2020). Activity in IC also appears to track outcome-specific expectancies, as stimulus-induced activity was found to extinguish with repeated reward omissions (M. P. H. Gardner & Fontanini, 2014).

Although evidence suggests that IC neural activity reflects Pavlovian learning and stimulus-induced general and specific expectancies, it remains unclear in the current literature whether this activity is functionally required for the acquisition or retrieval of S-O associations (M. P. H. Gardner & Fontanini, 2014; Kusumoto-Yoshida et al., 2015; Livneh et al., 2017; Samuelsen et al., 2012). For instance, Kusumoto-Yoshida and colleagues found that pharmacological inactivation of the IC during Pavlovian conditioning completely abolished the conditioned response, as demonstrated by levels of food port entries numerically lower than controls' baseline (Kusumoto-Yoshida et al., 2015). However, the near absence of any conditioned (or unconditioned) response following IC inactivation raises concerns about the general locomotor activity and/or motivation of these animals, an issue that was not addressed by the authors. Moreover, it is unclear whether this inactivation occurred during the early or later phases of training, which makes it difficult to ascertain whether the results reflect an impairment in acquisition or expression of the Pavlovian conditioned response. In a second set of experiments, Kusumoto-Yoshida et al. employed an optogenetic approach to inactivate the IC during the presentation of a reward-predictive stimulus (Kusumoto-Yoshida et al., 2015). Here, they found that IC inactivation significantly reduced the rate of Pavlovian conditioned responses, albeit modestly, suggesting that IC inactivation might attenuate, but does not abolish, the subjects' sensitivity to the general rewarding properties of a CS. This is consistent with our findings that the Pavlovian conditioned response is preserved in well trained animals following IC inhibition, in addition to the preserved ability to discriminate between CS+ and CS-.

Furthermore, electrophysiological recordings of IC activity during a stimulus-guided choice task confirmed its contribution to the encoding of specific stimulus-induced signals, reflecting anticipatory activity related to an impending choice (Vincis et al., 2020). Importantly,

perturbing IC during this anticipatory period significantly reduced performance in well trained animals (rewarded licks or correct trials), suggesting that GC's anticipatory activity participated in controlling taste-guided, reward-directed choice. While IC inactivation clearly impaired stimulus-guided choice behavior, it was not sufficient to completely abolish subjects' ability to perform the task. This suggest that stimulus-induced outcome expectancy was preserved to some extent, as the animals still performed above chance levels despite inactivation of IC. Interestingly, at first glance, this task may seem to share features reminiscent to the specific PIT protocol (**Figure 16**), where different actions are specifically biased by reward predictive stimuli. However, while specific PIT hypothetically operates through specific stimulus-action associations (S1-A1; S2-A2), each mediated by a specific outcome representation (S^{O1} -A1; S^{O2} -A2), these S-A associations are mediated by a common reward representation in the task developed by [Vincis and colleagues \(2020\)](#). Hence, the influence of a specific stimulus on a specific action was mediated by a general reward expectation, rather than an outcome-specific expectancy, perhaps mirroring a general transfer like-effect.

Our findings are also in accordance with studies showing that the gustatory region of IC is required to retrieve outcome value to guide action selection using the outcome devaluation task ([Balleine & Dickinson, 2000](#); [Parkes et al., 2015, 2018](#); [Parkes & Balleine, 2013](#)). In these experiments, rats are trained to perform two actions to earn two distinct rewards and then one of these rewards is devalued before a choice test between the two actions. Typically, rats will choose the action associated with the non-devalued outcome more than the action associated with the devalued outcome, indicating that they have both encoded the new value of the outcome and are able to retrieve this outcome representation to inform their instrumental responding. However, lesions or inactivation of NMDA receptors in the

gustatory region of IC renders rats insensitive to outcome devaluation and such that they fail to show this preference toward the action earning the non-devalued outcome (Balleine & Dickinson, 2000; Parkes et al., 2015, 2018b; Parkes & Balleine, 2013). Specifically, the evidence indicates that the IC is required not to encode but rather to *retrieve* the outcome representation (here, the outcome value) (Parkes & Balleine, 2013; Parkes et al., 2015; 2018).

Taken together, these studies demonstrate that the IC processes both general and specific outcome expectancies, whether they are guided by stimuli or value, to effectively influence instrumental actions. That is, the IC appears to play a critical role in the representation of both Pavlovian and instrumental outcomes.

3. Theoretical account of IC's role in transfer

We therefore propose that the deficits in transfer that we observed in rats with IC inhibition stem from the inability of these rats to use both general and specific outcome expectancies to influence instrumental actions. Indeed, our rats showed impaired transfer effects but intact Pavlovian and instrumental associations.

In our experiments, the IC was not inhibited during the acquisition of A-O and S-O associations, indicating that the deficit does not arise from a failure to learn these associations. Moreover, it appears that the retrieval of the S-O and A-O associations was also intact (although the extent to which the *specific* associations are recalled may be questioned). However, our IC inhibited rats showed a clear failure to use general and specific Pavlovian expectancies to invigorate or selectively bias instrumental actions.

As described in **Chapter I**, the associative cybernetic model of instrumental conditioning is currently the most comprehensive theoretical account for transfer effects (Balleine & Ostlund, 2007). A key feature of this model is that transfer is mediated by a stimulus-induced

representation of the outcome, which in turn serves as a stimulus that triggers the representation and subsequent performance of the associated instrumental action (see **Figure 6**). The theoretical emergence of this S^O -A chain results notably from the ability to use the backward A-O association (i.e., the O-A association), where the representation of the outcome (S^O) leads to a mental representation of the corresponding action, resulting in the activation of motor responses ($S1^{O1}$ -A1; $S2^{O2}$ -A2), thus reflecting specific PIT. In addition, the model proposes that reward predictive stimuli can prompt a rather general rewarding outcome expectancy, which in turn excite motor responses through a S^{reward} -A chain.

Interestingly, both forms of PIT rely on common mechanisms, such as the use of a S-O-A chain through an O-A association (Balleine & Ostlund, 2007). However, the associative cybernetic model predicts two distinct ways to produce S-O-A chains, both originating from associative memory (see **Figure 6**). The model suggests that the impairment observed in general PIT following IC inactivation may stem from the failure of stimulus-induced general reward expectancies in “reward memory” to invigorate instrumental actions through a S^{reward} -A associative chain, possibly reflecting the inability of using the reward-A association. In the case of specific PIT, the deficit we observed following IC inactivation may stem from either an inability to prompt stimulus-induced specific outcome expectancies in “S-A memory” ($S1^{O1}$; $S2^{O2}$), or from an inability to retrieve or use O-A associations stored in “S-A memory” ($O1$ -A1; $O2$ -A2). It is currently unknown if the IC is indeed required to use backward A-O associations but this could be assessed using the reinstatement test, whereby a previously extinguished instrumental action is reinstated by “free” delivery of its associated outcome, where the outcome serves as a stimulus signaling its availability, stimulating the performance of the associated action.

The theoretical integration of the IC into the neural associative cybernetic model of instrumental actions is illustrated in **Figure 21**. The lack of transfer effects observed in rats with a non-functional IC may result from either a failure to represent the outcome as a stimulus (S^O), which is required to activate the representation of the associated action (A), or an inability to use the triggered A-O representation in a backward manner. In both cases, there is a failure to use the S^O -A associative chain within S-A memory, reflecting an inability of stimulus-induced outcome expectations to influence instrumental actions.

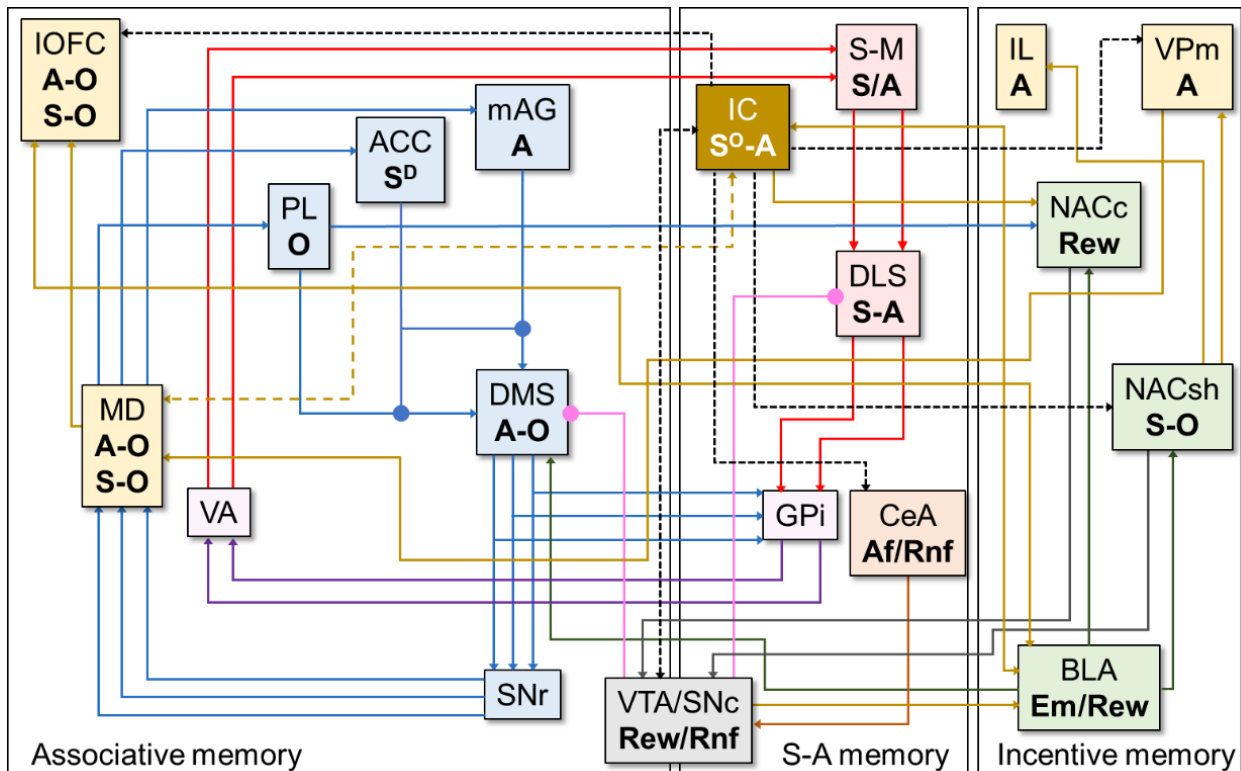


Figure 21. Proposed update of the neural implementation of the associative cybernetic model. The three modules of the model are presented (associative, S-A memory and incentive memory), and brain structures for which a role in associative learning is identified are placed in these modules. I have added several brain regions to the existing model from [Balleine and Ostlund \(2007\)](#) based on recent data (shown in gold) including: the lateral OFC (**IOFC**), the mediodorsal thalamus (**MD**), the infralimbic cortex (**IL**), the ventral medial pallidum (**VPm**) and the gustatory cortex (**GC**). Plain gold lines represent pathways known to be crucial for PIT expression. The gold dotted line represents the potential contribution of the IC-MD pathway to PIT. Black dotted lines represent connections between the IC and its anatomical partners, for which the contribution to PIT is currently unknown. The IC is highly connected within the associative network, and ideally placed to mediate the effects of Pavlovian expectancies on instrumental actions. Specifically, we propose that the IC promotes the transfer effect by allowing general and specific stimulus-induced outcome expectancies to prime a reward representation in S-A memory and trigger the backward use of the A-O association. **ACC**: anterior cingulate cortex; **BLA**: basolateral amygdala; **CeA**: central amygdala; **DLS**: dorsolateral striatum; **DMS**: dorsomedial striatum; **GPI**: pallidum; **mAG**: medial agranular area; **NACc**: core of nucleus accumbens; **NACsh**: shell of nucleus accumbens; **PL**: prelimbic area; **SNr**: substantia nigra pars reticula; **SNc**: substantia nigra pars compacta; **VA**: ventral anterior thalamus; **VTA**: ventral tegmental area. **Af**: affective; **Em**: emotional; **Rew**: reward; **Rnf**: reinforcement.

Our results indicate that the IC is not required for the acquisition or retrieval of A-O or S-O associations, as independent Pavlovian and instrumental responses of rats with IC inhibition do not differ from those of matched controls. While IC inactivation preserves the rats' sensitivity to the general rewarding properties of Pavlovian stimuli, as indicated by the maintained conditioned response in our PIT experiments, it remains unclear whether specific stimulus-induced outcome expectancies are also preserved. Inactivation of the IC only modestly attenuates performance in stimulus-guided tasks, suggesting that animals retain some sensitivity to general and specific-reward expectancies despite IC inactivation (Kusumoto-Yoshida et al., 2015; Vincis et al., 2020). If this assumption is correct, the deficits observed in our PIT experiments are likely not due to a failure in encoding or retrieving general and specific stimulus-induced outcome expectancies, but rather from an inability to use these expectancies to invigorate or selectively bias instrumental actions. According to the associative cybernetic model of instrumental actions, such a PIT effect relies on an intact S-O-A associative chain. Critically, the use of such an associative chain is a common mechanism to both forms of PIT. I propose that the use of this associative chain heavily depends on intact IC function.

4. Distinct contributions of insular pathways to general versus specific transfer

While we have shown that the IC is required for the expression of both forms of PIT, distinct insular pathways are likely at play. Notably, our preliminary data suggest that specific, but not general, PIT relies on an intact IC-MD pathway. Consistently, it has been proposed that the MD is crucial for generating outcome-specific, but not general, reward expectancies (Morceau et al., 2022; Ostlund & Balleine, 2008), see **Chapter III** for further discussion.

The IC is also reciprocally connected to the BLA (Gehrlach et al., 2020; Ponsérre et al., 2020; Schiff et al., 2018; Sripanidkulchai et al., 1984; Yamamoto, 2006; Yamamoto et al.,

1997), and the IOFC (Barreiros et al., 2021; Y. Chen et al., 2022; Mathiasen et al., 2023), and send inputs to the NAc shell (Allen et al., 1991; G. J. Lee et al., 2022), which are all regions that have been implicated in specific PIT (see **Appendix I**). One might therefore hypothesize that the IC-BLA, IC-shell and IC-IOFC pathways could support specific PIT, but not general PIT. This prediction is supported by a study from Samuelsen and colleagues suggesting that the BLA-IC pathway mediates some aspects of reward expectancies (Samuelsen et al., 2012). The authors found that pharmacological inactivation of the BLA significantly reduced the neural excitatory responses to predictive stimuli in the IC, confirming that the BLA-IC pathway underlies the anticipatory priming of the IC. Moreover, evidence from outcome devaluation studies also proposes that the BLA updates and encodes information about the value of the instrumental outcome and then sends this information to IC, where it is retrieved to guide behaviour (Parkes & Balleine, 2013). However, whether this pathway contributes to the specific transfer effect remains to be determined.

By contrast, general PIT may require interaction between the IC and the CeA and/or NAc core, as these regions are involved in mediating the general excitatory influences of reward-predictive stimuli on instrumental action (see **Appendix I**). This is supported by the crucial role of the CeA in generating stimulus-induced general motivation for natural rewards, as reviewed by Warlow and Berridge (2021). Again, whether IC inputs to the CeA or NAc core are required for reward-predictive stimuli to invigorate instrumental actions warrants further investigation.

Hence, it is hypothesized that a double dissociation may exist at the cortico-striatal and cortico-amygdalar (as well as cortico-thalamic) level, such that IC-core and IC-CeA may

support general (but not specific) transfer, and IC-shell and IC-BLA potentially supporting specific (but not general) PIT as illustrated in **Figure 22**.

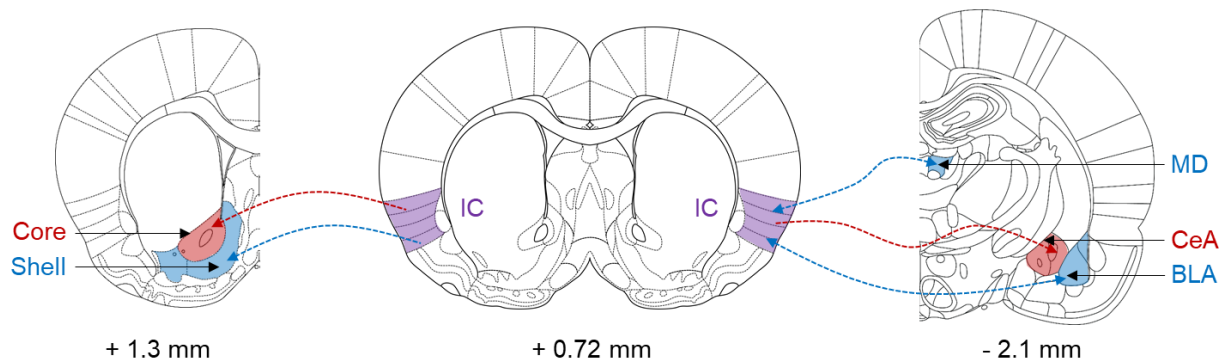


Figure 22. Hypothetical functional dissociation of insular pathways in general versus specific PIT. The insular cortex (IC; purple shade) is involved in both general and specific PIT. However, distinct cortico-amygdalar, -striatal and -thalamic pathways may support these dissociated forms of PIT. While IC-core and IC-CeA may be required for the expression of general PIT (red pathways), specific PIT might rely on IC-BLA and IC-MD pathways (blue pathways). Distance in mm from bregma is indicated under rats' coronal sections. *BLA*: basolateral amygdala; *CeA*: central amygdala; *MD*: mediodorsal thalamus.

5. Beyond food-related decision-making

The role of the gustatory region of IC in food-related decision-making is now well-established. However, a growing body of literature suggests a broader role for the IC in decision-making beyond food- or taste-related behaviours (for a review see [Rogers-Carter & Christianson, 2019](#)). For example, ecologically relevant evidence for a gustatory role in social behavior can be found in the social transmission of food preferences, where rats prefer to consume a certain food if they have recently interacted with a conspecific that has safely consumed that food ([Galef et al., 1985](#); [Galef & Wigmore, 1983](#)). More generally, social behaviors require the integration of external and internal sensory stimuli, and there is evidence that such integration occurs in both rodent and human IC ([Gu et al., 2012](#); [Ibañez et al., 2010](#); [Leigh et al., 2013](#); [Rogers-Carter et al., 2018](#)). The IC is therefore well-positioned to convey anticipatory social stimuli to the social decision-making network, supporting its

consideration as a crucial component of the social brain ([Rogers-Carter & Christianson, 2019](#)).

Recent studies have also found that the IC regulates approach and avoidance responses to social affective stimuli in rats ([Rogers-Carter et al., 2018](#)). In a social affective preference (SAP) test, rats could choose to approach or avoid either a juvenile or an adult stressed conspecific. In this setup, social stress signals from an adult are considered danger-predictive stimuli, prompting avoidance, while social stress signals from juveniles are perceived as prosocial stimuli, triggering approach. Optogenetic inactivation of the IC during the SAP test abolished these preferences, resulting in a significant decrease in approach behavior towards stressed juveniles and in avoidance behavior towards stressed adults. Evidence suggests that these social behaviors may be modulated by IC outputs to the NAc core ([Rogers-Carter et al., 2019](#)) and may rely on the transfer of emotional information from the BLA to the IC ([Djerdjaj et al., 2022](#)). Other social behaviors have been found to be impaired by IC inactivation, such as social recognition memory ([Min et al., 2023](#)) and social preference following social isolation ([Glangetas et al., 2024](#)). Importantly, it has been found that deficits in empathy in autistic patients have been linked to altered IC activity and connectivity ([Blanken et al., 2015](#); [Di Martino et al., 2009](#); [Morita et al., 2012](#); [Odriozola et al., 2016](#)), suggesting that the study of IC's contribution in rodent social behavior is clinically relevant.

In humans, IC lesions have been found to impair risky decision making, with an increase in betting behaviors when compared to healthy participants, suggesting a role of the IC in signaling the probability of aversive outcomes ([Clark et al., 2008](#)). By contrast, studies conducted in rats demonstrated that IC inactivation attenuates risk preference in gambling tasks ([Ishii et al., 2012](#); [Pushparaj et al., 2015](#)). Similarly, a more recent study conducted in

primates demonstrated that IC neurons encode decision-related variables that influence the animal's willingness to accept risk (Yang et al., 2022). Finally, the IC has been implicated in representing the internal state induced by drug use, and has therefore been studied for its potential contribution to drug-related decision-making (McGregor & LaLumiere, 2023). For example, IC inactivation has been found to decrease self-administration for nicotine, but not food, in rats (Forget et al., 2010; Pushparaj et al., 2013). This is particularly interesting as IC lesions have been found to disrupt smoking addiction in humans, by facilitating quitting, attenuating craving, and preventing relapses (Naqvi et al., 2007). IC manipulations have also been found to reduce alcohol self-administration in rats (De Oliveira Sergio et al., 2021; Haaranen et al., 2020; Pushparaj & Le Foll, 2015), as well as cocaine self-administration (Rotge et al., 2017), further expanding IC's contribution to broad decision-making processes.

6. Conclusion

Our results provide the first demonstration that the insular cortex (IC) mediates both general and specific forms of PIT. Preliminary findings also suggest that specific, but not general, PIT may depend on a functional cortico-thalamic pathway involving the mediodorsal thalamus, which is crucial for mediating stimulus-induced outcome expectancies. It is perhaps unsurprising that the IC is vital for mediating these PIT effects, given the extensive evidence showing that associative learning profoundly shapes taste-related activity in the IC. Indeed, this region is central to processing both general and specific stimulus-induced outcome expectancies, as well as retrieving outcome value to selectively bias action selection toward more desirable outcomes. This work contributes to the growing body of literature investigating the broad role of the IC in decision-making, with implications for social, risky, and addictive behaviors that all rely on the processing of outcome expectancies and internal body states.

APPENDIX I

SUMMARY TABLES: NEURAL BASES OF PIT IN RATS

Area	Req.	Inactivation			References
		Method	Phase	Result pattern	
Cortical areas					
ACC	✗	Lesion	Pre-	CS+ = PreCS	Cardinal et al., 2003
Subcortical areas					
BLA	✗	Lesion	Pre-	CS+ > PreCS	Hall et al., 2001
	✗	Lesion	Pre-	CS+ > CS-	Holland & Gallagher, 2003
	✗	Lesion	Pre-	General > PreCS	Corbit & Balleine (2005)
CeA	✓	Lesion	Pre-	CS+ = PreCS	Hall et al., 2001; Holland & Gallagher, 2003; Lingawi & Balleine, 2012
	✓	Lesion	Pre-	General = PreCS	Corbit & Balleine (2005)
NAcC	✓	Lesion	Pre-	CS+ = PreCS	Corbit & Balleine, 2011; Hall et al., 2001
	✓	Pharmacology	Post-	CS+ = PreCS	Corbit & Balleine, 2011
	✓	Pharmacology	Post-	CS+ = PreCS	Lex & Hauber, 2008
NAcS	✗	Lesion	Pre-	CS+ > PreCS	Corbit & Balleine, 2011; Hall et al., 2001
	✗	Pharmacology	Post-	CS+ = PreCS	Corbit & Balleine, 2011
	✗	Pharmacology	Post-	CS+ = PreCS	Lex & Hauber, 2008
NAcS	✓	Pharmacology	Post-	CS+ > PreCS	Wyvell & Berridge, 2000
VTA	✓	Pharmacology	Post-	CS+ = PreCS (= CS-)	Corbit et al., 2007; Murschall & Hauber, 2006
	✓	Chemogenetic	Post-	CS+ = PreCS (= CS-)	Halbout et al., 2019
Pathways					
VTA-CeA	✗	Lesion (contralateral)	Pre-	CS+ > PreCS & CS-	El-Amamy & Holland, 2007
VTA-mPFC	✗	Chemogenetic	Post-	CS+ > PreCS	Halbout et al., 2019
VTA-NAcC	✗	Chemogenetic	Post-	CS+ > PreCS	Halbout et al., 2019
VTA-SNpc	✓	Lesion (unilateral)	Pre-	CS+ = PreCS = CS-	El-Amamy & Holland, 2007
	✓	Lesion (contralateral)	Pre-	CS+ = PreCS = CS-	El-Amamy & Holland, 2007

Table 2. Neural substrates of general PIT identified using rat inactivation studies. Requirement (Req.) of a structure or pathway is indicated by “✓” (blue rows) while “✗” (white rows) indicates that the brain region is not required for general PIT. **ACC**: anterior cingulate cortex; **BLA**: basolateral amygdala; **CeA**: central amygdala; **mPFC**: medial prefrontal cortex; **NAcC**: Core of the NAc; **NAcS**: Shell of the NAc; **SNpc**: substantia nigra pars compacta; **VTA** : ventral tegmental area.

Area	Req.	Inactivation			References
		Method	Phase	Result pattern	
Cortical areas					
aMO	✓	Lesion	Pre-	Same = Diff. (> PreCS)	Bradfield et al., 2018
IL	✓	Lesion	Pre-	Same = Diff. = PreCS	Keistler et al., 2015
IOFC	✓	Optogenetic	Post-	Same = Diff. (> PreCS)	Leung et al., 2024
MO	✓	Lesion	Pre-	Same = Diff. (> PreCS)	Bradfield et al., 2015
PL	✗	Lesion	Pre-	Same > Diff. & PreCS	Corbit & Balleine, 2003
pMO	✗	Lesion	Pre-	Same > Diff. & PreCS	Bradfield et al., 2018
VO-LO	✗	Lesion	Pre-	Same > Diff. & PreCS	Ostlund & Balleine, 2007
vIOFC	✓	Lesion	Post-	Same = Diff. = PreCS	Ostlund & Balleine, 2007
Subcortical areas					
aDMS	✓	Pharmacology	Pre-	Same = Diff. (> PreCS)	Corbit & Janak, 2010
BLA	✓	Lesion	Pre-	Same = Diff. (> PreCS)	Blundell et al., 2001
	✓	Lesion	Pre-	Same = Diff. = PreCS	Corbit & Balleine (2005)
	✓	Lesion	Post-	Same = Diff. = PreCS	Ostlund & Balleine, 2008
	✓	Chemogenetic	Post-	Same = Diff. = PreCS	Derman et al., 2020
	✓	Optogenetic	Pavlovian	Same = Diff. = PreCS	Sias et al., 2021
CeA	✗	Lesion	Pre-	Same > Diff. & PreCS	Corbit & Balleine (2005)
DLS	✓	Pharmacology	Post-	Same = Diff. = PreCS	Corbit & Janak, 2007
	✓	Pharmacology	Pre-	Same = Diff. (> PreCS)	Corbit & Janak, 2010
DMS	✓	Pharmacology	Post-	Same = Diff. (> PreCS)	Corbit & Janak, 2007
MD	✓	Lesion	Post-	Same = Diff. = PreCS	Ostlund & Balleine, 2008
NAcC	✗	Lesion	Pre-	Same > Diff. & PreCS	Corbit et al., 2001; Corbit & Balleine, 2011
	✗	Pharmacology	Post-	Same > Diff. & PreCS	Corbit & Balleine, 2011
NAcS	✓	Lesion	Pre-	Same = Diff. = PreCS	Corbit et al., 2001; Corbit & Balleine, 2011
	✓	Pharmacology	Post-	Same = Diff. = PreCS	Corbit & Balleine, 2011
pDMS	✓	Pharmacology	Pre-	Same = Diff. (> PreCS)	Corbit & Janak, 2010
VTA	✓	Pharmacology	Post-	Same = Diff. = PreCS	Corbit et al., 2007
Pathways					
BLA-NAcC	✗	Lesion (contralateral)	Pre-	Same > Diff. & PreCS	Shiflett & Balleine, 2010

BLA-NAcS	✓	Lesion (contralateral)	Pre-	Same = Diff. = PreCS	Shiflett & Balleine, 2010
BLA-IOFC	✓	Chemogenetic	Post-	Same = Diff. = PreCS	Lichtenberg et al., 2017
	✓	Optogenetic	Pavlovian	Same = Diff. = PreCS	Sias et al., 2021
IOFC-BLA-IOFC	✓	Optogenetic; Chemogenetic	Pavlovian; test	Same = Diff. (> PreCS)	Sias et al., 2021
MD-IOFC	✓	Chemogenetic	Post-	Same = Diff. (> PreCS)	Leung et al., 2024
NAcS-IL	✓	Lesion (contralateral)	Pre-	Same = Diff. = PreCS	Keistler et al., 2015
NAcS-VPm	✓	Pharmacology (contralateral)	Post-	Same = Diff. = PreCS	Leung & Balleine, 2013
IOFC-BLA	✗	Chemogenetic	Post-	Same > Diff. & PreCS	Lichtenberg et al., 2017
VPm-MD	✓	Lesion; pharmacology	Post-	Same = Diff. (> PreCS)	Leung & Balleine, 2015
	✓	Optogenetic; Chemogenetic	Post-	Same = Diff. (> PreCS)	Leung et al., 2024
VPm-MD-IOFC	✓	Chemogenetic	Post-	Same = Diff. (> PreCS)	Leung et al., 2024
VPm-VTA	✗	Lesion; pharmacology	Post-	Same > Diff. & PreCS	Leung & Balleine, 2015
VTA-BLA	✓	Optogenetic	Pavlovian	Same = Diff. (> PreCS)	Sias et al., 2024

Table 3. Neural substrates of specific PIT identified in rat inactivation studies.

Requirement (Req.) of a structure or pathway is by “✓” (blue rows) while “✗” (white rows) indicates that the region/pathway is not required for specific PIT. **aDMS**: anterior dorsomedial striatum; **aMO**: anterior medial OFC; **BLA**: basolateral amygdala; **CeA**: central amygdala; **DLS**: dorsolateral striatum; **DMS**: dorsomedial striatum; **IL**: infralimbic cortex; **IOFC**: lateral OFC; **MD**: mediodorsal thalamus; **MO**: medial OFC; **NAcC**: Core of the NAc; **NAcS**: Shell of the NAc; **OFC**: orbitofrontal cortex; **pDMS**: posterior dorsomedial striatum; **PL**: prelimbic cortex; **pMO**: posterior medial OFC; **vIOFC**: ventrolateral OFC; **VPm**: medial ventral pallidum; **VTA**: ventral tegmental area.

APPENDIX II

PIT IN FEMALE RATS

1. GENERAL PAVLOVIAN-TO-INSTRUMENTAL TRANSFER IN FEMALE SUBJECTS

We first attempted to apply the general PIT protocol we successfully used in males to female rats (**Table 4**) but it did not produce a general transfer effect (Experiment 1). The failure to observe a transfer effect appeared to be due to very low levels of instrumental responding in female rats during the PIT test. Visual comparison of the male and female data revealed that females appeared to extinguish their instrumental responding to a greater extent than males during the instrumental extinction that was conducted prior to the PIT test. As a reminder, instrumental extinction is typically performed prior to PIT tests to reduce baseline responding ([Holmes et al., 2010](#)). That is, instrumental extinction is performed to avoid a ceiling effect in instrumental responding such that it is not possible to observe increased responding during the stimulus presentation. Thus, we reduced the instrumental extinction session that precedes the test session for the female rats. Initially, we reduced the extinction session from 30 minutes to 20 minutes (Experiment 2) and then we tested the protocol without any instrumental extinction session (Experiment 3). All three experiments used a single outcome design, that is, all animals received grain pellets during both Pavlovian and instrumental training phases.

(A) Pavlovian Training	(B) Instrumental training	(C) Instrumental extinction	(D) Pavlovian extinction	(E) Test (extinction)
(CS+) S1 → O1 (CS-) S2 → ∅	(Active) A1 → O1 (Inactive) A2 → ∅	A1 → ∅ A2 → ∅	S1 → ∅ S2 → ∅	S1: A1 > baseline S2 : A1 = baseline
8 sessions	12 sessions	1 session	1 session	1 session

Table 4. General PIT protocol used in males. The protocol begins with a habituation session where rats are exposed to two auditory stimuli (S1 and S2), each presented six times in a pseudo-random sequence. **(A)** The following day, Pavlovian conditioning begins and continues for 8 days, with one session per day. During each session, one auditory stimulus (either a tone or clicker, counterbalanced across subjects) is paired with the delivery of grain pellets (CS+), while the other stimulus (CS-) is not paired with any reward. Each session includes 9 presentations of the CS+ and 3 of the CS-, with each stimulus lasting 2 minutes. The CS+ is paired with 4 rewards, delivered every 30 seconds on average. **(B)** Then, subjects undergo 12 instrumental training sessions, with two sessions per day. In these sessions, rats learn that performing one of two actions (active lever, e.g., A1) results in the same outcome (O1) as in Pavlovian conditioning (grain pellets). The other lever (inactive lever, e.g., A2) does not earn reward. At the start of each session, both levers are extended for 30 minutes or until 30 rewards are earned. In the first session, every response on the active lever (counterbalanced for right or left) is reinforced. In the subsequent 11 sessions, reinforcement occurs on average every 30 seconds. The inactive lever is never reinforced. **(C)** The animals are then subjected to a 30-minute instrumental extinction session, during which no responses are reinforced. This is followed by a Pavlovian extinction session **(D)**, similar to the initial habituation session. **(E)** Finally, animals undergo an unrewarded PIT test. During this test, it is expected that the Pavlovian reward-predictive stimulus S1 (CS+) will increase responding on the lever associated with the same outcome (A1), while responding during S2 (CS-) should not exceed baseline levels. The inactive lever is expected to be largely ignored.

1.1. Experiment 1: Same general PIT protocol as males

We initially trained a cohort of 15 female Long-Evans rats using the same protocol that reliably produced robust general PIT effects in males (see **Table 1** and **Figure 23.A**). The results of this experiment are presented in **Figure 23.B-F**. Following a single session of habituation to auditory stimuli without reward delivery (data not shown), Pavlovian conditioning progressed smoothly. Throughout training, there was a consistent increase in the number of food port entries during the CS+ compared to the CS-, relative to baseline (**Figure 23.B**; main effect of Session $F_{1,14} = 33.16$, $p < 0.0001$; main effect of CS $F_{1,14} = 76.02$, $p < 0.0001$; Session \times CS interaction $F_{1,14} = 39.24$, $p < 0.0001$; simple effect for CS+ $F_{1,14} = 49.8$, $p < 0.0001$, but not for CS- $F_{1,14} = 2.13$, $p = 0.17$). Instrumental training

also proceeded smoothly, with a progressively higher rate of lever presses on the active lever compared to the inactive lever across training sessions (**Figure 23.C**; main effect of Session $F_{1,14} = 92.6$, $p < 0.0001$; main effect of Lever $F_{1,14} = 214.1$, $p < 0.0001$; Session \times Lever interaction $F_{1,14} = 98.46$, $p < 0.0001$; simple effect for active lever $F_{1,14} = 97.37$, $p < 0.0001$, but not for inactive lever $F_{1,14} = 0.004$, $p = 0.95$). Subsequently, subjects underwent a 30-minute instrumental extinction session to reduce baseline levels of lever pressing (**Figure 23.D**; main effect of Time $F_{1,14} = 111.25$, $p < 0.0001$; main effect of Lever $F_{1,14} = 117.43$, $p < 0.0001$; Time \times Lever interaction $F_{1,14} = 75.43$, $p < 0.0001$; simple effect for active lever $F_{1,14} = 80.55$, $p < 0.0001$, but not for inactive lever despite approaching significance $F_{1,14} = 4.14$, $p = 0.06$). Following this, subjects underwent a single session of Pavlovian extinction, mirroring the habituation session (data not shown).

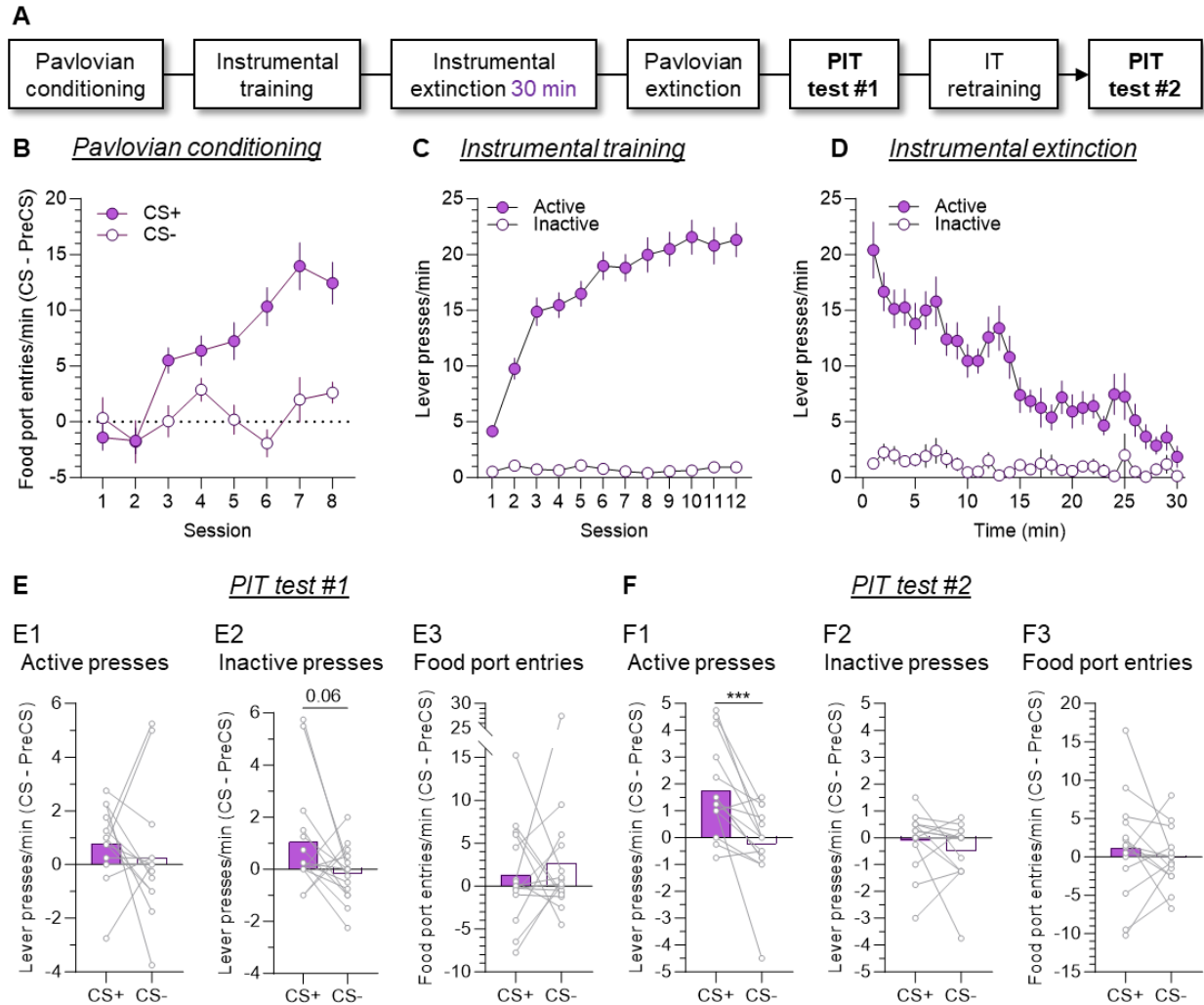


Figure 23. Experiment 1 – General PIT protocol that was used in males did not produce reliable general PIT in females. (A) Experimental overview. **(B)** Rate of food port entries (\pm SEM) during Pavlovian conditioning was obtained by subtracting performance during baseline period from performance during CS presentations (CS – PreCS). Rats discriminated between CS+ (tone or clicker counterbalanced) versus CS- and entered the food port more during CS+. **(C)** Rate of lever pressing (\pm SEM) on the active (purple circles) and inactive lever (white circles) across instrumental conditioning. Rats responded on the active lever to earn the food reward. **(D)** Rate of lever pressing (\pm SEM) during the instrumental extinction session. Rats reduced their performance on the active lever across the 30-minute session. **(E)** Rate of lever pressing (CS-PreCS, i.e. presses during CS presentations minus presses during baseline period) on the active lever during the general PIT test. Rats failed to show a transfer effect on the active lever such that their instrumental responding during CS+ versus CS- did not differ **(E1)** while it approached significance on the inactive lever **(E2)**. Food port entries (CS-PreCS) did not differ between CS+ and CS- during the PIT test **(E3)**. Animals were briefly retrained to lever press and were then subjected to a second PIT test without prior extinction sessions **(F)**. A significant transfer effect was now observed on the active lever **(F1)** but not the

inactive lever (**F2**). Food port entries did not differ between CS+ and CS- (**F3**). $N = 15$ female subjects. $***p < 0.001$.

On the subsequent day, subjects underwent a PIT test, but they did not exhibit a transfer effect on the active lever (**Figure 23.E1**; $F_{1,14} = 0.53$, $p = 0.48$). There was however a trend towards significance on the inactive lever (**Figure 23.E2**; $F_{1,14} = 4.23$, $p = 0.06$). Food port entries did not significantly differ between CS+ and CS- (**Figure 23.E3**; $F_{1,14} = 0.3$, $p = 0.59$).

Due to the absence of a transfer effect, animals were given two instrumental re-training sessions (data not shown) and then we conducted a second PIT test the following day, without prior extinction sessions. This time, rats demonstrated a significant transfer effect on the active lever (**Figure 23.F1**; $F_{1,14} = 19.3$, $p < 0.001$), while the rate of lever pressing on the inactive lever was similar between CS presentations ($F_{1,14} = 1.32$, $p = 0.27$) and to baseline (one sample t-test $p_{CS+} = 0.51$, $p_{CS-} = 0.9$) as shown in **Figure 23.F2**. Food port entries during CS+ versus CS- did not significantly differ (**Figure 23.F3**; $F_{1,14} = 0.56$, $p = 0.47$).

These findings indicate that the protocol designed for male subjects fails to yield a significant general transfer effect in female subjects. This discrepancy could be attributed to a heightened sensitivity to instrumental extinction among female subjects, evidenced by substantial pressing on the inactive lever at test despite its lack of reinforcement. Notably, female subjects exhibited a near-significant transfer effect on the inactive lever. However, after undergoing instrumental retraining sessions followed directly by a PIT test (i.e., without instrumental extinction session), female subjects demonstrated a significant

transfer effect on the lever previously associated with reward, while performance on the inactive lever remained comparable to baseline levels.

1.2. Experiment 2: Reducing the duration of instrumental extinction

In the next experiment, we sought to test the hypothesis that slightly reducing the duration of the instrumental extinction session (from 30 to 20 minutes) would enable us to observe a general transfer effect in female subjects while also maintaining the general PIT effect in males. We thus trained 8 naïve male and female Long-Evans rats as described above (**Figure 24.A**). Pavlovian conditioning progressed smoothly and rats entered the food port more during CS+ than during CS- (**Figure 24.B**; main effect of Session $F_{1,14} = 85.04$, $p < 0.0001$; main effect of CS $F_{1,14} = 120.5$, $p < 0.0001$; Session \times CS interaction $F_{1,14} = 16.85$, $p = 0.001$). There was no main effect of Sex ($F_{1,14} = 0.39$, $p = 0.54$) or interactions between Sex and the other factors (largest $F_{1,14} = 2.73$). Food port entries increased during both CS+ and CS- across sessions (main effect of CS $F_{1,14} = 120.5$, $p < 0.0001$; CS \times session interaction $F_{1,14} = 16.85$, $p = 0.001$) but the effect was greater during CS+ (CS+ $F_{1,14} = 49.5$, $p < 0.0001$; CS- $F_{1,14} = 17.47$, $p = 0.001$). Instrumental training also proceeded smoothly, with a higher rate of lever presses on the active lever compared to the inactive lever across the training sessions (**Figure 24.C**; main effect of Session $F_{1,14} = 98.57$, $p < 0.0001$; main effect of Lever $F_{1,14} = 391.8$, $p < 0.0001$; Session \times Lever interaction $F_{1,14} = 98.69$, $p < 0.0001$). Inspection of the figure suggest that male subjects pressed the active lever at a higher rate across training compared to females. This was confirmed by a significant main effect of sex ($F_{1,14} = 6.38$, $p = 0.024$) and a Sex \times Lever interaction Sex ($F_{1,14} = 5.39$, $p = 0.036$) while other Sex interactions did not reach significance (largest $F_{1,14} = 1.27$). Both males ($F_{1,14} = 43.58$, p

< 0.0001) and females ($F_{1,14} = 64.57$, $p < 0.0001$) increased responding on the active lever, but not on the inactive (largest $F_{1,14} = 3.34$, $p = 0.09$). Rats then underwent a 20-minute instrumental extinction session during which they significantly reduced their level of responding (**Figure 24.D**; main effect of Time $F_{1,14} = 43.57$, $p < 0.0001$; main effect of Lever $F_{1,14} = 103.5$, $p < 0.0001$; Time \times Lever interaction $F_{1,14} = 30.04$, $p < 0.0001$). There was no main effect of Sex ($F_{1,14} = 3.56$, $p = 0.08$), or interactions between sex and any other factor (largest $F_{1,14} = 1.34$). Indeed, both males ($F_{1,14} = 22.17$, $p < 0.0001$) and females ($F_{1,14} = 40.26$, $p < 0.0001$) significantly reduced their responding on the active lever, while responding on the inactive lever remained stable (largest $F_{1,14} = 2.04$) and low. Following this, rats underwent a single session of Pavlovian extinction, mirroring the habituation session (data not shown).

The next day, subjects underwent a PIT test. Unfortunately, as in Experiment 1, female rats did not exhibit a general transfer effect nor did male rats (**Figure 24.E1**). Statistical analysis confirmed that there was no main effect of CS on active lever presses ($F_{1,14} < 0.001$, $p > 0.98$). Active lever presses during test also did not differ between female and male rats as shown by the absence of main effect of sex ($F_{1,14} = 0.071$, $p = 0.79$) and no sex \times CS interaction ($F_{1,14} = 0.002$, $p = 0.97$). Performance on the inactive lever did not differ during CS+ versus CS- presentations (**Figure 24.E2**; $F_{1,14} = 1.36$, $p = 0.26$) or between sexes (no main effect of Sex $F_{1,14} = 0.66$, $p = 0.43$; no CS \times Sex interaction $F_{1,14} = 1.47$, $p = 0.25$). Moreover, performance on the inactive lever was not different from baseline (females: $p_{CS+} = 0.21$, $p_{CS-} = 0.39$; males: $p_{CS+} = 0.82$, $p_{CS-} = 0.65$). Finally, food port entries were similar between CS+ and CS- presentations (**Figure 24.E2**; no main

effect of CS $F_{1,14} = 2.06$, $p = 0.17$), and did not differ between sexes (no main effect of Sex $F_{1,14} = 1.01$, $p = 0.33$; no CS \times Sex interaction $F_{1,14} = 0.64$, $p = 0.44$).

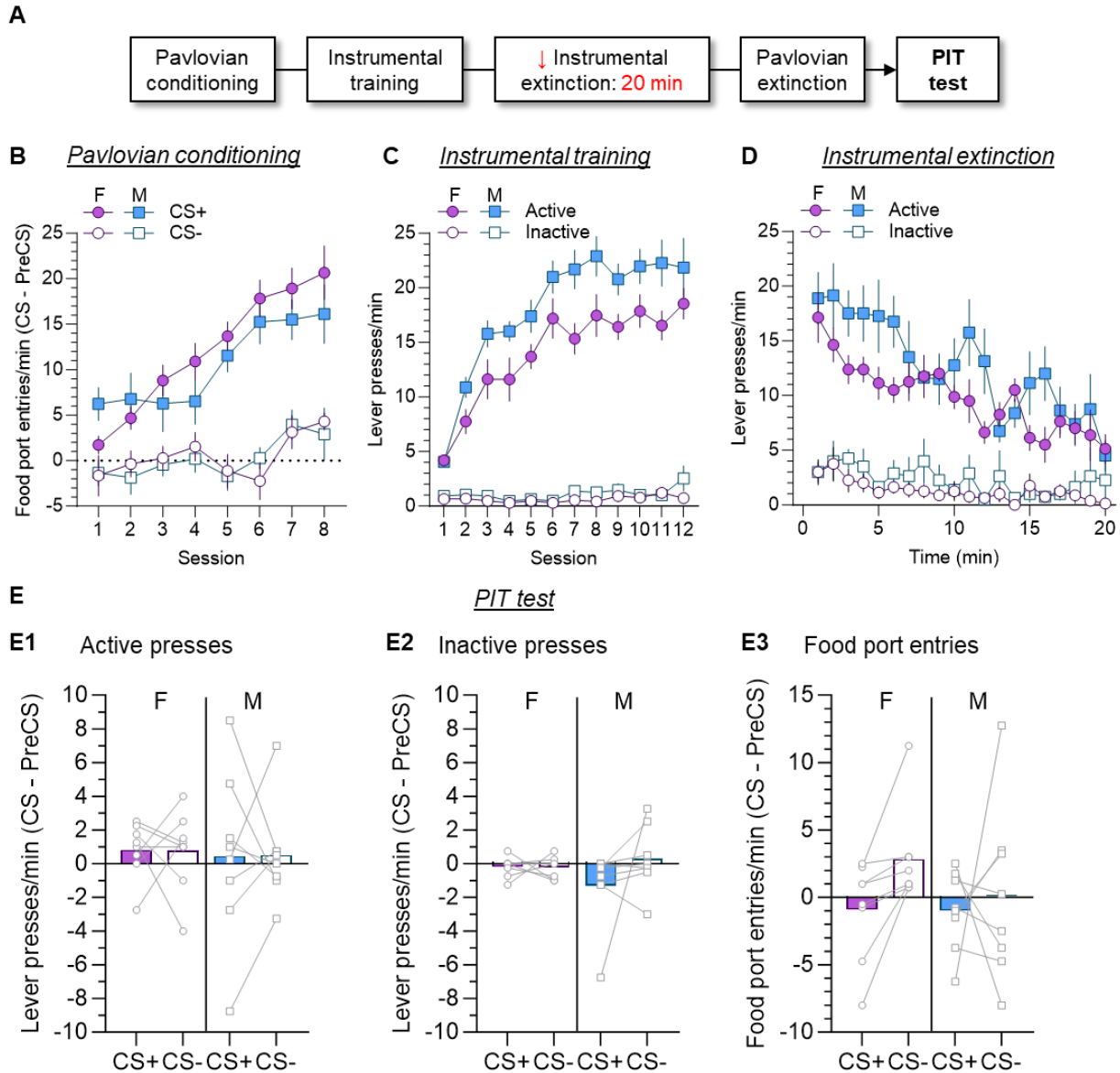


Figure 24. Experiment 2 - Reduced instrumental extinction session. (A) Experimental overview: instrumental extinction reduced from 30 to 20 mins. **(B)** Rate of food port entries (\pm SEM) during Pavlovian conditioning (CS – PreCS). Rats entered the food port more during CS+ than during CS-. **(C)** Rate of lever pressing (\pm SEM) on the active (Females: purple circles, Males: blue squares) and inactive lever (Females: white circles, Males: white squares) across instrumental conditioning. Rats responded more on the active than inactive lever. **(D)** Rate of lever pressing (\pm SEM) during the instrumental extinction session. Rats reduced their performance on the active lever across this 20-minute session. **(E)** Rate of lever pressing (CS-PreCS, i.e. presses during CS minus presses

during baseline) on the active lever during the general PIT test. Both female and male rats failed to show a transfer effect on the active lever such that their instrumental responding during CS+ versus CS- did not differ (**E1**) nor on the inactive lever (**E2**). Food port entries (CS-PreCS) did not differ between CS+ and CS- during the PIT test for either sex (**E3**). *F*: females ($n = 8$); *M*: males ($n = 8$).

Collectively, these findings indicate that reducing the duration of the instrumental extinction session from 30- to 20-minutes was not sufficient to observe a general transfer effect in females. Moreover, it also abolished the transfer in males.

1.3. Experiment 3: Omission of the instrumental extinction session

Finally, we tested if removing the instrumental extinction session would enable us to observe a transfer effect in female subjects. We trained 8 females as described above (**Figure 25.A**) except that the instrumental extinction session was omitted. Across Pavlovian conditioning, rats entered the food port more during the CS+ than during CS- (**Figure 25.B**; main effect of CS $F_{1,7} = 26.88$, $p = 0.0013$) and responding increased across sessions (main effect of Session $F_{1,7} = 18.15$, $p = 0.004$; Session \times CS interaction approached significance $F_{1,7} = 4.6$, $p = 0.06$). Instrumental training also proceeded smoothly, with a progressively higher rate of lever presses on the active lever compared to the inactive lever across training sessions (**Figure 25.C**; main effect of Session $F_{1,7} = 38$, $p < 0.0001$; main effect of Lever $F_{1,7} = 378.9$, $p < 0.0001$; Session \times Lever interaction $F_{1,7} = 55.09$, $p < 0.0001$). Notably, performance increased on both active ($F_{1,7} = 47.16$, $p < 0.0001$) and inactive levers ($F_{1,7} = 8.54$, $p = 0.022$). Rats then underwent a single session of Pavlovian extinction, mirroring the habituation session (data not shown).

The next day, rats underwent a PIT test and, while it appears that instrumental responding was greater during CS+ than during CS-, unfortunately this did not reach statistical significance (**Figure 25.D1**; $F_{1,7} = 4.36$, $p = 0.075$). Performance on the inactive

lever did not differ during CS+ and CS- presentations (**Figure 25.D2**; $F_{1,7} = 0.34$, $p = 0.58$) or between CS presentation and baseline ($p_{CS+} = 0.076$, $p_{CS-} = 0.06$). Food port entries did not significantly differ between CS+ and CS- (**Figure 25.D3**; $F_{1,7} = 0.01$, $p = 0.92$).

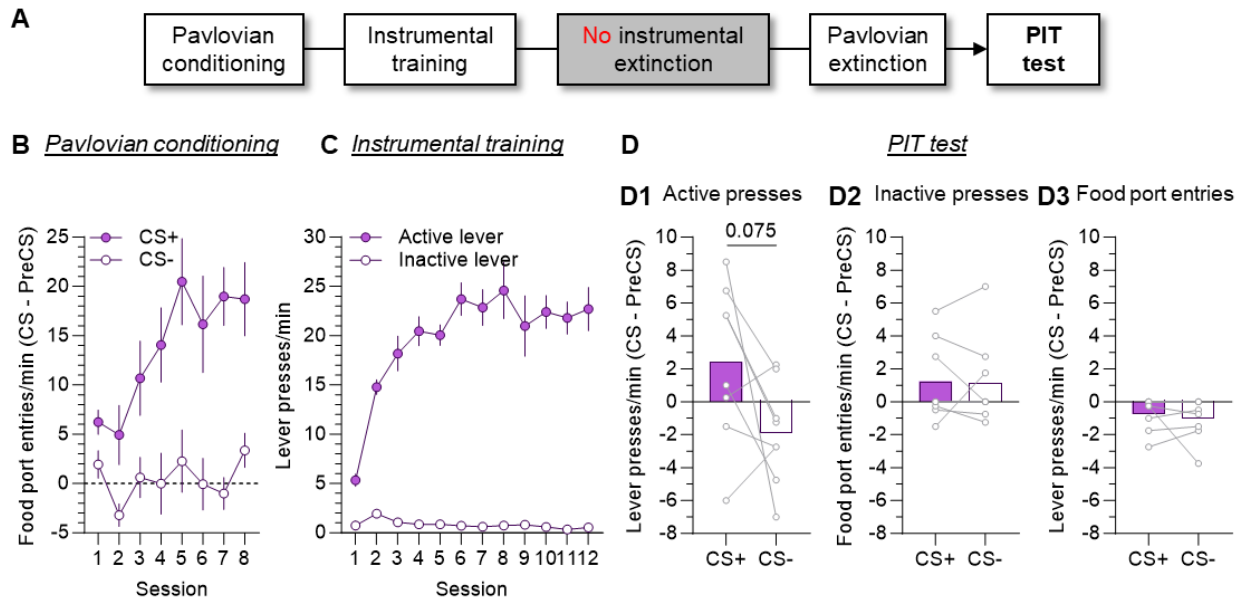


Figure 25. Experiment 3 - Omission of the instrumental extinction session. (A) Experimental overview: no instrumental extinction session. **(B)** Rate of food port entries (\pm SEM) during Pavlovian conditioning was obtained by subtracting performance during baseline period from performance during CS presentations (CS – PreCS). Rats entered the food port more during CS+ than during CS-. **(C)** Rate of lever pressing (\pm SEM) on the active (purple circles) and inactive lever (white circles) across instrumental conditioning. Rats learned to respond on the active lever to earn the food reward. **(D)** Rate of lever pressing (CS-PreCS, i.e. presses during CS presentations minus presses during the preceding baseline period) on the active lever during the general PIT test. Unfortunately, the general transfer effect on the active lever was not statistically significant **(D1)**. Inactive lever presses were similar between stimulus presentations and to baseline. **(D2)**. Food port entries (CS-PreCS) did not differ between CS+ and CS- during the PIT test **(D3)**.

In summary, while the initial protocol used in males did not produce a clear transfer effect in females, the modified protocol without the instrumental extinction session (Experiment 3) approached statistical significance. Given the high variability observed between subjects, it is likely that increasing the sample size could reveal a significant transfer effect in females. To date, there is no evidence to suggest that females are more or less susceptible to instrumental extinction than males, nor are there documented sex differences in PIT expression. For instance, [Wyvell and Berridge \(2000\)](#) conducted general PIT experiments using only female subjects and demonstrated robust PIT effects. Their study, which used Sprague Dawley rats, employed slightly different conditioning parameters: the variable interval (VI) reinforcement schedule during instrumental training was extended to 45 seconds, and the CS duration was reduced to 30 seconds, compared to our protocol's 30 seconds and 2 minutes, respectively. Although they included an instrumental extinction session before the PIT test, they did not specify its duration, and there was no mention of a Pavlovian extinction session. More recently, [Halbout and colleagues \(2019\)](#) conducted general PIT experiments with both sexes. Their Pavlovian conditioning protocol differed from ours in that it involved exposure only to a CS+, with one or two additional separate sessions with a CS- for habituation. Their instrumental training also differed, with the VI increasing to 60 seconds by the end of training. Following a 30-minute instrumental extinction session and a Pavlovian reminder session (CS+ only), the PIT test was conducted on the following day. This protocol yielded robust PIT effects, although these effects were not analyzed by sex, and the authors did not report any sex differences.

In conclusion, the protocol that effectively demonstrated a general transfer effect in male subjects did not yield the same results in females. However, when the instrumental extinction session was omitted, the transfer effect in females approached statistical significance, suggesting potential sex differences in response to the protocol. Although few studies in the literature have used females for general PIT, those that did reported robust transfer effects, indicating that, with the right approach, such effects can be observed in female subjects. Additionally, different outcomes may be achieved by using alternative conditioning parameters, as variations in these factors have shown to influence PIT in previous studies (see **Chapter I**).

2. SPECIFIC PAVLOVIAN-TO-INSTRUMENTAL TRANSFER IN FEMALE SUBJECTS

Concurrently with our efforts to establish a general PIT protocol suitable for female rats and to incorporate them into our research, we also assessed the applicability of our specific PIT protocol (**Figure 26.A**), originally developed for male subjects, to female rats.

In our specific PIT protocol, rats first undergo Pavlovian conditioning in which they learn that two distinct auditory stimuli (S1 and S2) signal the delivery of two different rewards (O1 and O2). Then, during instrumental training, rats learn to perform two distinct actions (A1 and A2) to obtain the same outcomes (O1 and O2) that were associated with the auditory stimuli. Similar to the general PIT protocol, animals then undergo a single instrumental and Pavlovian extinction session, followed by an unrewarded test on the subsequent day.

As for the male rats, females were first given a single session of habituation to the auditory stimuli without reward delivery (data not shown) before undergoing Pavlovian conditioning. Across Pavlovian conditioning, rats increased their number of food port entries during the CSs relative to baseline (**Figure 26.B**; main effect of Session $F_{1,15} = 49.76$, $p < 0.0001$). Instrumental training also proceeded smoothly, with a progressively higher rate of lever pressing observed across training sessions (**Figure 26.C**; main effect of Session $F_{1,15} = 471$, $p < 0.0001$). Subsequently, rats underwent a 30-minute instrumental extinction session to reduce baseline levels of lever pressing rate (**Figure 26.D**; main effect of Time $F_{1,15} = 126.23$, $p < 0.0001$). Following this, rats were given a single session of Pavlovian extinction (data not shown).

On the subsequent day, subjects underwent a specific PIT test. Unfortunately, we did not observe a specific PIT effect. Indeed, during the CS presentations, there was no difference in responding on the action associated with the same outcome versus the action associated with the different outcome (**Figure 26.E1**; $F_{1,15} = 0.74$, $p = 0.4$). Food port entries were significantly higher during CS presentations compared to the baseline period (**Figure 26.E2**; $p = 0.018$).

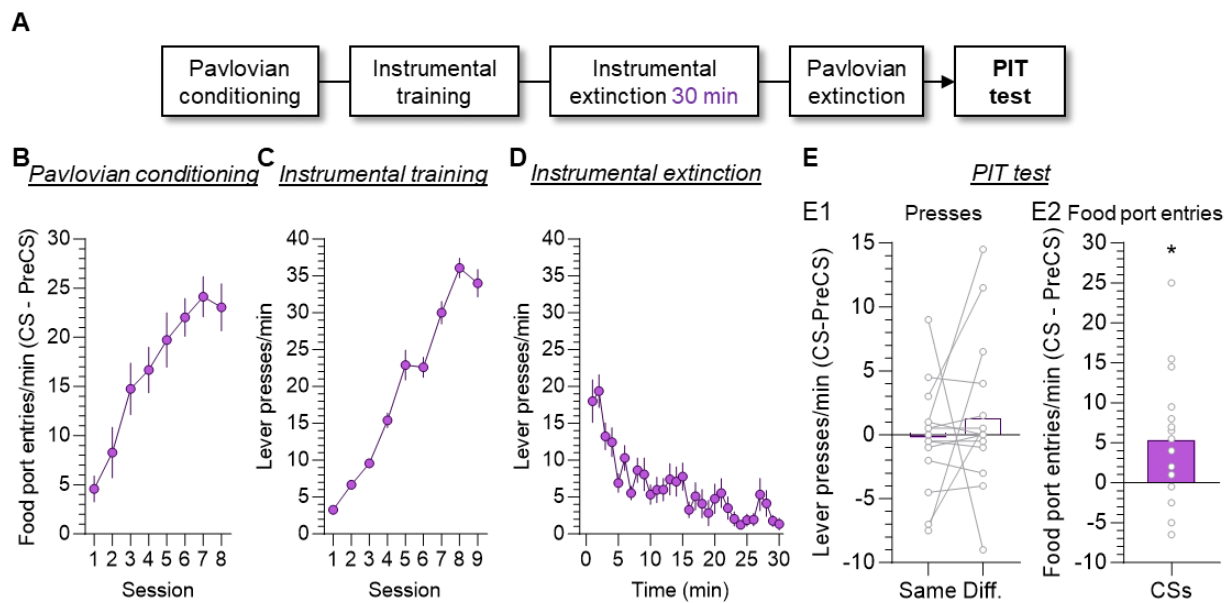


Figure 26. Experiment 4 - Specific PIT protocol that was used in males did not produce reliable general PIT in females. (A) Experimental overview. **(B)** Across Pavlovian conditioning, rats learned that the CSs (average of tone and clicker) predicted food outcomes (grain and sugar pellets, counterbalanced). Data are presented as average food port entries per minute (\pm SEM), which was calculated as performance during the CS presentation minus performance during the preceding 2-minute baseline period (CS – PreCS). **(C)** Instrumental conditioning was successful with rats increasing their lever press performance (right and left levers averaged) across sessions. Data are presented as average lever presses per minute \pm SEM. The first three sessions were under a Fixed-Ratio 1 schedule, followed by three sessions of Random-Ratio (RR) 5 schedule, followed by three sessions of RR10. **(D)** Rats were then subjected to an instrumental extinction session for 30 minutes during which performing A_1 and A_2 were not rewarded (average lever presses per minute \pm SEM). **(E)** Results of the specific PIT test. **(E1)** Subjects failed to show a transfer effect, with a similar pressing rate during both the Same and Different (Diff.) periods relative to baseline (CS – baseline). **(E2)** Average rate of food port entries relative to baseline (\pm SEM) was significantly higher than zero, indicating responding levels above baseline. $N = 16$; $*p < 0.05$.

These findings suggest that the specific PIT protocol optimized for male subjects fails to produce a significant transfer effect in female subjects. Again, this discrepancy may be attributable to an increased susceptibility to instrumental extinction in females, consistent with observations from earlier general PIT experiments. Further research is necessary to identify the optimal parameters for eliciting a specific PIT transfer effect in female subjects within our experimental framework. Among the specific PIT studies reviewed in this thesis, a few have exclusively used female subjects (Corbit et al., 2001; Ostlund & Balleine, 2007, 2008), while others have included both males and females (L. A. Bradfield et al., 2018; Leung et al., 2024; Park et al., 2024; Sias et al., 2024), consistently demonstrating robust PIT effects.

In the study by Corbit and colleagues (2001), Long-Evans female rats underwent instrumental training sessions, where the random ratio (RR) schedule progressively increased to RR20 (reward after 20 responses in average), compared to RR10 in our protocol. This was followed by eight Pavlovian conditioning sessions similar to ours. By the end of instrumental training, subjects in Corbit's study were pressing at an average rate of 25 to 30 presses per minute, while our female rats pressed around 35 times per minute, despite using a lower RR schedule. Unlike our approach, which includes a single PIT test with both levers available simultaneously, Corbit et al. conducted two PIT tests, each with only one lever available, without any prior extinction session. In the studies conducted Ostlund and Balleine (2007, 2008), female Long-Evans rats underwent Pavlovian conditioning using a protocol similar to ours. However, their instrumental training differed by increasing the reinforcement schedule to RR20, as in Corbit et al. (2001). Despite the higher reinforcement ratio, Ostlund and Balleine reported that female

rats pressed at an average rate of approximately 22 presses per minute during the RR20 block (three sessions), whereas our female rats responded at a higher rate of 33.37 ± 1.31 presses per minute during the final block of three days of instrumental training (RR10). Additionally, after training, Ostlund and Balleine subjected the animals to a satiety-induced outcome devaluation task (two 5-minute extinction sessions) before conducting a PIT test 48 hours later, allowing any temporary effects of satiety to dissipate. Although they did not include extinction sessions specifically aimed at reducing baseline responses prior to PIT testing, it is possible that the devaluation extinction test contributed to lowering baseline instrumental responding.

In more recent studies involving both male and female Long-Evans rats, [Bradfield, Hart and Balleine \(2018\)](#) also observed robust PIT effects. Although they employed the same instrumental training protocol as ours, it resulted in a lower response rate, with rats pressing around 20 times per minute, compared to over 30 presses per minute in our female subjects by the end of training. The Pavlovian conditioning protocol used in their study was similar to ours. Following the completion of training phases, the animals were subjected to a PIT test without any preceding extinction session. Similarly, [Park and colleagues \(2024\)](#), as well as [Leung and colleagues \(2024\)](#), employed protocols comparable to ours, except that they did not include any extinction session before the PIT test. In contrast, [Sias and colleagues \(2024\)](#) employed different training protocols. They subjected both male and female Long-Evans rats to 12 Pavlovian conditioning sessions (compared to 8 in our protocol) and 11 days of instrumental training, during which the reinforcement schedule was progressively increased to RR20 (versus RR10 in our protocol). These conditions resulted in a lever pressing rate of approximately 25 presses

per minute. Before the PIT test, the rats underwent a 30-minute instrumental extinction session, with the PIT test conducted the following day.

Different protocols have been employed across laboratories, consistently revealing robust specific PIT effects in female subjects. However, none of the studies that included both male and female rats provided sex-specific statistical analyses ([L. A. Bradfield et al., 2018](#); [Leung et al., 2024](#); [Park et al., 2024](#); [Sias et al., 2024](#)). Interestingly, our female rats exhibited often higher pressing rates than those documented in the literature, even though we employed lower reinforcement schedules. Additionally, instrumental extinction sessions are not consistently used across studies, and there is no mention of Pavlovian extinction sessions. Importantly, Pavlovian S-O associations demonstrate a notable resistance to treatment efforts aimed at undermining them as they appear immune to the effects of a simple extinction treatment ([Delamater, 1996](#); [Park et al., 2024](#)). Therefore, we do not believe that omitting the Pavlovian extinction session in our protocol would facilitate the observation of a transfer effect, as this treatment is designed to reduce Pavlovian conditioned responses in favor of instrumental responses. As observed in our general PIT experiments with females, omitting the instrumental extinction sessions may be sufficient to produce robust PIT effects within our laboratory setup.

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