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The establishment of invariable behaviour of rats in novel one-trial trace association task (OTTAT)

Ustanovení neměnného chování potkanů v nové úloze asociativního učení na jeden pokus (OTTAT)

Diploma thesis

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PROHLÁŠENÍ

Prohlašuji, že jsem závěrečnou práci vypracovala samostatně na základě konzultací s vedoucím práce a konzultantem, a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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ABSTRACT

Animal episodic-like memory tasks represent important component of episodic memory research. However, currently available episodic-like memory tasks are not based on episodic-like memory or encompass important caveats. In our laboratory, we recently devised a novel one-trial trace association task (OTTAT) to examine one-time associations of temporally discontinuous stimuli. This thesis deals with the improvement of OTTAT protocol by rat strain and compartment divider ('doors') selection which optimally promote the establishment of invariable behaviour of rats in OTTAT. Moreover, the accuracy of one-trial associations is also assessed by determining specificity of "rapid escape" response to conditioned stimulus of given sound characteristics. In Experiment 1, rats (Sprague-Dawley (SD), n = 36; Wistar (WI), n = 17; Long-Evans (LE), n = 8) were habituated 15 min daily for 3 days with standard doors (9 x 11 cm opening) to modified light and dark apparatus. The number of transfers between compartments and values of time spent in dark compartment obtained from 3rd habituation session were evaluated as indicators of invariable behaviour of rats. We found WI rats spend significantly more time in dark compartment than LE (p = 0.002) and SD rats (p = 0.001) and have significantly fewer transfers than LE rats (p = 0.001). In experiment 2, rats (WI, n = 34) were habituated 15 min daily for 3 days with doors with wide opening (3 x 40 cm). The number of transfers between compartments and values of time spent in dark compartment obtained from 3rd habituation session were evaluated as indicators of invariable behaviour of rats. We found WI rats habituated with wide doors spend significantly more time in dark compartment (p = 0.003) and have fewer transfers than WI rats habituated with standard doors. In Experiment 3 WI rats (n = 16) were subjected to standard OTTAT protocol. During testing session, half of the rats received shock-paired sound (2.4 kHz), the other half received novel sound (5 kHz) and numbers of rats with "rapid escape" response were obtained. There was no difference in frequency of rats with "rapid escape" response following 5 kHz and 2.4 kHz sound during testing session (42.9 % in both cases). We conclude Wistar rats habituated with wide doors display the most invariable behaviour and thus are the optimal combination for OTTAT. Additionally, the results suggest rats are not able to distinguish between novel and shock-paired sounds differing by 2.6 kHz during memory recollection in OTTAT.

KEY WORDS: episodic-memory, one-trial learning, associative learning, rat, hippocampus

ABSTRAKT

Animální modely paměti epizodického typu představují důležitou složku výzkumu epizodické paměti. Současně dostupné úlohy testující paměť epizodického typu však zcela nereflektují epizodickou paměť nebo vykazují důležité metodologické nedostatky. V naší laboratoři jsme nedávno vytvořili novou úlohu asociativního učení na jeden pokus (OTTAT), která testuje asociaci časově oddělených stimulů prezentovaných pouze jednou. Tato práce se zabývá zdokonalením protokolu OTTAT prostřednictvím výběru kmene potkanů a charakteristik děliče přihrádek aparátu ("dvířka"), které vedou k optimálnímu ustanovení neměnného chování potkanů v OTTAT. Dále byla testována přesnost asociací vytvořených při OTTAT určením specifity reakce "rychlý únik" vůči podmíněnému podnětu dané zvukové charakteristiky. V experimentu 1 byli potkani (Sprague-Dawley (SD), n = 36; Wistar (WI), n = 17; Long-Evans (LE), n = 8) denně 15 minut po dobu 3 dnů habituováni modifikovanému aparátu se světlou a tmavou přihrádkou (L/D) a se standardními dvířky (otvor 9 x 11 cm). Počty přechodů mezi přihrádkami a časy strávené ve tmavé přihrádce získané ze 3. habituačního sezení byly vyhodnoceny jakožto indikátory neměnného chování potkanů. Zjistili jsme, že potkani WI strávili signifikantně více času ve tmavé přihrádce než potkani LE (p = 0.002) a SD (p = 0.001) a přecházeli mezi přihrádkami signifikantně méně než potkani LE (p = 0.001). V experimentu 2 byli potkani (WI, n = 34) denně 15 minut po dobu 3 dnů habituováni modifikovanému L/D aparátu se širokými dvířky (otvor 3 x 40 cm). Počty přechodů mezi přihrádkami a časy strávené ve tmavé přihrádce získané ze 3. habituačního sezení byly vyhodnoceny jakožto indikátory neměnného chování potkanů. Zjistili jsme, že potkani WI habituovaní se širokými dvířky tráví signifikantně více času ve tmavé přihrádce (p = 0.003) a méně přechází mezi přihrádkami než potkani WI habituovaní se standardními dvířky. V experimentu 3 podstoupili potkani (n = 16) OTTAT dle standardního protokolu. Během testovacího dne byl polovině potkanů přehrán nový zvuk (5 kHz) a druhé polovině potkanů byl přehrán zvuk dříve spárovaný s elektrickým šokem (2,4 kHz) a následně byly zjištěny počty potkanů s reakcí "rychlý únik" na dané zvukové stimuly. Nezjistili jsme rozdíl ve frekvencích reakcí "rychlý únik" po přehrání zvuku o frekvenci 5 kHz a 2,4 kHz (42,9 % v obou případech). Na základě našich výsledků docházíme k závěru, že potkani WI habituovaní s širokými dvířky vykazují optimálně neměnné chování a jsou tak nejvhodnější kombinací pro OTTAT. Z našich výsledků dále usuzujeme, že při vybavování paměťové stopy v OTTAT nejsou potkani schopni rozlišit mezi novým zvukovým stimulem a zvukovým stimulem dříve spárovaným s elektrickým šokem, liší-li se frekvence zvukových stimulů o 2,6 kHz.

KLÍČOVÁ SLOVA: epizodická paměť, učení na jeden pokus, asociativní učení, potkan, hipokampus

LIST OF ABREVIATIONS

5-HT	5-hydroxytryptamine
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CA	*Cornu Ammonis*
CFC	conditioned fear conditioning
CS	conditioned stimulus
CTA	conditioned taste aversion
DAID	"Do as I do"
DC	delay conditioning
DEC	delay eyeblink conditioning
DFC	delay fear conditioning
DG	dentate gyrus
DMS	delayed matching-to-sample
DR	dopamine receptor
DNMS	delayed non-matching-to-sample
HPA	hypothalamic–pituitary–adrenal
L/D	light and dark
LE	Long-Evans
Mdn	median
MTL	medial temporal lobe
mPFC	medial prefrontal cortex
NMDA	N-methyl-D-aspartate
NOR	novel object recognition
OLL	odour-location learning
OTPM	one-trial place memory
OTTAT	one-trial trace association task
SD	Sprague-Dawley
SEM	standard error of the mean
TC	trace conditioning
TEC	trace eyeblink conditioning

TFC	trace fear conditioning
US	unconditioned stimulus
WI	Wistar
WIN	Wistar rats habituated with normal doors
WIW	Wistar rats habituated with wide doors

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1 INTRODUCTION

Learning and memory are crucial abilities for both human and animals. Both permit adaptive behaviour and promote a survival of both individuals and species. Episodic memory represents memory system of the utmost importance for one's everyday life. Episodic memory allows encoding and retrieval of personal experiences - episodes. A decrease in episodic memory functioning is associated with lower quality of life (Shin et al., 2005) and with a considerable financial burden to the health systems through the world (Lopez, 2011). Currently, thorough understanding of episodic memory formation and circuitry is lacking. Since episodic memory is of the declarative nature, i.e. it is possible to verbally 'declare' if the memory is present, it is most advantageous to test episodic memory on human subjects. Unfortunately, options of episodic memory research conducted in human patients are limited.

The well-established and defined animal models of episodic-like memory are potentially fruitful in episodic memory research as they allow use of more precisely targeted manipulations and of techniques not deployable in human subjects. Although it is conundrum to assess a presence of declarative memory in a-verbal animals, several episodic-like memory tasks are currently in place. Each of these episodic-like memory tasks present themselves with unique advantages, but, importantly, they also encompass considerable drawbacks. Importantly, these drawbacks limit an ability of these tests to model episodic-like memory in animals.

This diploma thesis deals with the optimisation of a novel episodic-like memory task for rats recently developed in our laboratory. One-trial trace association task (OTTAT) is a feasible and rapidly established task to study one-trial associations of temporally discontinuous events. To our knowledge, such task was not yet employed anywhere as indicated by published literature. Since reproducibility of many behavioural tests is problematic, detailed understanding of the novel OTTAT test was needed. With a goal to produce the most run-to-run stable OTTAT protocol, we explored behavioural differences between three common rat strains (Wistar, Long-Evans, Sprague-Dawley) and behavioural differences following custom modification of behavioural apparatus. Based on our observations, we selected measures that promote the invariable behaviour during OTTAT task. Additionally, to test the accuracy of trace associations formed during pairing session of OTTAT, we examined the effect of sound cue of different frequency on induction of conditioned reaction during OTTAT.

2 EPISODIC MEMORY

2.1 General overview

Episodic memory is a form of long-term declarative memory defined as ability to recall and mentally reexperience specific episodes from one's personal past. Memory of an episode includes information specific to the time and the place of memory acquisition (Tulving, 2002; Tulving, 1985). For example, the ability to describe details of past events such as one's wedding day or recent office meeting relies on intact episodic memory function. Importantly, episodic memory is often composed of many items which do not necessary co-occur in time. The process of formation of associations between temporally discontinuous events is called *temporal binding* (Nombre & Coull, 2010; Sellami et al., 2017). In fact, association of temporally discontinuous events has been for a long time considered a hallmark feature of episodic memory (Tulving, 1983, 2002).

Episodic memory is contrasted with semantic memory, another type of declarative memory, which includes memory for generic, context-free knowledge, such as facts, skills or concepts (Hudson et al., 2011) (see Figure 1 for schematic division of memory). It is assumed episodic and semantic memory represent distinct neurobiological systems which operate independently and in parallel both during encoding and information retrieval (Atkinson & Juola, 1974; Dobbins et al., 1998; Tulving, 1985). The notion of distinction between episodic and semantic memory is supported by neuropsychological investigations of amnestic patients who show profound impairments in episodic memory but at the same time their semantic memory is largely spared (Manns et al., 2003). Additionally, patients with semantic dementia show large deficits in semantic memory (verbal identification of stimuli), while their episodic memory is mostly unaffected (Chan et al., 2001).

The capacity to form both episodic and declarative memories is measured by a level of successful retrieval. According to 'dual-process' memory model, retrieval from memory can be carried by either recollection-based (episodic retrieval) or familiarity-based (semantic retrieval) processing (Wixted & Mickes, 2010). Recollection signal involves the recollection of an event-specific details (*remembering* the event), while the familiarity signal elicits an acontextual feeling the event has been already experienced (the general feeling of *knowing*) (Tulving, 1985; Yonelinas, 2002).

In episodic memory tests, it is important to distinguish between these quantitatively distinct kinds of mnemonic processing as both familiarity and recollection can support performance in memory tasks (e.g. object recognition) (Eichenbaum et al., 2010; Fortin et al., 2004). Since recollection-based retrieval is associated with episodic memory, it is important to exclude a possibility that during episodic-like memory tests recall relies on familiarity-based retrieval.

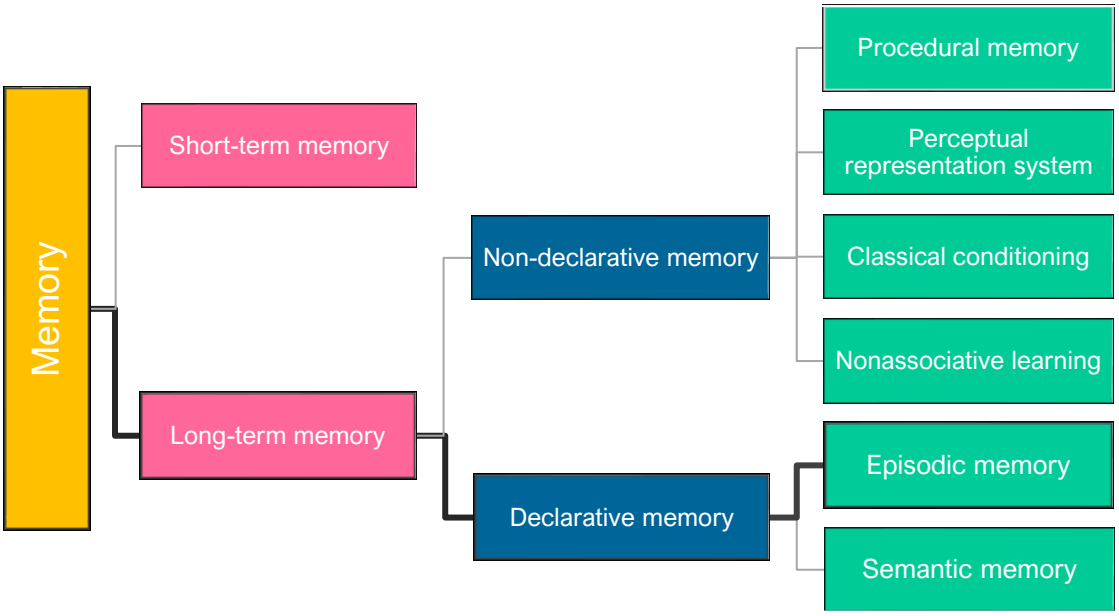


Figure 1 | **The Division of memory**. Episodic memory (thick black branch) is a subdivision of long-term declarative (explicit) memory and contains memories of specific personal experiences from a particular time and space.

Familiarity and recollection differ in several important aspects. First, familiarity was shown to be faster than recollection (Hintzman et al., 1998). Next, familiarity and recollection are presumably mediated by different neural circuits (Aggleton & Brown, 1999). This notion is supported by findings of distinct neurobiological correlates exhibited by recollection and familiarity during electrophysiological testing (Curran, 2000), magnetic resonance imaging (Rugg & Vilberg, 2013) and by findings that some brain injuries severely disrupt recollections, while familiarity is only mildly disrupted (Duzel et al., 2002). Additionally, the ability of recollection develops in human later in age than familiarity (Koenig et al., 2015). Moreover, the recollection- and familiarity-based decisions reflect a memory strength; recollection-based decisions were shown to be based on strong memories, while familiarity-based decisions on weak memories (Wiesmann, 2008).

Number of neuropsychiatric and neurodegenerative disorders are associated specifically with a decline in episodic memory functioning. For example, disruptions to the episodic memory system are among the earliest signs and symptoms of Alzheimer's disease. A selective impairment in episodic memory is observed up to 3 years prior diagnosis of Alzheimer's disease (Bäckman et al., 2001). Moreover, the deficits in episodic memory are observable in patients with hippocampal sclerosis (Dickson et al., 1994), Huntington's disease (Montoya et al., 2006), variety of amnesia-associated focal lesions (Lim & Alexander, 2009; Vargha-Khadem et al., 1997), epilepsy (Kapur, 1993) or transient global amnesia (Hodges & Warlow, 1990). Aging is generally related to deterioration of episodic memory, with a notable decline in temporal binding (Disterhoft & Oh, 2007; Sellami et al., 2017). Contrarily, the temporal binding window of audio-visual information might be expanded in patients with mild cognitive impairment, resulting in abnormal audio-visual experiences (Chan et al., 2015). Moreover, patients with schizophrenia show deficit in binding contextual cues together to form a coherent representation of an event in memory ("hyper-associations") (Waters et al., 2004). All of these clinical conditions have in common impairment of hippocampal function (Dickson et al., 1994; Heckers et al., 1998; Sloviter, 1987; Spargo et al., 1993; Sze et al., 1997; West, 1993).

Most of our current knowledge about episodic memory circuitry emerged from human case-studies and, more recently, from application of neuroimaging techniques in humans. Currently, there is a general consensus that regions of the medial temporal lobe (hippocampal formation, perirhinal and parahippocampal cortices) are the primary anatomic substrates of episodic memory (Kramer et al., 2007; Nyberg et al., 1996). Additionally, the performance in episodic memory tasks is supported by prefrontal (Shallice et al., 1994), medial and lateral-parietal (Cabeza et al., 2008; Rugg & King, 2018), posterior-temporal (Wiggs et al., 1999) and medial-frontal cortical regions (Kramer et al., 2007). Despite consistent implication of these regions, studies of pattern of activity during encoding-related activity (Pihlajamäki et al., 2003; Zeineh et al., 2003) or activity of specific subregions during recollection and familiarity (Eichenbaum et al., 2007; Squire et al., 2007) produced conflicting results. Unearthing circuits and molecular mechanisms of formation of episodic memories using animal models might possibly allow better insight in this phenomenon.

2.2 Do animals possess episodic memory?

Our ability to research episodic memory using animal models was for a long time limited by a notion that animals do not possess episodic memory (Tulving, 1972, 2002; Tulving & Markowitsch, 1998). According to Tulving (2002), concepts unique to human such as language, sense of self, autonoetic consciousness, subjective sense of time and mental time travel are all crucial to episodic memory. While it is clear these elements cannot be assessed in nonhuman animals, some argue their absence does not necessarily invalidate the concept of episodic memory in animals (see Morris, 2001). Famously, Clayton and Dickinson (1998) first shown that animals are able to form episodic-like memory. They have shown that birds are able to recall where certain type of prey was cached and estimated current availability of the prey according to the time elapsed since caching (Figure 2). Even though Clayton's and Dickinson's results convincingly suggest that episodic memory may exist in nonhuman animals, several authors criticized the validity of this task. Specifically, some argued the food caching task relies on naturally occurring behaviour of birds mediated by genetically fixed learning mechanisms (Dere et al., 2006). Others argued that birds form some kind of internal map of cache locations with estimated time of prey edibility (Jeffery & O'Keefe, 1998) or that birds simply learn the rule of the edibility/inedibility of certain food types later in time as food-caching generally requires extensive training (Easton & Eacott, 2008).

Figure 2 | A western scrub jay caching way worms in a compartment of sand-filled ice-cube tray. Reprinted from *Can animals recall the past and plan for the future?* (Clayton et al., 2003).

In follow-up to Clayton and Dickinson's experiments, the presence of episodic-like memory was many times demonstrated in other species of animals, such as dogs, primates and rodents (Babb & Crystal, 2006; Fugazza et al., 2016; Martin-Ordas et al., 2010).

To circumvent absence of verbal declaration of episodic-like memories in animals, many scientists proposed sets of behavioural criteria to assess episodic memory in animals. Some of these criteria focus on the content (i.e. what happened, where did it take place and when did it occur, shortened to *what-when-where*), other on integrity (i.e. representation of an event must be integrated) or on the flexibility (i.e. the information must be flexibly deployed in other situations) of the formed memory trace (Clayton et al., 2003; Dere et al., 2006; Morris, 2001). However, despite the proposed behavioural criteria, the direct comparison to human episodic memory is still a subject of the debate (Babb & Crystal, 2006; Raby et al., 2007; Suddendorf et al., 2009). Due to ongoing controversy and possible confusion, this type of memory displayed by animals is commonly referred to as *episodic-like* memory or *what-where-when* memory. Up to this day, plethora of episodic-like animal paradigms exist, but most of them involve important caveats (for more details see Chapter 4). The novel task developed in our laboratory, which is a subject of this work, addresses several of these important shortcomings.

To summarize, the concept of episodic memory is an important subject with biomedical, social and behavioural implications. Especially, the relevance of episodic memory to aging and number of neuropsychiatric and neurodegenerative diseases calls for well-established and relevant animal models that could be used in future research, e.g. for testing of possible therapeutical interventions. It is no coincidence that hippocampus, one of the brain regions first affected in neurodegenerative diseases, was consistently implied in episodic memory.

3 THE HIPPOCAMPUS

The hippocampus is a region within limbic system of mammals and birds, located bilaterally underneath the cerebral cortex. The gross anatomy of hippocampal formation and hippocampal connectivity will be discussed in this chapter.

3.1 Gross anatomy of hippocampal formation

The hippocampus is one of the several brain regions that together form hippocampal formation. The hippocampal formation is a prominent C-shaped structure bulging in the floor of the temporal horn of the lateral ventricle (Schultz & Engelhardt, 2014). According to literature, hippocampal formation consists of hippocampus proper, the dentate gyrus (DG), subicular complex (subiculum, presubiculum and parasubiculum) and entorhinal cortex (Andersen et al., 2007).

In some ways, such as neuronal organization or presence of pyramidal neurons, the hippocampal formation resembles cortical areas. However, thanks to its three-dimensional organization of intrinsic associational connections and the largely unidirectional passage of information through hippocampal circuits, the neuroanatomy of hippocampal is unique. It is noteworthy that anatomical organization of hippocampal formation is conserved across mammalian species (Andersen et al., 2007; Clark & Squire, 2013). The comparison of human and rat hippocampal formation is displayed in Figure 3.

When viewed in coronal section, hippocampal archicortex consists of three *Cornu Ammonis* (CA) areas named based on the nomenclature of Lorente de Nó: CA1, CA2 and CA3 (Lorente De Nó, 1934). The CA1 is located next to subiculum, CA3 lies next to DG, while CA2 is located in between zones CA1 and CA3. The hippocampal CA1, CA2 and CA3 fields and DG are illustrated in Figure 3.

The hippocampal archicortex comprises of three distinct layers. The molecular layer, the deepest of the three layers, comprises of apical dendritic trees of pyramidal cells and axon terminals of granule cells and merges with the molecular layer of the DG and neocortex. The pyramidal layer, located superficially, is the most prominent part of hippocampal archicortex and consists of small and large pyramidal cells which form Schaffer collaterals to reach molecular layer and form synaptic connections with other pyramidal cells. Superficially to the pyramidal cell layer lays the polymorphic layer.

This layer structurally resembles the deepest neocortical layer and consists of interneurons, pyramidal cell dendritic trees and axon collateral branches (Figure 4).

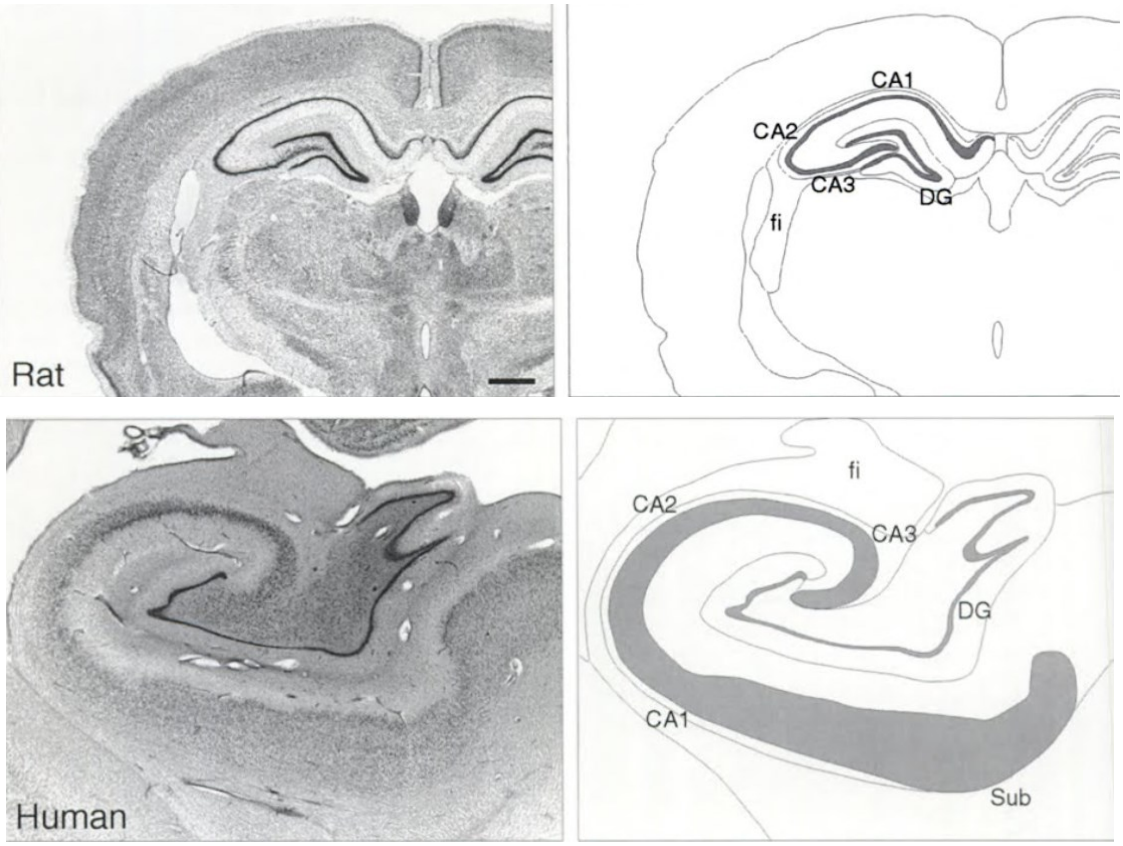


Figure 3 | **The hippocampal formation of human and rat**. The comparison of Nissl-stained sections and line-drawings illustrating the general organization and the anatomical similarities of rat and human hippocampal formation. Of note is particularly similarity in shape and structure of CA regions (CA1, CA2, CA3), DG and the subiculum. Adapted from *The Hippocampus Book* (Andersen et al., 2007).

The structure of the subiculum is nearly analogous to the structure of hippocampus proper while the structure of the entorhinal cortex differs from that of hippocampus proper. Similarly as hippocampus proper, the subiculum consists of three layers: the molecular layer and internal and external pyramidal sublayer (Braak, 1972). On the contrary, six laminar layers (Layer I – Layer VI) can be distinguished in the entorhinal cortex (Amaral et al., 1987). Superficially, there is a plexiform layer forming Layer I. Layer II consists of modified pyramidal and stellate cells. Beneath the Layer II, there is a population of pyramidal cells forming Layer III. The lamina dissecans located underneath the Layer III is indicative

of the virtual absence of Layer IV. Lastly, Layers V and VI located below the lamina dissecans are not easily distinguished (Schultz & Engelhardt, 2014).

3.2 Hippocampal circuitry

The hippocampal formation forms number of intrinsic and extrinsic connections. The intrinsic hippocampal circuit forms mostly unidirectional excitatory (glutaminergic) path with DG as the information gateway. The DG receives its major input from entorhinal cortex via perforant path which descends primarily from entorhinal cortex layer II (Freundl & Buzsáki, 1996; Witter & Amaral, 1991). Next, DG granule cells project via mossy fibres to subjacent dentate polymorphic layer and onto CA3 pyramidal neurons. The polymorphic layer neurons give rise to local and associative projections, whereas CA3 pyramidal cells form collaterals to other levels of CA3, to septal nuclei and, importantly, to II layer of CA1 via Schaffer collaterals, a major input system to CA1 (Ishizuka et al., 1990). CA1 receives also direct input from layer III entorhinal cortex via temporoammonic and alvear path (Witter & Amaral, 1991). Pyramidal cells of CA1 project predominantly to the subiculum which, in turn, projects to presubiculum and parasubiculum. Moreover, CA1 projects directly to prefrontal cortex, amygdala, nucleus accumbens and thalamic nucleus reuniens (Pitkänen et al., 2006; Swanson, 1981; Trouche et al., 2019; Varela et al., 2014) All three regions of subicular complex process and relay CA1 input to the entorhinal cortex (layer III-IV), closing the circuit (Beckstead, 1978). Despite several above-mentioned direct output regions of CA1, entorhinal cortex is a major output structure of hippocampus. The diagram of intrinsic circuit of hippocampal formation is displayed in Figure 5.

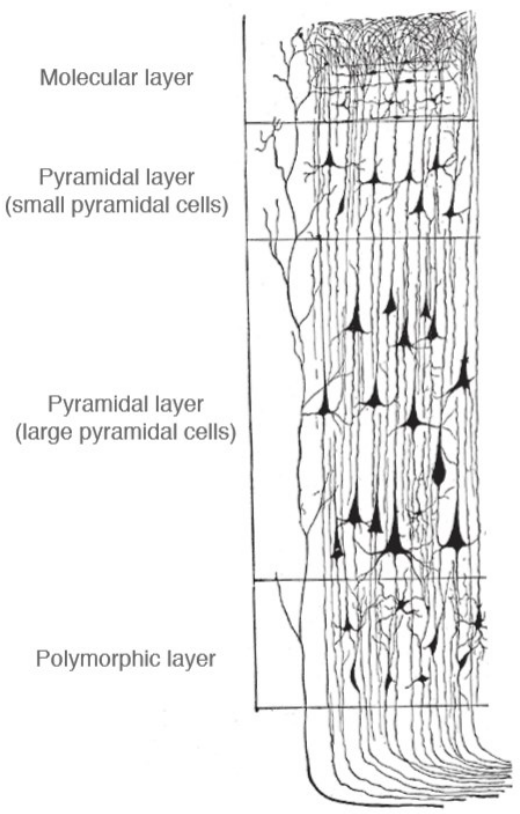


Figure 4 | Column representation of hippocampal archicortex cell body layers. Molecular layer is located most superficially, while polymorphic layer is the deepest of the hippocampal archicortex layers. Adapted from *Anatomy of the Human Body* (Gray, 1918).

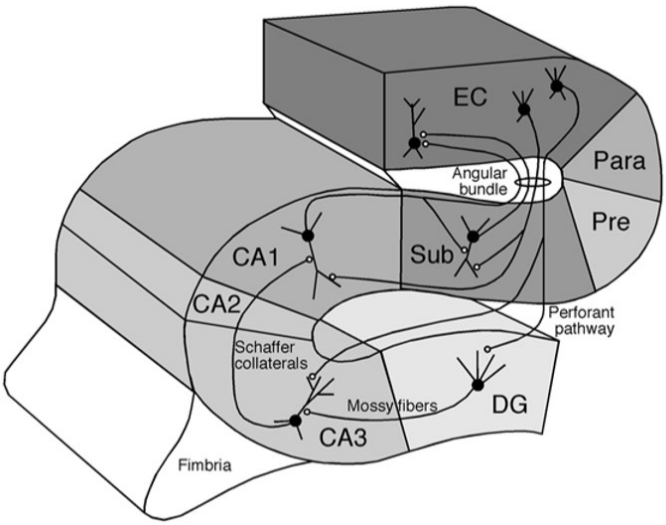


Figure 5 | **The intrinsic hippocampal circuit**. Neurons of the entorhinal cortex layer II project to the DG and the CA3 via perforant pathway. Neurons in the entorhinal cortex layer III project to the CA1 and the subiculum via temporoammonic and alvear pathway. Pyramidal neurons in CA3 field project to the CA1 via Schaffer collaterals. Pyramidal neurons in CA1 project to the subiculum. Subiculum projects back to the deep layers of the entorhinal cortex. Reprinted from *The Hippocampus Book* (Andersen et al., 2007).

As described above, the flow of information within hippocampus is generally unidirectional. This is also reflected by input structures being very different from output structures.

The hippocampal formation forms extrinsic input or/and output connections with cortical areas the hypothalamus, the amygdaloid complex, the septum pellucidum, the thalamus, the nucleus accumbens and the monoaminergic brainstem nuclei (Schultz & Engelhardt, 2014) as following:

- Corticohippocampal connections primarily convey the multimodal sensory information from medial temporal lobe through hierarchical chain represented by perirhinal cortex, posterior parahippocampal and entorhinal cortex to the hippocampal formation (Figure 6), (Van Hoesen, 1995). Importantly, the structures of this chain form reciprocal connections to respective cortical areas, e.g. various regions of temporal, parietal and frontal lobes (Van Hoesen, 1995).
- The major hypothalamic input to the hippocampus arises from supramamillary nuclei. The supramamillary projections are mostly excitatory and terminate

directly in DG and CA2 area of hippocampus (Barbas & Blatt, 1995; Maglóczky et al., 1994).

- The vast majority of amygdala connections to the hippocampal formation originates in lateral and basal nuclei of amygdala. The lateral nucleus of amygdala provides input mainly to the entorhinal cortex layer III neurons, while the basal nucleus of amygdala project to layer III neurons of CA1 and CA3 subfields (Pikkarainen et al., 1999). The most substantial efferent connections to the amygdala originate in rostral entorhinal cortex, temporal subiculum and the ventral part of CA1 area (Pitkänen et al., 2006).
- Septal fibres are most prominent in the DG and CA3, arising from medial septal nucleus and the diagonal band of Broca (Milner & Amaral, 1984). Additionally, CA1 and CA3 pyramidal cells project to the lateral septal complex (Risold & Swanson, 1997).
- The most prominent projections to the nucleus accumbens are unidirectional connections originating in subiculum (Groenewegen et al., 1987). Additionally, hippocampus projects to the nucleus accumbens via direct connections originating from dorsal CA1 (Trouche et al., 2019).
- The hippocampal formation receives projection arising from anterior thalamic complex and lateral dorsal thalamic nucleus via connections that form part of Papez circuit (Insausti et al., 1987). For example, the nucleus reuniens, located on the midline of anterior thalamic complex, gives rise to prominent projections to the layer I neurons of CA1 (Dolleman-Van der Weel et al., 1997). Feedback to the nucleus reuniens originates primarily in the dorsal and ventral subiculum (Varela et al., 2014).
- With respect to brain stem connections, the hippocampal formation receives major noradrenergic input from locus coeruleus (Segal & Landis, 1974), major serotoninergic input from raphe nuclei (Köhler & Steinbusch, 1982) and dopaminergic input from ventral tegmental area (Insausti et al., 1987). Of dopamine receptors (DR), the highest D1R density was observed in the DG, CA1 and CA3 regions (Gangarossa et al., 2012) and the highest D2R receptor density was observed in the DG and the subiculum (Goldsmith & Joyce, 1994). All of the serotonin (5-hydroxytryptamine, 5-HT) receptor families are expressed in the hippocampus (Berumen et al., 2012). The most abundant

are 5-HT_1 type receptors, most of which belong to the 5-HT_{1A} subtype in the DG (Hoyer et al., 1994).

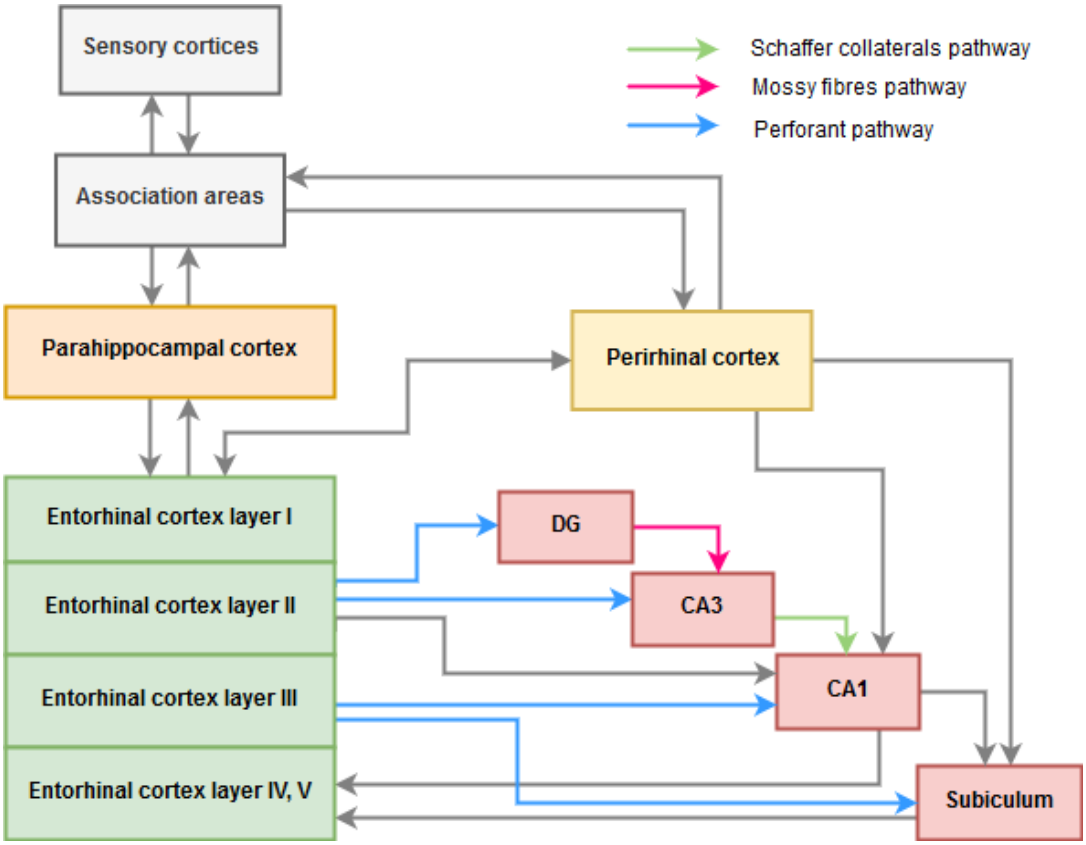


Figure 6 | The diagram of selected intrinsic and extrinsic connections of hippocampal formation and important pathways. Multimodal sensory information from sensory cortices is conveyed to hippocampus by hierarchical chain consisting of perirhinal, parahippocampal and entorhinal cortex. Perforant pathway (blue arrow) provides connection from entorhinal cortex (layer II and III) and other fields of hippocampal formation including DG, CA1 and subiculum. Mossy fibres (red arrow) originating from DG granule cells project to CA3 region. Schaffer collaterals (green arrow) provide connectional route from CA3 to CA1 pyramidal cells.

To summarize, hippocampal formation displays a vast intrinsic and extrinsic connectivity. How these connections and regions aid the formation of episodic memory is explored using animal models.

4 TESTING EPISODIC-LIKE MEMORY

Animal models allow us to study episodic-like memory formation and retrieval using methods unavailable or with limited availability in human research. These include targeted lesions of selected brain regions, inactivation of selected pathways, targeted receptor blockage, electrophysiological recording during task execution, or visualisation of immediate-early genes expression following the task execution. Currently, several episodic-like behavioural tasks exist. These tasks either relate particularly to animal's experience of the context in which learning takes place or to the memory of events which took place prior to animal's experience. Although most currently available episodic-like memory tasks require multiple trials, it is noteworthy one-trial tasks exist when an animal can learn to change their behaviour after a single learning trial. Examples of such rapid learning are conditioned taste aversion, recognition memory tasks, spatial learning and food caching (Morris, 2001).

The most widely used one-trial and multiple-trial tasks of episodic-like character will be discussed in this chapter with the emphasis on their advantages and drawbacks, as we believe each task substantially differs in its ability to assess episodic-like memory. We consider the involvement of hippocampus essential in a test to be a good episodic memory test, as it was shown the hippocampus represents crucial component of episodic memory formation in humans (Gaffan, 1992). Among others, we consider one-trial nature of the task and the presence of temporal gap advantageous due to higher potential to mimic naturalistic episodic memory formation as real world experiences usually happen only once and encompass events or stimuli not necessarily overlapping in time. Furthermore, as previously noted by Zentall et al., (2008) animals should not know beforehand what 'questions' they will be asked since when animals are trained to learn rules or contingencies, they likely develop expectations about the upcoming memory test. When the information is encoded incidentally it is not possible to transform it into action plan because the nature of subsequent memory is not yet known (Zhou et al., 2012). For this reason, we consider unexpectedness of trial 'question' advantageous. Complementarily, as disadvantageous or limiting we consider especially long trainings, ambiguous interpretation of behavioural outcome and only partial or unclear dependency of hippocampus. Additionally, we consider it limiting when a task can be solved

by non-episodic mechanism and the involvement of non-episodic mechanism was not yet disproven.

4.1 Conditioned taste aversion

4.1.1 General overview

Conditioned taste aversion (CTA, also known as *poison avoidance*) is a learning and memory paradigm in rats and mice, which is considered a special form of classical conditioning. This paradigm is based on notion that after the consumption of a novel food, the memory trace of the novel taste will be associated with either a nauseous feeling and avoided in future or it will be considered safe. Acquisition of taste aversion is a biologically meaningful paradigm as it can be readily observed under the natural conditions outside the laboratory (Rozin & Kalat, 1971; Welzl et al., 2001).

In CTA task, rodents are given access to a bottled liquid substance of a specific novel taste (conditioned stimulus, CS) (usually saccharin enriched solution) for a limited period of time. Later, the rats receive an injection of nausea-inducing drug. Most commonly used drug in CTA task is lithium chloride (LiCl), which is known to have nausea-inducing properties by activating the vagal and splanchnic afferent nerves (Niijima & Yamamoto, 1994). In the course of several hours, the animal becomes sick (unconditioned stimulus, US), showing signs of nausea (e.g. lying-on-belly in case of rats). If animal associates the substance ingestion with the nausea, it will avoid substance of this specific taste for a long time in future (Garcia & Koelling, 1966). The successful formation of CS-US association and subsequent memory trace extinction is tested using taste preference test (e.g. saccharine preference) usually during 20 consecutive days. The schematic procedure of the CTA task is displayed in Figure 7.

The interval between CS and US resulting in taste aversion can be as long as 6 hours, with the aversion against CS gradually decreasing with CS-US delay (Misanin et al., 2002; Welzl et al., 2001). Interestingly, no avoidance can be observed if the ingestion of the substance is followed by injection of a toxic substance not directly affecting gastro-intestinal system (Ionescu & Burešová, 1977) or by an electric shock (Garcia & Koelling, 1966). Some attribute such findings to the "generality of laws of learning" according to which one conditioned stimulus (e.g. gustatory cue)

is more associable than the other (e.g. shock) with one unconditioned stimulus (nausea) (Krane & Wagner, 1975).

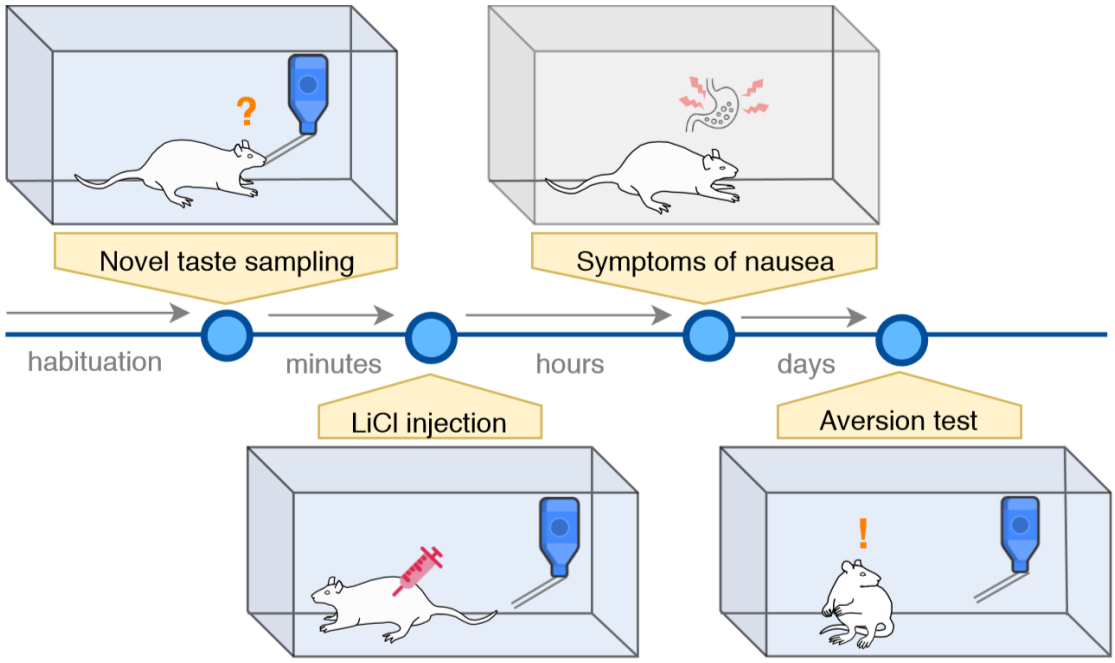


Figure 7 | **The general CTA procedure.** (A) The rat is given access to bottled liquid of novel taste. Shortly after novel taste sampling, rat is injected with nausea inducing substance. (B) Few hours later, rat develops nausea. During the second exposure to taste, the avoidance to the sampled taste is assessed.

Some studies modified the original CTA paradigm by using different CS modalities. Hayashi et al. (2002) shown the confinement of a rat in a running wheel results in the rats' subsequent avoidance to the taste consumed before the confinement Similarly, Masaki and Nakajima (2005) demonstrated aversion to the taste solution can be established by forced swimming in a water pool following the solution consumption. The energy expenditure (Nakajima et al., 2000) or gastrointestinal discomfort yielded by physical exercise (Lett et al., 1999) were suggested for underlying mechanisms for CTA induced by physical activity. However, the neurobiological mechanism of CTA induced by exercise presumably differs from CTA induced by gustatory cue, as the interval between CS and US successfully leading to taste aversion is only approximately 1 hour (Hayashi et al., 2002).

4.1.2 Neurobiology of conditioned taste aversion

There are only scarce studies available concerning the neural substrate and molecular mechanisms of memory trace formation in CTA task. The pattern of activity changes

in response to CS in CTA task were detected in *nucleus tractus solitarii*, *parabrachial nuclei*, the amygdala and the hypothalamus using electrophysiological techniques, immediate-early genes expression mapping as well as lesion studies (Houpt et al., 1994; Yamamoto et al., 1995, 1994). Using lesions studies, insular cortex was shown to play important role in CTA memory consolidation and retention (Stehberg & Simon, 2011). The investigations of possible involvement of hippocampus yield conflicting results. Hippocampal and/or fornix lesions resulted in mild acquisition of taste aversion or no acquisition of taste aversion at all (Best & Orr, 1973) but in other case rats with hippocampal lesions showed normal acquisition but slower extinction of the taste aversion memory (Kimble et al., 1979). Additionally, muscimol-induced hippocampal inactivation during training session strongly enhanced CTA learning (Stone et al., 2005).

4.1.3 Advantages and limitations of conditioned taste aversion

The main advantage of CTA task is it can rapidly established and completed in a single trial (Verendeev & Riley, 2012). Even the interval of several hours between CS-US leads to aversion to previously tasted substance (Misanin et al., 2002). Another advantage of CTA task is the formed memory trace is long-lasting. Even after 49-days long retention interval an averted taste does not return to the status prior poisoning (Rosas & Bouton, 1996). Moreover, the CTA task is relatively independent of motor behaviour (Welzl et al., 2001).

Albeit listed advantages, there are several limitations to CTA task. One of the drawbacks of the original CTA task is it relies on the ability of rodent to distinguish between specific tastes. The ability of certain rodent strains to discriminate between specific tastes varies, and so does their preference to specific or novel tastes (i.e., taste neophobia) (Blizard, 2007; Fuller, 1974). Another potential limitation is that the signs of nausea are sometimes not clearly visible - especially in case of mice – which causes difficulties to assess whether nausea was successfully induced (Welzl et al., 2001). Moreover, rapid food aversion is considered evolutionary conservative specialization (Rozin & Kalat, 1971), which rises question whether CTA task is a good model situation of episodic-like memory. According to some, the observed “preparedness” of animals in CTA task is given by the selective nature of CTA that facilitates biologically relevant stimuli (i.e., taste and sickness)

but minimize associations between biologically irrelevant stimuli (i.e., electric shock and sickness) (Verendeev & Riley, 2012). Most importantly, an argument against CTA task as a model of episodic-like memory is that neurobiological substrates of CTA appear to be very different from brain regions involved in human episodic-like tests (for review see Rugg & Vilberg, 2013).

Taken together, albeit visually strongly mimicking episodic-like memory, conditioned taste aversion represents specific type of memory connected to involvement of distinct brain regions and a presumably being a conserved evolutionary specialization. For this reason, we consider CTA task in rodents not an ideal animal model of episodic-like memory.

4.2 Novel object recognition task

4.2.1 General overview

The *novel object recognition* (NOR) task evaluates an ability of rodents to recognize novel object in an environment. NOR task exploit a natural tendency of increased exploration of novel objects compared to familiar ones.

Although there are many methodological variations, the common basic procedure of NOR involves habituation, familiarization and test phase (see Figure 8) (Antunes & Biala, 2012). During the habituation phase (lasting seconds to minutes), the animal is allowed to freely explore the open-field arena in absence of any objects. On the same day, the familiarization phase occurs (lasting seconds to minutes), during which the animal is returned to the open-field arena enriched of two or more identical sample objects fixed to the specific locations of the arena. After the retention interval (lasting minutes to hours), an animal is once again placed to the enriched open-field arena, but this time one of previously encountered objects is replaced with a new one (Bevins & Besheer, 2006).

It was shown the rats can retain a memory of an object encountered during familiarization phase for up to 4 hours (King et al., 2004). The successful recollection of a previously experienced event is evaluated by the differences in the exploration time between novel and familiar objects (Antunes & Biala, 2012). When animals are exposed to familiar and novel object, usually they visit novel object more frequently and spend more time exploring the novel object compared to the familiar one (Ennaceur, 2010; Silvers et al., 2007). The increased preference of the novel object

is given by the association of the environment with the familiar object and by an appetitive effect of the access to the novel stimuli as rats show natural propensity to the novelty (Bevins et al., 2002; Dellu et al., 1996).

The NOR task is widely used to test memory alteration in rodents and the effect of retention interval[1] manipulations on short-term and long-term memory, however it can also be used to test working memory, attention, anxiety and preference to novelty in rodents (Goulart et al., 2010; Taglialatela et al., 2009). Methodologically similar tasks to NOR task exist, in which case the animal is required to select novel object (*delayed non-matching-to-sample* task, DNMS) or to select the already familiar object (*delayed matching-to-sample* task, DMS) while the correct choice is rewarded with food pellets in both tasks. Due to similarities in methodology to NOR task, the DMS and DNMS task will not be described in further detail.

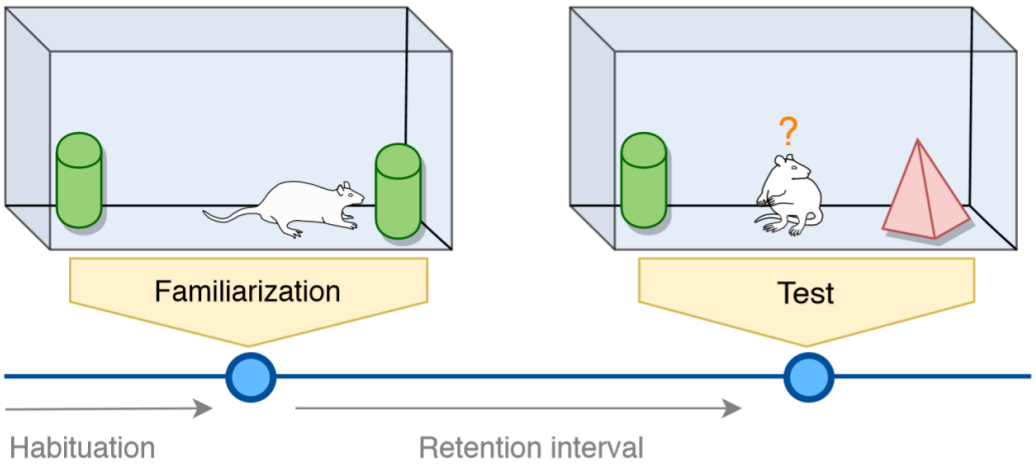


Figure 8 | **The novel object recognition task overview**. In the familiarization phase, rat is given chance to explore identical sample objects (green). During the test phase, rats are presented with already encountered object (green) and a novel object (red) and the exploration activity of rat is assessed.

With goal to closely model the episodic-like memory in rodents, several authors substantially modified the original NOR task. For example, Dere et al. (2005) introduced a 3-trial object recognition task based on the ability of rodents to distinguish between objects according to the level of their familiarity (objects encountered later in time are considered more familiar) and their spatial properties.

[1] The retention interval is in this context understood as the amount of time an animal must retain the memory of an object encountered during familiarization phase before the commencement of the test phase where the animals has to recall which objects were previously encountered.

During the third trial of this procedure, animals are placed in the open-field with two objects from the first trial ("old familiar objects") and two objects from the second trial ("recent familiar objects") with one of old familiar objects being placed to a new location (Dere et al., 2005). Kart-Teke et al. (2006) later modified the NOR paradigm by Dere et al. (above) by additional spatial displacement of one of the recent familiar objects to evaluate the structural integrity of what-where-when memory in animals (Figure 9). According to the authors, animals' exploration/preference pattern in both these tasks is indicative of its ability to recall previous experience and of the structural integrity of what-where-when memory (Dere et al., 2005; Kart-Teke et al., 2006). *What* is represented by the object (novel object should be preferred), *where* by the location (novel location should be preferred) and *when* is represented by the exploration of least recently presented object (Kart-Teke et al., 2006).

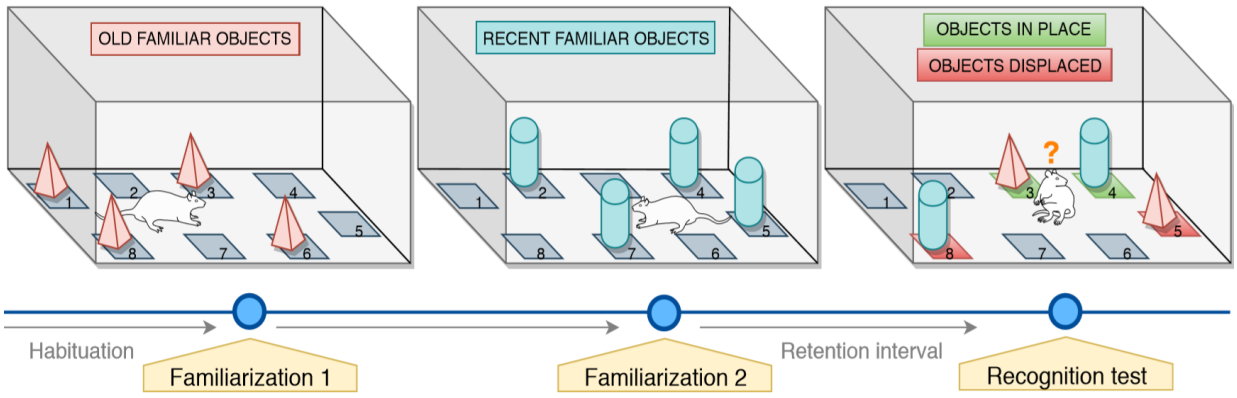


Figure 9 | **The overview of NOR task by Kart-Teke at el.** In the first familiarization session, rat explores four novel identical sample objects (light red pyramids; old familiar objects) located in their defined positions (e.g. 1, 3, 6, 8). Later, there is a second familiarization session, during which rat explores four novel identical objects (light blue cylinders; recent familiar objects) located in their defined positions (e.g. 2, 4, 5, 7). During the test phase, rat encounters four objects - one recent familiar and one old familiar object located in correct positions (pos. 3 and 4; highlighted in green) and one old familiar and one recent familiar object displaced from familiarization session positions (pos. 5 and 8; highlighted in red). Figure created according to procedure described by Kart-Teke et al., 2006.

4.2.3 Neurobiology of novel object recognition

Perirhinal cortex, entorhinal cortex and amygdala were consistently implied in NOR while evidence of hippocampal involvement is less consistent. Specifically, lesions of perirhinal cortex were shown to produce severe deficits in NOR performance (Meunier et al., 1993). Entorhinal cortex was demonstrated to be critical in integration

of object with the contextual features of environment (i.e. object-context association). This was demonstrated by an increased c-fos expression in the entorhinal cortex following performance in NOR task and by impaired performance in NOR task in rats with entorhinal cortex lesions (Wilson et al., 2013). Moreover, post-training noradrenergic activation of basolateral amygdala enhances consolidation of object recognition memory (Roozendaal et al., 2008).

Early experiments shown MTL damage causes severe impairment in performance in NOR task in monkeys (Mishkin, 1978). However, the role of hippocampus in NOR task is still unclear as number of conflicting results exist. Specifically, de Lima et al. (2006) have shown reversible hippocampal inactivation impairs early and delayed consolidation of NOR memory of up to 3 hours after training. Following inactivation of hippocampus, others found moderate impairment in NOR task performance (Broadbent et al., 2010), or no impairment in NOR task performance at all (Barker & Warburton, 2011). Furthermore, Oliveira et al. (2010) shown animals exposed to shorter habituation intervals following hippocampal inactivation have their long-term object recognition memory enhanced. Inconsistent observations of hippocampus involvement in NOR task might be caused by substantial procedural differences in NOR task used across studies - such as differences in exploration criterion and delay (Cohen & Stackman, 2015). The standardization of NOR task procedure is therefore needed to clearly assess the hippocampus involvement in NOR task.

4.2.2 Advantages and limitations of novel object recognition tasks

The advantage of NOR task is it can be completed in relatively short time as it requires only little habituation and no training. Importantly, the recognition memory can be assessed after a single trial (Ennaceur, 2010). Moreover, the NOR task is clinically relevant as it evaluates the ability of recognizing previously presented stimuli in a similar way as in case of visual paired comparison tasks in patients (Manns et al., 2000). With regard to modified procedure proposed by Kart-Teke, the fact that rodents prefer objects which they have not seen very recently over those explored more recently might be considered a measure of temporal order memory described previously by Mitchell and Laiacona (1998). The clear advantage of NOR task

is that it requires no positive or negative reinforcers such as external motivation, reward or punishment (Nemanic et al., 2004)

The NOR task encompasses several limitations. The main limitation of NOR task is the object recognition memory can be explained by implicit processes such as judgements of familiarity, as the task only requires animal to recognize the stimulus without requiring information about when and where was the stimulus previously encountered (Clayton et al., 2003; Morris, 2001). A simpler non-episodic memory explanation is an animal learns to choose the most familiar (in case of DMS) or unfamiliar (in case of NOR) object (Griffiths et al., 1999). Another limitation of NOR task is it takes advantage of the natural preference of novelty in rats (Berlyne et al., 1966; Dellu et al., 1996) and thus might not be characteristic of a more generally occurring *what-where-when* event. In addition, the NOR task suffers from the number of potential methodological issues. Specifically, the inherent differences in preference for novelty were shown depending on strain, sex and age of animals (Pisula, 2003; Stansfield & Kirstein, 2006) The object *affordance*[2] needs to be considered carefully as the preference for object might not be a result of novelty seeking or familiarity but of its affordance to animal. According to Chemero and Heyser (2005), the rats show preference for objects that have affordances for common rat activities (e.g. object rats can climb on or hide in). Moreover, the differences between to-be discriminated object need to be maximized due to limited perceptual abilities of rodents (Carter-Dawson & Lavail, 1979; Jacobs et al., 2001).

In overall, due to inconsistent results of hippocampus involvement in NOR, possible non-episodic mechanism and possibility that NOR task evaluates familiarity and not recollection *per se,* we consider NOR task not an ideal model of episodic-like memory.

4.3 Contextual fear conditioning

4.3.1 General overview

Contextual fear conditioning (CFC) represents a form of associative learning (Rescorla, 1968) of a painful stimulus (US) in a specific spatial context. The measure of memory

[2] Object affordance is generally described as relations between the abilities of animals and the properties of objects (Chemero & Heyser, 2005).

recall used in contextual fear conditioning is a freezing[3] response that takes place following the pairing of an US with a context.

A standard CFC procedure consists of 2 trials. During the first day, the animal is transferred to a conditioning chamber and allowed to explore it freely for few minutes. Following habituation, two foot-shocks (0.17-0.8 mA, generally 0.6 mA) are administered to the animal for 1-2 sec separated by an intertrial interval of 60-120 sec (Curzon et al., 2009). Twenty-four hours later, animal is placed into the environment in which an aversive event was previously experienced. At this point, animals will display a freeze response if they associated the context with the foot-shock (US) (Figure 10) (Phillips & Le Doux, 1992). The duration of freezing behaviour lasts from seconds to minutes depending on the various variables, such is number of foot shock presented during first day, the strength of aversive stimuli or the degree of learning achieved by an animal (Curzon et al., 2009). The fear response elicited by an animal can be further accompanied by other emotional and physiological responses such as defecation, piloerection, stereotyped increases in arterial pressure and heart rate and the release of adrenal hormones into the circulation (Smith & DeVito, 1984; Vianna & Carrive, 2005) Most of contextual fear conditioning (CFC) tasks can be modified for almost all animal species, but tasks for rats and the mouse are most widely used (Kim & Jung, 2006).

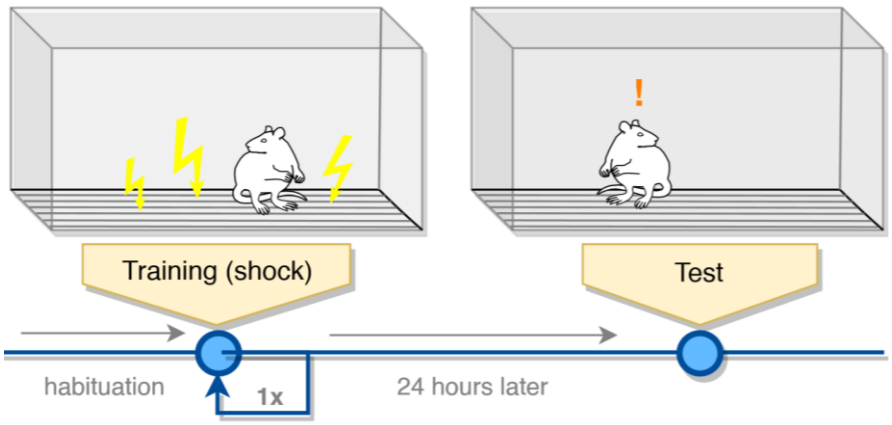


Figure 10 | **Contextual fear conditioning general overview**. The animal is placed in conditioning chamber for a subtle habituation of several minutes. Next, sudden mild shock is administered to an animal. After the interinterval of 1-2 minutes, shock of identical length and intensity is given to an animal again (recurring arrow). The animal is returned to the chamber on following day and its freezing response is assessed.

[3] Freezing behaviour is a species-specific defence reaction defined as the absence of movement except for respiration.

4.3.3 Neurobiology of contextual fear conditioning

Amygdala, hippocampus and medial prefrontal cortex are three brain regions consistently implicated in CFC.

Amygdala is regarded as structure with pivotal role in contextual fear conditioning (Fendt & Fanselow, 1999). Specifically, inactivation of amygdala impairs freezing following shock administration in original training context (Chang & Liang, 2017; Phillips & Le Doux, 1992). Similarly, pre-training lesions of the entire basolateral amygdala results in deficits in contextual fear conditioning (Kochli et al., 2015). NMDA receptor signalling in amygdala appears to be critical to acquisition, consolidation and retrieval of contextual fear (Johansen et al., 2011). Synaptic plasticity of cortical and thalamic inputs to the basolateral nucleus of amygdala is necessary for normal fear conditioning (Blair et al., 2001; Maren, 2005).

Hippocampus, too is involved in CFC in rodents (Chen et al., 1996; Maren et al., 2013). Hippocampal lesions produce deficits in freezing in rats in a shock-paired context (Phillips & Le Doux, 1992). Moreover, temporal inactivation of dorsal hippocampus (contrary to amygdala inactivation) immediately after shock administration impairs conditioned freezing in both direct and mediated [4]pattern completion test (Chang & Liang, 2017). However, hippocampal lesions do not necessarily prevent the acquisition of CFC if the number of conditioning trials is increased (Maren et al., 1997; Wiltgen, 2006). Additionally, the acquisition of CFC is spared if hippocampal lesions are induced after pre-exposition to the context. If a rat is pre-exposition to the context up to month before hippocampal lesions it is protected from contextual fear amnesia (Matus-Amat, 2004; Young et al., 1994). Therefore it has been suggested that the role of hippocampus in CFC is related to integration of contextual features and not to the formation of context-shock association (Fanselow, 2000). Moreover, the fact CFC can be acquired despite hippocampus inactivation rises a possibility of alternative hippocampus-independent mechanism of conditioned contextual fear acquisition.

[4] Memory trace can be reactivated fully by presenting part of its original stimulus configuration as a cue (direct pattern completion) or can be retrieved by stimuli that are not contained in the original memory episode but share common elements with it (mediated pattern completion) (Chang & Liang, 2017).

Medial prefrontal cortex presumably plays role both in long-term memory storage and fear responses related to conditioned context. Specifically, temporal inactivation of mPFC activity impairs contextual fear responses in rats (Kesner, 2005). Neurotoxic lesions of the mPFC disrupt remotely encoded contextual memories (Quinn et al., 2008). Additionally, ventromedial prefrontal cortex inhibition reduces expression of freeze response, heart rate and mean arterial pressure (Resstel et al., 2006) during CFC.

4.3.2 Advantages and drawbacks of contextual fear conditioning

The advantage of CFC is that fear conditioning to the context is established very rapidly (with the single context-US pairing) and has lasting effects of up to several months (Kim & Jung, 2006). Next, CFC paradigm is widely used and highly translational as similar tests can be used across range of species, including non-human primates (Kazama et al., 2013), rodents (Davis, 1990) and humans (Jovanovic et al., 2006).

Concerning the limitations of CFC, fear conditioning to the context can be explained by familiarity judgements (Pause et al., 2013). Another potential limitation of CFC paradigm is the incomplete dependency on hippocampus (see above). As the acquisition of contextual fear is possible in animals with hippocampal lesions, alternative non-hippocampal learning mechanisms (simple cue learning) were proposed (Gerlai, 1998). Moreover, CFC does not involve trace interval which limits ability of this task to mimic naturalistic episodic memory formation.

To summarize, despite being highly translational and rapidly established learning task, the association between foot-shock and context in CFC may not be dependent on hippocampus. Moreover, CFC task lacks a trace interval, a situation, which is not representative of many events that later serve as substrates for episodic memories.

4.4 Odour-location learning tasks

4.4.1 General overview

Odour-location sequence learning (OLL) paradigm is exemplified by group of procedurally different tasks with similar concept. The basic common principle of OLL tasks is assessment of temporal memory using rats' natural proficiency in odour discrimination (Uchida & Mainen, 2003) in combination with emotional experience

due to proposed emotional nature of olfactory stimuli (Masaoka et al., 2012; Pause et al., 2008). Despite the common concept, there are substantial differences in OLL procedures including distinct trial lengths and total number of trials, differences in stimuli presentation (digging in sand, sampling scented lids, …) and varied complexity. With goal to gain a better insight into OLL paradigm, selected OLL tasks will be briefly described below.

In OLL procedure deployed by Fortin et al. (2004), rats initially sampled several scents by digging for food in a scented sand-filled cups (5 cups each pre-training trial). Following 30 min long retention interval, old and new odours were individually presented to rat in random order. In case the scent was new, the rat was rewarded for digging in the scented cup, if the rat recognized the scent, it was rewarded for approaching an empty cup placed behind the home-cage (Fortin et al., 2004). The procedure of OLL task by Fortin et al. consisted of 3 consecutive daily sessions with 6 trials per session (Fortin et al., 2004) and the procedure overview is pictured in Figure 11. Similar task was proposed by Ergorul et al. (2004) who assessed whether rats remember the sequence of presented odours by using spatial and olfactory cues.

Another alternative of OLL task was proposed by Panoz-Brown et al. (2018). In their approach, the rats are sequentially presented with the list of trial-specific odours (5-12 items, the number of to-be-presented items is chosen randomly). Later, rats are moved to a different context and are given choice between two odours from the presented list. One odour is from a required ordinal position (fourth to last; correct choice), while the other odour is from a different ordinal position in the list (incorrect choice). Correct identification of previously encountered item of required ordinal position is awarded with food pellets (Panoz-Brown et al., 2018).

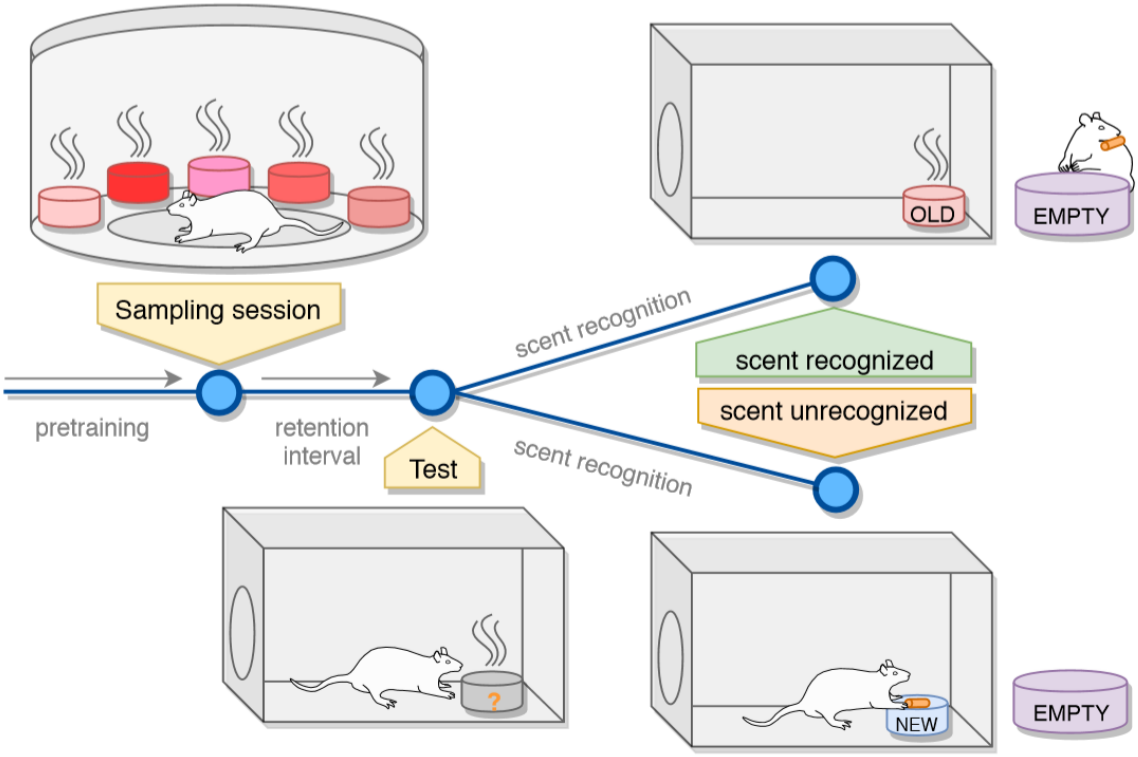


Figure 11 | **The overview of odour-location task developed by Fortin et al**. During sampling session, rats sample several scents by digging for food in scented sand-filled cups (shades of red). After the retention interval, rats are individually presented with scented sand-filled cups. These cups contain either novel (blue; NEW) or already encountered scent (red; OLD). When rat encounters the novel scent, it digs in sand-filled cup for a reward (food-pellet). If rat recognizes already known scent, it approaches an empty cup located behind home-cage and receives reward. Figure created according to procedure described by Fortin et al., 2004.

4.4.3 Neurobiology of odour-location tasks

Only few studies exploring neural circuitry of memory formation in OLL tasks were published. All these studies found a hippocampal dependency of OLL tasks. Specifically, Panoz-Brown et al. (2018) reported both selective and reversible memory impairment in OLL task performance following hippocampus suppression by chemogenetic activating drug (clozapine-N-oxide). Similar results were previously obtained by radiofrequency lesions of hippocampus in OLL task (Fortin et al., 2002). By comparing [5]receiver operating characteristics curves in animals with and without selective hippocampus damage, Fortin et al. (2004) reported presence of both

[5] Receiver operant characteristics analyses assess the relative contribution of recollection and familiarity depending on the pattern of correct and incorrect responses across different levels of confidence or bias (Yonelinas, 2001).

familiarity and recollection component of OLL task, with hippocampal damage specifically reducing the capacity for recollection. Complementary results were reported by Panoz-Brown et al. (2018) who shown new-old odour recognition and an odour discrimination tasks (presumed familiarity mechanism) are unaffected by hippocampus suppression.

4.4.2 Advantages and drawbacks of odour-location tasks

There are several advantages of OLL tasks. Importantly, as noted above, OLL tasks were shown to be dependent on hippocampus (Fortin et al., 2002). The memory trace formed is long-lasting, surviving 45-60 minutes long retention interval (Panoz-Brown et al., 2016, 2018). Moreover, the memory trace of odour-location was shown to be resistant to interference from new memories (Panoz-Brown et al., 2018).

Despite advantages, considerable limitations to OLL tasks exist. A possible non-episodic memory mechanism was proposed the rats are sensitive to the length of time elapsed between information-coding and the memory assessment and thus the different ordinal positions in the list are connected to position-specific levels of relative familiarity. In relation to this mechanism (i.e. decaying memory trace strength), animal might select items matching the relative familiarity memory trace of second to last or fourth to last item (Panoz-Brown et al., 2018). However, it was suggested that recollective process is involved when repeated expositions to odours are utilized. Wright et al. (2007) proposed that in situation when all presented items are already encountered numerous times (and thus familiar), subject may be likely to change its memory strategy from familiarity process to more recollective processes. According to another proposed non-episodic mechanism, the rats might discriminate cups using the traces of odour left on whiskers and fur (Clayton et al., 2003). No matter the modifications, OLL usually requires extensive training to perform the test (e.g. “shaping” the animal to dig in cups with sand) or number of stimuli presentations (Ergorul & Eichenbaum, 2004), which does not well parallel naturally occurring conditions.

Altogether, OLL are promising tasks with evidence of episodic-like memory involvement. However, the intensive training and “shaping” of animal required for performance limits relevance of OLL tasks to generally occurring conditions of episodic-memory formation.

4.5 Delay and trace conditioning

Delay conditioning (DC) and trace conditioning (TC) are associative learning tasks which are part of forward conditioning. Delay conditioning refers to a situation when US is administrated to co-terminate with or co-occur immediately after CS, while in TC the CS and US separated by stimulus-free interval ("trace") that separates the cessation of CS from the onset of US (see Figure 12 for visual representation of differences in CS-US timing). As CS, a tone, light or other somatosensory stimuli are generally used, while foot shock or air puff to the eye serve as US. Based on the dependent outcome, both DC and TC paradigms are subdivided into *fear conditioning* paradigms (measuring freezing; delayed fear conditioning (DFC) and delay trace conditioning (TFC)) and *eyeblink conditioning* paradigms (measuring presence of eyeblink; delay eyeblink conditioning (DEC) and trace eyeblink conditioning (TEC)). The overview of basic trace/delay fear conditioning paradigms is displayed in Figure 13.

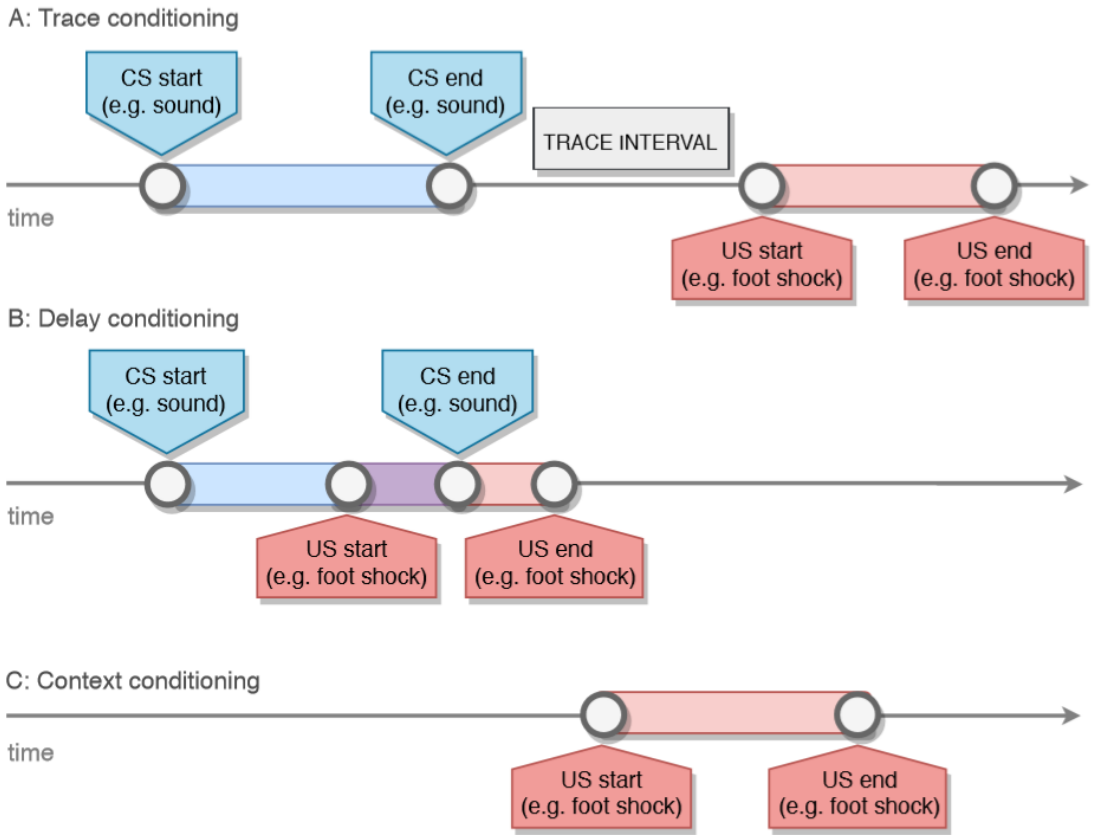


Figure 12 | A visual representation of differences in timing of the US and the CS presentations in conditioning tasks. In trace conditioning paradigm, CS and US are separated by an empty interval (trace interval). Contrarily, in delay conditioning paradigm, there is no trace interval and the US and the CS are partially overlapping in time. Lastly, in context conditioning paradigm, there is no CS preceding onset of US.

4.5.1 Delay conditioning

Standard DFC procedure is comprised of two trials. On the first day, the animal is habituated to a conditioning chamber (generally few minutes). After that an auditory cue (CS) is presented lasting several seconds (usually 15-30 sec) at the intensity 70-80 dB. During the last 2 sec of the tone presentation, a mild foot-shock (0.17-0.8 mA) is administered to the animal for 1-2 sec. Following the shock presentation, there is an intertrial interval of 60-120 sec which precedes the second identical tone-shock pairing. The animal is allowed to recover from shock administration and transferred to home cage 30-60 sec after the second shock administration (Curzon et al., 2009). On the second day, animals are either transferred to a novel environment (60 min prior testing for habituation) or transferred to an "altered conditioning chamber" from previous day with different odour (for example conditioning chamber cleaned with a diluted vanilla extract; 3 min prior testing for habituation). The sound cue of the same intensity used in a conditioning session is presented for the next 3 min (Curzon et al., 2009) and the freeze response is measured.

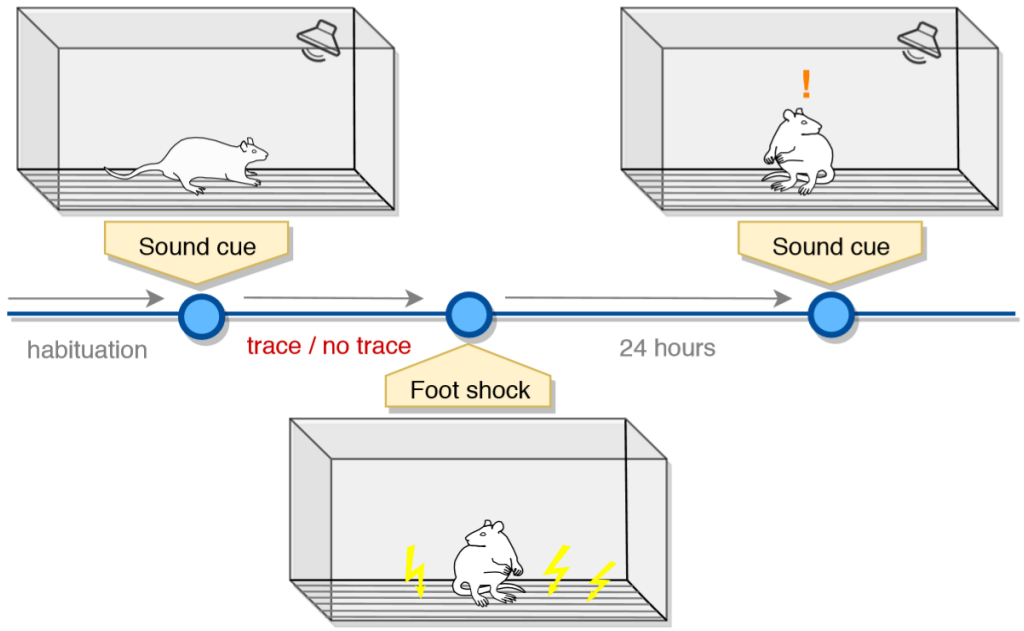


Figure 13 | **Basic trace/delay fear conditioning paradigm.** The rat is put into conditioning chamber for a subtle habituation of several minutes. Next, the sound cue of specific length and intensity is presented to an animal. Depending on the presence or absence of trace interval (red), mild electric shock is given to an animal few seconds after sound termination (TFC) or before the sound is terminated (DFC). Upon the placement in the conditioning chamber following day, the identical sound cue is administered, and the freeze response of rat is assessed.

The basic DEC procedure greatly resembles the DFC procedure with exception the auditory cue (CS) is followed by a short interval air puff (approximately 100 msec) directed to either eye of animal or a short periocular electric shock (usually 100msec) (US). In the trial session next day, the electromyographic signals from eyelid (measure of an eyeblink) of animal are sampled and analysed (Ivkovich & Stanton, 2001).

4.5.2 Trace conditioning

As stated above, in trace conditioning (TC), the offset of CS and onset of US are separated by a trace interval. The trace interval length used in TFC paradigm ranges from a relatively short (100 msec – 5 sec) to very long intervals (45-60 sec) (Cole et al., 1995; Curzon et al., 2009). Short trace intervals are generally considered to utilize similar associative learning mechanism as in long-delay conditioning (Barnet & Hunt, 2005) while longer trace intervals lead to weaker associations (Ellison, 1964) and are considered to be mediated by a different mechanism (Gerwig et al., 2008; Walker & Steinmetz, 2008). Usually, for longer delays 5 pairings suffice for a successful tone-shock association (Burman et al., 2014; Lugo et al., 2014). On the contrary, trace intervals successfully leading to CS-US association formation in TEC are much shorter, with the most rapid conditioning occurring for trace intervals ranging between 250-500 milliseconds (Woodruff-Pak et al., 2007). Yet, even them TEC requires many (e.g. 180 trials) repetitions of tone-air puff pairings (Siegel et al., 2015).

The presence of trace interval is generally the only substantial difference between TC and DC procedures. However, the presence of trace interval leads to a recruitment of similar brain regions as are observed in human episodic memory tasks. For this reason, neurobiology of both TC and DC will be described in detail below.

4.5.3 Neurobiology of delay and trace conditioning

Neural substrates, circuitry and memory trace mechanisms of eyeblink and fear conditioning are amongst those studied in most detail and well understood. Especially, the roles of cerebellum in eyeblink conditioning, amygdala in fear conditioning and hippocampus in both paradigms have been widely researched.

The essential role of cerebellum in learning and retention of eyeblink response in eyeblink conditioning have been implied for some time (for more ref. see Christian & Thompson, 2003). Deep cerebellar interpositus nucleus is important for both TEC

and DEC (Halverson et al., 2010; Pakaprot & Thompson, 2005). On the other hand, the cerebellar cortex appears to be more critical for DEC than for TEC. The disruption of cerebellar cortex function by lesions hampers the performance in DEC task (Kishimoto et al., 2001). On the contrary, following cerebellar cortex inactivation, only mild to no impairment in performance can be observed in TEC (Kishimoto et al., 2001; Woodruff-Pak et al., 2006). There is currently no evidence pointing on the involvement of cerebellum in TFC or DFC.

Amygdala has been consistently reported as key structure in DFC (see Pape & Pare, (2010) for a more detailed review), however the role of amygdala in TFC is unclear. Specifically, amygdala lesions disrupt the acquisition and expression of DFC (Kochli et al., 2015; Phillips & Le Doux, 1992) In contrast, there are conflicting reports of amygdala involvement in TFC. Rats with lesions of amygdala show robust deficits in TFC (Kochli et al., 2015; Selden et al., 1991), however, opposite findings were reported in mice (Raybuck & Matthew Lattal, 2011). Amygdala has not been implicated in either TEC or DEC paradigm.

It is noteworthy hippocampal activation has been observed in both trace (TFC, TEC) and delay (DEC, DFC) conditioning tasks (Thompson, 2005; Weiss et al., 1999). The role of hippocampus, however, appears to be different in each paradigm. Hippocampal lesions or hippocampectomy block both acquisition and retention of learning in trace conditioning (McEchron et al., 1998; Moyer et al., 2015), but not in basic delay conditioning paradigms (Quinn, Wied, et al., 2008; Woodruff-Pak et al., 1997). Moreover, the hippocampal involvement differs with the length of trace. Specifically, a marked involvement of NMDA receptors of dorsal hippocampus was shown for long trace intervals between 15 and 30 seconds (Chowdhury et al., 2005; Misane et al., 2005). Hippocampal NMDA receptors have been shown to be required for temporal memory formation in trace conditioning (Huerta et al., 2000). Some suggest hippocampal involvement in delay paradigms is due accompanying contextual fear conditioning (Raybuck & Lattal, 2014), as contextual learning presumably recruits the hippocampus for the encoding of complex multi-modal features (Rudy et al., 2004). Nonetheless, such speculations remain to be experimentally tested.

Concerning other brain regions, medial prefrontal cortex (Beeman et al., 2013; Gilmartin & Helmstetter, 2010; McLaughlin et al., 2002; Sierra-Mercado et al., 2011)

were additionally implied in both TFC and TEC paradigms. Moreover, perirhinal cortex have been involved with in TFC, TEC and DFC paradigms (Kholodar-Smith et al., 2008; Lindquist, 2004; Suter et al., 2013). Additionally, anterior cingulate cortex and entorhinal cortex have been implicated in TFC but not in DFC, TEC or DEC paradigm (Esclassan et al., 2009; Han et al., 2003; Li et al., 2018).

Two neural circuits originating from dorsal subiculum that independently regulate freezing behaviour and stress hormone response to conditioned cues were recently identified in fear conditioning paradigms using Cre recombinase expressing mice. First circuit, the CA1 - dorsal subiculum – entorhinal cortex layer V circuit, mediates freezing behaviour during memory retrieval. Second circuit, the dorsal subiculum – mamillary bodies circuit was shown to be essential for memory retrieval-induced stress responses (Roy et al., 2017).

4.5.4 Advantages and limitations of trace and delay conditioning tasks

Both trace and delay conditioning paradigms are considered experimentally tractable models which can be studied in both human and animals (Clark & Squire, 1998). Trace conditioning appears to be more advantageous than delay conditioning due to presumed involvement of higher cognitive processes. Specifically, trace conditioning requires individuals to acquire and retain knowledge of the task structure and thus to be aware of temporal relationships among the stimuli (Clark & Squire, 1998). Similarly, it has been shown trace conditioning but not delay conditioning requires attention (Han et al., 2003) and that delay conditioning is more resistant to interfering distractor stimuli (Carter et al., 2003). Moreover, as described above, the TC paradigm has been shown to be clearly dependent on the activity of hippocampus. To compare fear and eyeblink paradigms, it is noteworthy that fear conditioning occurs more rapidly, that is, requires fewer CS-US pairings than eyeblink conditioning (Woodruff-Pak & Disterhoft, 2008) and utilizes longer trace intervals (see above). On the other hand, the advantage of TEC is a dichotomous outcome (i.e. the presence or absence of blink).

There are several limitations to the trace and delay conditioning tasks. Despite its non-invasive nature, the measurement of freeze response itself is potentially limiting due to continuous nature of this outcome since it might confound the differentiation between learners and non-learners. Moreover, currently available

trace and delay conditioning tasks generally require repeated training trials (Lugo et al., 2014; Weiss et al., 1999) which hampers the descriptiveness of the paradigm to the naturally occurring conditions. Additionally, repeated training trials rise the possibility animal learns a rule: an electric shock follows a sound. Another challenge is to distinguish freezing to the context from freezing to the cue due to the frequent presence of contextual fear conditioning, which remains strong especially in TFC with longer trace intervals (Marlin, 1981). Delay conditioning have been by some considered as having automatic and reflexive features that are characteristic of nondeclarative memory (Clark et al., 2001).

Taken together, trace conditioning tasks appear to be more suitable for modelling episodic-like memory than delay conditioning tasks. Regarding eyeblink and fear conditioning paradigms, both TFC and TEC represent considerable methodological advantages but also present some drawbacks.

4.6 "Do as I do" imitation task

4.6.1 General overview

In order to explore the ability of dogs to recall past events with no expectation of the recall test, Fugazza et al. (2016) employed "Do as I do" (DAID) imitation which relies on ability of dogs to imitate human actions after a delay. This task may be a most direct proof of declarative episodic memory in animals. It relies on lower communication barrier between humans and dogs compared to humans and other animals.

The DAID imitation procedure consists of preliminary DAID training, baseline imitation test, "Lie down" training and the unexpected imitation test. During preliminary DAID pretraining, dogs are trained with regular DAID task to imitate human actions on command "Do it!". Subsequently, dogs are tested in the baseline imitation test during which they are initially exposed to two novel actions performed by the owner (e.g. touching umbrella with hand, standing on chair, walking around bucket etc.) and afterwards given command to imitate ("Do it!") these actions. Successful imitation of demonstrated actions is rewarded with treat. In the next phase, dogs are not required to imitate anymore, instead, they are requested to perform simple training task: lying down. Next, during the unexpected imitation test, dogs are exposed to two novel actions performed by the owner similarly as during baseline

imitation test. This time, however, dogs are only allowed to observe, not motor practice demonstrated actions. Next, there is a retention interval (1 minute or 1 hour) during which the dog is kept behind an opaque screen to prevent it from interacting with or watching objects. After the predetermined retention interval, dogs are given command to imitate ("Do it!") both actions performed by the owner before the retention interval (unexpected recall test). (Fugazza et al., 2016). The overview of unexpected recall test is displayed in Figure 14.

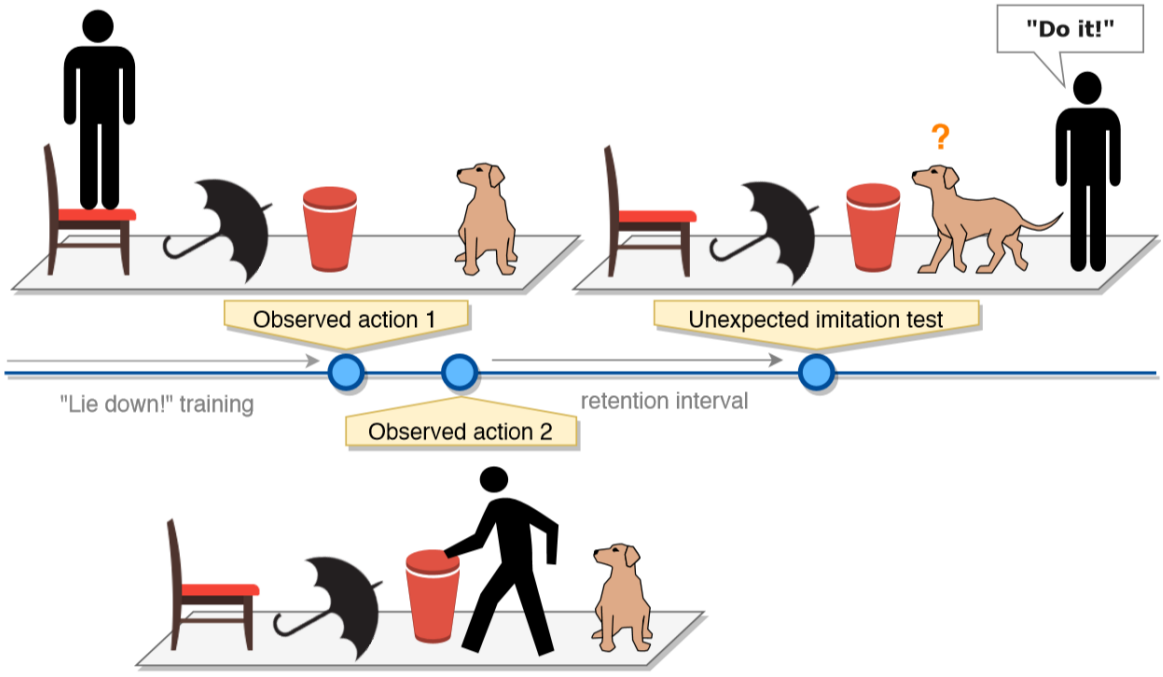


Figure 14 | Overview of unexpected imitation procedure of "Do as I do" imitation paradigm developed by Fugazza et al. At the start of experiment, dogs are trained to lie down on command ("Lie down!") to ensure subsequent imitation test is unexpected. Next, dogs are allowed to observe but not motor-practice two actions performed by owner (e.g. standing on chair as action 1 and placing hand on bucket as action 2). After the retention interval, dogs are commanded to imitate two observed actions in unexpected imitation test. Created according to procedure described by Fugazza et al., 2015, 2016.

4.6.2 Advantages and limitations of "Do as I do" modified imitation

There are several advantages to "Do as I do" imitation task. Specifically, dogs trained with DAID training are able to imitate the action sequence on the basis of observation alone in terms of the initial state, the means of action performance and the final goal, with no need to motor-practice the actions (Fugazza et al., 2015; Topál et al., 2006). Next, Fugazza et al. have shown the command to imitate the action after retention

interval is truly unexpected by dogs, as shown by [6]violation of expectation paradigm (Fugazza et al., 2016). The memory trace of observed actions is long-lasting, lasting up to 12 hours (Fugazza et al., 2015). Moreover, memory traces of actions in unexpected imitation decay faster than memory traces of actions in expected imitation over identical retention intervals (Fugazza et al., 2015). This is considered advantageous since episodic memories were proposed to decay faster over time than other types of memory (Talamini & Gorree, 2012).

Probably the most important limitation of DAID imitation task is that there are currently no studies aimed at the neurobiological processes underpinning action imitation in dogs, hence it is unclear whether DAID imitation is dependent on the activity of hippocampus. Moreover, despite Fugazza et al. (2016) claim the encoding of observed actions in dogs is incidental, the alternative explanation exists the dogs could have encoded observed actions with prior expectation of future "Do it!" command as previously all demonstrated actions were followed by command to repeat these actions. Yet, "Do it!" command was preceded by repeated "Lie down" command ensuring that "Do it!" command was not expected. Additionally, a minor methodological setback is the need of long, individualized and intensive pretraining of dogs to imitate human actions on command which limits the applicability of this task in regular research conditions.

Taken together, DAID imitation task is relatively new and promising task which implies evidence of episodic-like memory involvement. For future deployment in episodic memory research, however, the neurobiological substrate of DAID imitation needs to be clarified.

4.7 One-trial place memory task

4.7.1 General overview

To study encoding and retrieval of single-experience allocentric place memory in rats, Bast et al. (2005) developed one-trial place memory (OTPM) task, a food-reinforced arena paradigm analogous to paired-associate tasks used to examine episodic memory in humans (Holdstock et al., 2000; Shallice et al., 1994).

[6] Violation of expectation paradigm predicts a longer duration of looking toward the source of violation of expectation (Wang et al., 2004), e.g. dogs looking longer at the owned if command is unexpected.

The standard OTPM task protocol comprises a single daily trial (separated by at least 24 hours) consisting of encoding and retrieval phase (Figure15). Before the start of the experiment, rats (held on a food-restriction diet) are pretrained to dig for food in sandwells placed in their home cages and are habituated to the test environment. In the encoding phase of OTPM task, rats search arena for food of trial-specific flavour (different flavours used across days) in a trial specific place (sandwell, different sandwells are used across days) to encode the locations of this place in the memory. Upon eating the food, the rat is placed back to its home cage for a delay interval of 5 – 360 minutes. Next, retrieval phase starts with rat being placed to different starting location of the test arena from the one used in the encoding phase. Once again, the rat searches for food located in the same sandwell as during encoding phase ("correct" location), but this time four other sandwells with no reward are opened as well ("novel" locations). The rat is returned to its cage 60 second after successful food retrieval.

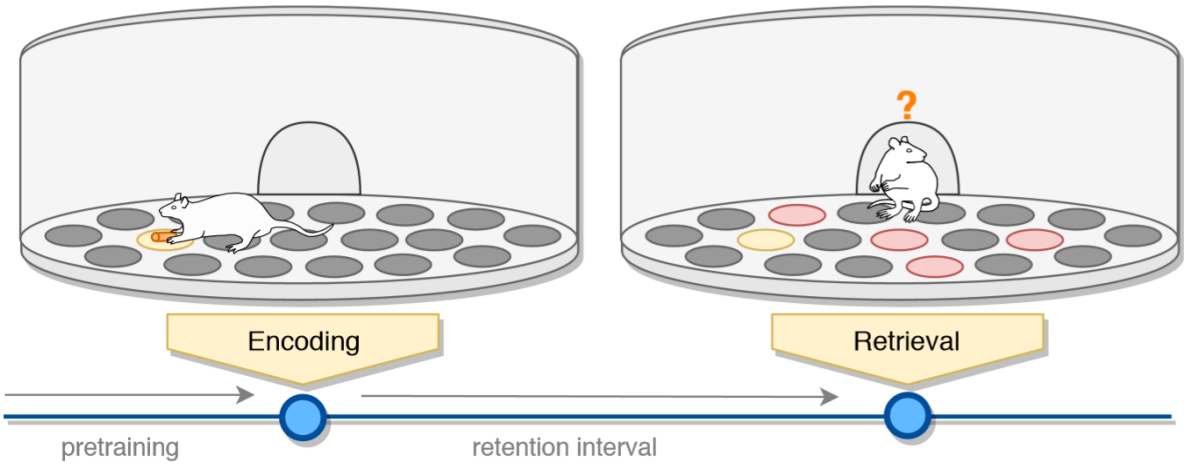


Figure 15 | Basic overview of one-trial place memory task developed by Bast et al. In the encoding phase, rats search arena for food pellets of trial-specific flavour hidden in trial specific sandwell (yellow). Upon eating the food pellet, rat is returned to its home-cage for the duration of retention interval. During subsequent retrieval phase, rat is returned to the arena to search for flavoured food pellet again. The food pellet is located in the same sandwell as during encoding phase (yellow), however, four additional empty sandwells are opened (red). Created according to procedure described by Bast et al., 2005.

4.7.2 Neurobiology one-trial place memory

Learning of food-place associations in OTPM task was shown to be hippocampus-dependent with the memory encoding being mediated by hippocampal N-methyl-D-

aspartate (NMDA) receptors and the memory retrieval being dependent on hippocampal alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors (Bast et al., 2005; Day et al., 2003). Apart from hippocampus, the involvement of other brain regions in OTPM have not yet been explored.

4.7.3 Advantages and limitations of one-trial place memory task

The clear advantage of OTPM is the rapid establishment of flavour-place association which is available after only one encoding trial. Furthermore, the memories of flavour-place associations are long-lasting (still significant after 6 hours) and decline with increasing length of retention interval (Bast et al., 2005). Next, Day et al. (2003) shown there are no differences in rat's preference of individual flavours used across trials, hence the choice of different flavours across trials does not affect the paradigm reliability. Additionally, animal's performance in OTPM is clearly dependent on the presence visuospatial cues while odour or idiothetic cues are not required for optimal performance in OTPM task (Bast et al., 2005). Next, as stated above, both encoding and retrieval of food-place association in OTPM task were shown to be dependent on activity of hippocampus. Another advantage is the OTPM task meets the content criterion of episodic-like memory by having "what" (flavoured food), "where" (sandwell) and "when" (during previous pairing session) component. Lastly, it is of note the OTPM task protocol resembles viewpoint-independent place memory tasks developed to research episodic memory in humans (Holdstock et al., 2000).

Concerning disadvantages of OTPM task, main one is that OTPM task is time-consuming, as it involves several weeks to months of pretraining, habituation and test trials (usually 18 trials are conducted) (Bast et al., 2005). Additionally, with rising number of conducted test trials it becomes very likely the animals expect beforehand what 'question' they will be asked in future and thus consciously remember the flavour-place association. Moreover, the combination of external motivation (food) and food-restricted diet could be appetitive enough to further drive animal's attention to conscious remembering of the association presented during encoding trial since it was shown trait reward drive leads to selective attention to food-related stimuli in hunger (Mogg et al., 1998; Tapper et al., 2010).

Taken together, one-trial place memory task represents a potentially fruitful task to apply in episodic-like memory research since it encompasses strong evidence

of episodic-like memory involvement. However, for regular use in research conditions, the protocol of OTPM task should be additionally reviewed and adjusted so the task could be more readily deployed and less time-consuming.

4.8 One-trial trace association task (OTTAT)

For the sake of testing recollection of temporally separated stimuli without any previous training we combined concepts from light-dark avoidance and active avoidance paradigms in rat to develop a novel one-trial trace association task (OTTAT) (Radostova et al., unpublished results). The OTTAT procedure is divided into two steps. In the first step, invariable behaviour of animals is established. The desired invariable behaviour is to voluntarily remain in a dark compartment for the duration of experiment (until disturbed). Second step of OTTAT consists of pairing session and testing session. During pairing session, two stimuli are presented to an animal (CS and US); reaction to second (US) requires a specific behaviour of the animal (escape to the light compartment) which later serves as an indicator of successful memory retrieval. In the testing session later, we test if first of two stimuli (CS) elicits the same exceptional behaviour (escape to the light compartment) as in pairing session.

OTTAT procedure will be described more in detail in Chapter 7. Additionally, advantages and limitations of OTTAT task will be discussed in Chapter 9.

4.9 Summary

To summarize, several episodic-like memory paradigms currently exist. Considerable progress has been done in the field of memory formation and retrieval neurobiology in trace and delay conditioning paradigms. According to our criteria, we consider TFC, OTPM task and DAID imitation to be the most promising attempts to model episodic-like memory developed up to this day. However, similarly as other reviewed episodic-like memory tasks, they encompass number of important drawbacks such as need of repeated training to perform the task. Importantly, most of available episodic-like memory tasks can be explained by a non-episodic mechanism, e.g. relative familiarity judgements. Moreover, the role of neural substrates involved in episodic-like memory formation in some of currently used episodic-like memory tasks is inconclusive or the mechanism of memory trace formation was not yet appropriately assessed. Therefore, we conclude the research of the relevant models representing episodic-like memory in animals is still ongoing.

6 AIMS OF THE THESIS AND EXPERIMENTAL QUESTIONS

We developed a novel one-trial trace association task which tests the ability of rats to form associations between temporally distinct events upon single trial presentation. The establishment of invariable behaviour of rats preceding the stimulus presentation is crucial for interpretation of results from OTTAT testing sessions and is essential for replicability of procedure between laboratories. The main goal of this work is to develop an experimental setup which leads to an invariable behaviour of rats. In order to develop optimal and replicable task protocol, we sought answers to the following experimental questions:

- Which of the common rat strains (Wistar, Sprague-Dawley and Long-Evans rat strain) displays the least number of transfers between compartments and spends the most time in dark compartment of multi-conditioning shuttle box during last habituation session of OTTAT?

- Does the instalment of compartment divider with wide opening ("wide doors") lead to reduction of transfers between compartments and/or to increase in time spent in the dark compartment by rats during last habituation sessions of OTTAT?

Moreover, we assessed CS cue specificity. To this aim we assessed whether conditioned reaction ("rapid escape" to the light compartment) is specific to identical acoustic stimulus or is organized to broader spectrum of acoustic stimuli. In order to address this issue, we sought an answer to the following experimental question:

- During OTTAT testing session, is the number of "rapid escapes" elicited by rats after hearing a sound of non-paired frequency (5000 Hz) decreased in comparison to number of "rapid escapes" elicited by sound paired with shock administration previous day (2400 Hz)?

7 MATERIAL AND METHODS

7.1 Animals

Altogether 103 adult male rats of Sprague Dawley (n = 36), Wistar (n = 51) and Long-Evans (n = 16) strain were used for the experiment. At the start of the experiment, rats were between 12 – 15 weeks old and weighted 300 – 400 g. The rats were obtained from the breeding colony of the Institute of Physiology of the Czech Academy of Sciences, Prague. The rats were housed in transparent laboratory polyethylene terephthalate boxes (50 x 25 x 25 cm), two animals per cage. Boxes were kept in animal room of stable temperature (22 °C) and humidity (50 %). Water and standard laboratory chow pellets for rodents were distributed *ad libitum*. The animals were kept on 12hours light cycle, with lights being turned on daily at 6 am.

Three days before the commencement of experiments, the rats were handled daily for 2 minutes by the experimenter. The experiments were conducted during the light phase of the day and in accordance with the Animal protection Code of the Czech Republic and a corresponding directive of the European Community Council on the use of laboratory animals (2010/63/EC).

7.2 Apparatus

The experimental apparatus were two modified TSE multi-conditioning shuttle boxes (TSE-systems GmbH, Germany) (Figure 16). Apparatus consisted of two interconnected 24 x 47 cm compartments. One of the compartments had transparent Plexiglas walls (“light compartment”) while the other was constructed using black opaque Plexiglas walls. Black opaque colour of Plexiglas walls of the second compartment reduced the amount of light transmitted to the compartment (“dark compartment”) but transmitted laser beams that registered position of animal. Light and dark compartments were divided by a 34 cm high and 44 cm wide black opaque divider with 9 x 11 cm or 3 x 40 cm opening (“doors”). Continuous background illumination (approximately 150 lx) was provided by two house lights located on the right side of housing roof. In order to reduce the amount of light transmitted inside the dark compartment, it was covered by a black lid resulting in light intensity of less than 3 lx in the centre of the compartment. Light compartment, on the other hand, was not covered and an extra light source (a lamp with 240V lightbulb)

was attached right above it, resulting in light intensity of 1090 lx in the centre of the compartment (higher than in standardly equipped TSE apparatus). This modification was done to make the light compartment uncomfortable for the animals as rats tend to avoid bright light and prefer to stay in darker areas (Campbell & Messing, 1969). Movement of animal was detected by horizontal laser beams, which, when interrupted by animal body, registered animal's location. Both shuttle boxes were equipped with a speaker delivering sound at 2 – 22 kHz frequency. When prompted by experimenter, the sound of desired frequency (2400 Hz was generally used) was delivered for 2 seconds at 80 dB, serving as the CS during both pairing and trial sessions. Floor of shuttle boxes consisted of metallic grid with 0.5cm diameter stainless steel rods, with centres spaced 1.5 cm apart. Metallic grid was used to deliver alternating current electric shock, which we applied at 1.0 mA, serving as the unconditioned stimulus (US).

Figure 16 | Photography of TSE multi-conditioning shuttle box. The shuttle box consists of dark and light compartment divided by black opaque compartment divider ("doors"). The dark compartment is covered with black opaque lid and the light compartment is equipped with lamp as an extra light source.

The behaviour of animals during sessions was videotaped using cameras located above each shuttle box and connected to the computer situated in the same room. The apparatus was cleaned and disinfected with alcohol-based cleaning liquid (Incidine) each time animal was removed from it to eliminate residual scents of animal and left to air dry before next animal was placed into it.

7.3 Behavioural testing

7.3.1 Description of OTTAT standard protocol

7.3.1.1 Establishment of invariable behaviour

To establish the invariable behaviour of animals, modified light-dark place preference task is utilized. In light-dark test animal is free to explore interconnected light and dark compartments. The task exploits a natural tendency of rodents' preference for a dark places. The amount of time spent in a light compartment is a measure of exploratory behaviour and low anxiety levels. In OTTAT there are, altogether, three 15-minute daily habituation sessions (1^{st} – 3^{rd} experimental day) in light-dark apparatus. During the habituation sessions, animals have a freedom to explore both compartments (light and dark) but are motivated to remain exclusively in dark compartment by white light intensity in the light compartment which was increased in comparison to commercially bought TSE apparatus. In fact, the light is in average 2.18 times brighter than light reported to be used in classic light-dark test (Bourin & Hascoët, 2003; Sirohi et al., 2017).

7.3.1.2 CS-US pairing

Association of CS-US separated by a trace interval were utilized using one-trial trace conditioning. During pairing session, an animal is placed into the apparatus for 15 minutes as if for another habituation session (pairing session, 4^{th} day). After 15 minutes elapsed, when an animal is present in dark compartment, a sound cue is presented (CS, sound frequency 2400 Hz), followed by electric shock (CS) after 2 second delay (trace interval). Shock is terminated when animal transferred to light compartment or after 25 seconds of shock administration without transfer to light compartment (in that case, the animal was excluded from study). After approximately one minute following foot-shock administration, the animal

is returned to its home-cage. The simplified overview of pairing session is displayed in Figure 17.

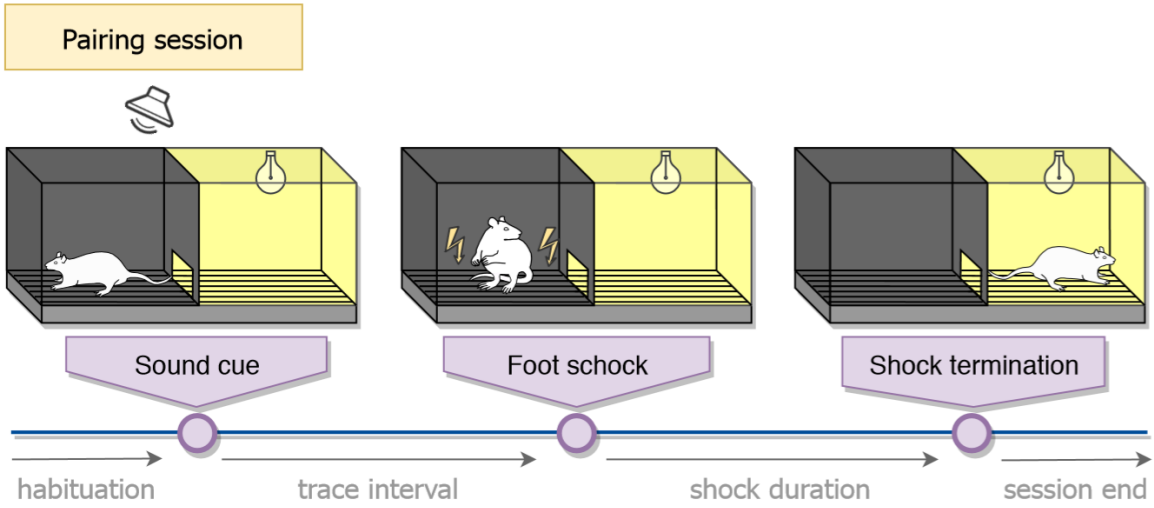


Figure 17 | Simplified overview of OTTAT pairing session. After a subtle habituation (15 min), the animal present in the dark compartment is presented with a sound cue (CS) followed by an empty trace interval of 2 seconds. Following immediately after trace interval, a mild foot shock (US) is given to the animal which is terminated once animal transfers to light compartment.

7.3.1.3 Assessment of trace associations

During testing session 24h later (5th day), the presence of trace association between CS - US is tested (i.e. if sound alone elicited an escape response in an animal). Animal is again placed into the dark compartment if multi-conditioning shuttle box and sound is presented after 10 minutes of habituation. The 10 minutes delay interval was chosen to later assess the presence of contextual association between dark compartment and the shock from training session (assessment of contextual freezing is not part of this work). Following the sound presentation, rats either voluntarily transfer to the light compartment and the time interval of escape response is assessed (active avoidance) or, alternatively, remain in the dark compartment (no escape). The simplified overview of testing session of OTTAT is displayed in Figure 18.

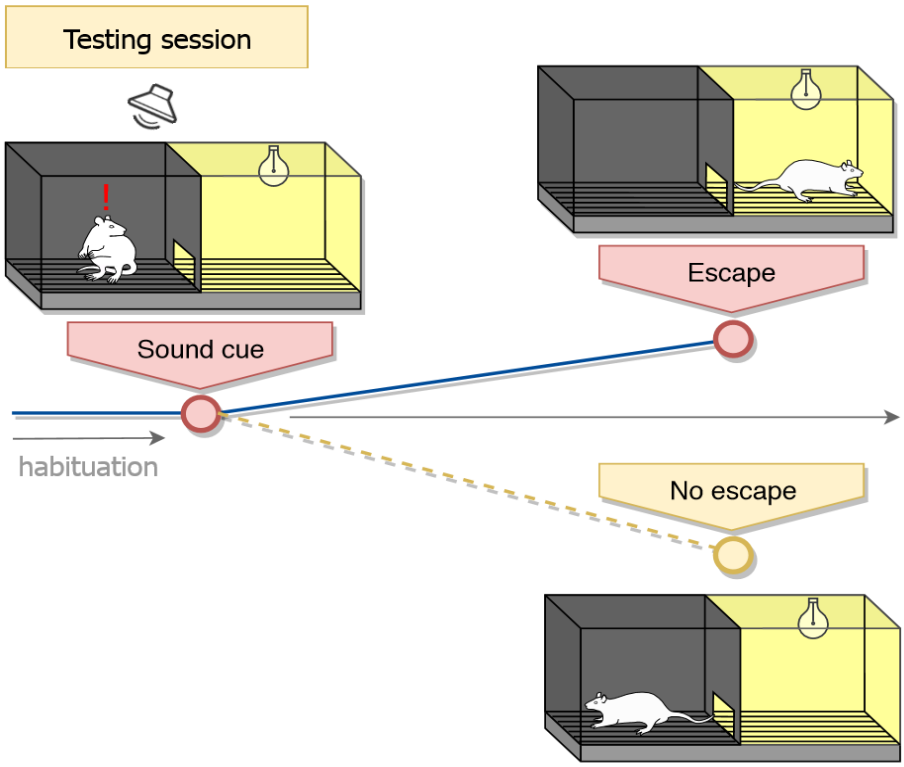


Figure 18 | Simplified overview of OTTAT testing session. The animal receives a subtle habituation (10 minutes) which is followed by the administration of sound cue (CS). As sound cue was previously followed by a foot shock which was terminated only by transfer to the light compartment (pairing session, see above), the rats escape to the light compartment once they hear the sound. Alternatively, as in case of unsuccessful association between CS-US, no escape response is observed.

7.3.2 Experimental design

Altogether, three experiments were conducted. In all three experiments, we used the apparatus and the standard OTTAT procedure described above with the exception of few experiment-specific modifications described below:

In experiment I, the differences in acquisition of invariable behaviour across three selected rat strains (Wistar, Sprague-Dawley, Long-Evans) were examined. Invariable behaviour, for purpose of this experiment, was a constant presence of animals in a dark compartment with no transfers to the light compartment (despite free access to the light compartment). For the experiment, we used 36 Sprague-Dawley rats, 17 Wistar rats and 8 Long-Evans rats. All rats underwent the standard protocol for habituation sessions of OTTAT described above. The rats were habituated with the standard (11 x 9 cm) opening in the divider ("normal doors").

In experiment II, we examined the effect of wider, but lower, opening between compartments on time spent in the dark compartment and number of transfers to the light compartment. To compare the effect of wider opening between compartments to a normal type of door divider, 34 Wistar rats were habituated with low-and-wide (3 x 40 cm) opening between compartments ("wide doors"). We followed the standard protocol for habituation sessions of OTTAT described above.

In experiment III, we examined whether escape reaction is specific to paired sound or of animals display a more generalized response. Altogether, 16 Wistar rats were in the experiment. We utilized the habituation and pairing session with accordance to the standard OTTAT procedure described above with all animals. On the testing day (5^{th} day), the animals were randomly separated into two groups. Following 10 minutes of habituation, first group (n = 8) received an acoustic cue of 5000 Hz (novel acoustic stimulus) and the second group (n = 7) received an acoustic cue of 2400 Hz (used during pairing session). Both length of acoustic stimuli administration and its intensity was kept identical for both used frequencies (2 s sound length and 80 dB sound intensity).

7.4 Parameters used and statistical analysis

In experiment I and II, statistical analysis focused on the number of transfers between light and dark compartments and on amount of time spent in each compartment during three habituation sessions (1^{st} – 3^{rd} day). We believe these parameters most straightforwardly reflect the willingness of animals to stay in dark compartment. The videos obtained from habituation sessions were semi-automatically analysed using BORIS software (version 6.3.8, released 2018) (Friard & Gamba, 2016). The transfers between compartments were manually determined by experimenter, while the time spent in each compartment were subsequently generated by the software.

The reaction to sound cue during testing session in experiment III was assessed by Two Compartments software (TC, part of TSE multi-conditioning system (TSE-Systems GmbH)) which both delivered the sound stimulus and registered the transfer of animal between compartments, while the time limit of transfer was registered by the experimenter. The main parameter of interest was a "rapid escape" following sound cues of respective frequencies. We designated the threshold

for “rapid escape” to the light compartment to be counted as a rapid retrieval of the trace memory following sound presentation to be less than 2 seconds after CS termination. The rest of the rats that did not fulfilled the “rapid escape” criterion was labelled “no escape,” “slow escape” (for escape in interval between 3 to 7 seconds after CS termination).

Statistical analysis was performed on the data collected from experiment 1 and 2 using SPSS software (IBM SPSS Statistics, version 25, released 2017). We tested normality of data distribution by Shapiro-Wilk test and homogeneity of variances by Levene’s test. Depending on the normality of variables distribution, the variables obtained from Day 3 of experiment 1 and 2 (time spent in dark compartment, number of transfers between compartments) were tested either by Kruskal-Wallis H test (parametric assumption not met), Mann-Whitney U test (parametric assumption not met) or one-way ANOVA (parametric assumption met). The level of significance was universally set at alpha = 0.05.

8 RESULTS

8.1 Establishment of invariable behaviour

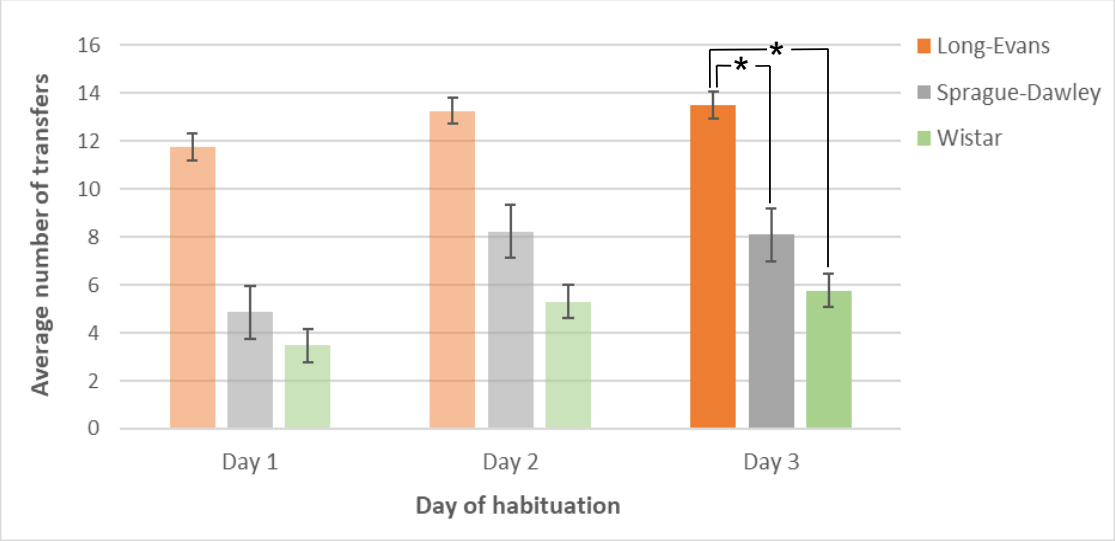
8.1.1 Strain-dependent differences in induction of invariable behaviour

Our aim was to select optimal rat strain for OTTAT experiment. Optimal strain should make as little transitions as possible into the light compartment, because transfer to the light compartment should ideally occur only following electric shock or memory recollection of electric shock. Since most data groups did not meet parametric assumptions (see below), independent samples Kruskal-Wallis H test, a non-parametric equivalent of one-way ANOVA, was used to compare the number of transfers between compartments and the time spent in dark compartment between Wistar (WI), Sprague-Dawley (SD) and Long-Evans (LE) rats during 3rd habituation session (Day 3).

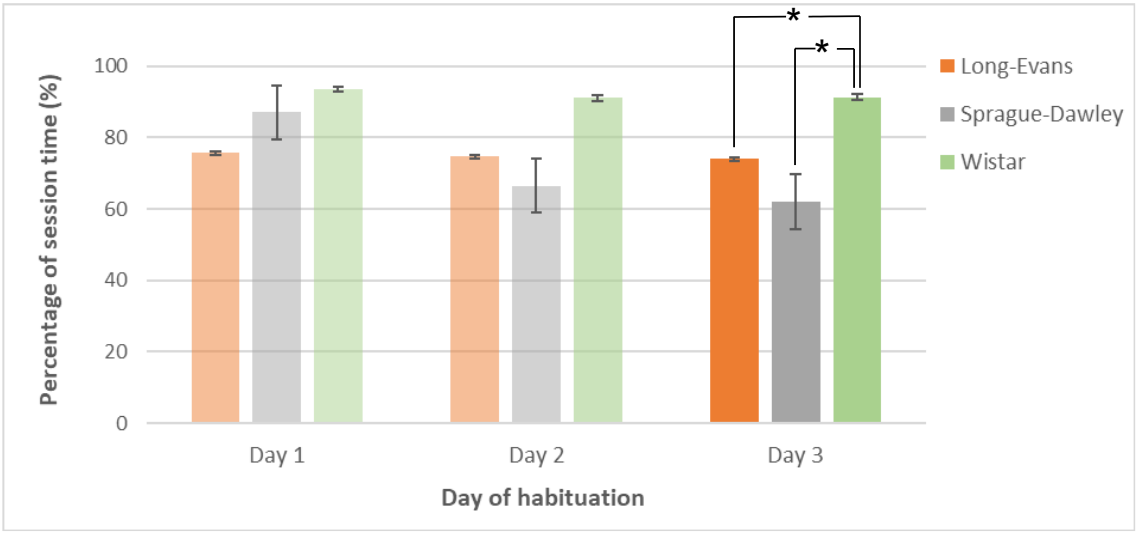
The number of **transfers** between compartments on the 3rd habituation day was not normally distributed in both SD and WI groups (Shapiro-Wilk test; all $p < 0.001$) and was normally distributed in LE group (Shapiro-Wilk test; $p = 0.229$). A Kruskal-Wallis H test showed that there was a statistically significant differences in number of transfers between strains [$\chi^2(2) = 13.119$, $p = 0.01$]. Post hoc analysis with Mann-Whitney U test was conducted with a Bonferroni correction applied, resulting in a significance threshold set at $p < 0.017$. We found LE rats (Median (Mdn) = 13.5) made significantly more transfers during Day 3 than SD (Mdn = 9) [U = 52.000, $p = 0.005$ (2-tailed)] and WI rats (Mdn = 5) [U = 11.500, $p = 0.001$ (2-tailed)]. There was no significant difference in number of transfers between SD and WI strain [U = 201.500, $p = 0.060$ (2-tailed)]. Average numbers of transfers between compartments by WI, SD and LE rats during habituation sessions are shown in Graph 1.

Time spent in dark compartment is a complementary measure of behavioural stability. The values are in seconds out of 900 s habituation session (15 minutes). Values of the **time spent in dark** compartment by Long-Evans rats (Shapiro-Wilk test; $p = 0.557$) met parametric distribution, however, values of the time spent in dark compartment by SD and WI rats did not meet parametric distribution (Shapiro-Wilk test; all $p < 0.001$). A Kruskal-Wallis H test showed that there was a statistically

significant differences in values of the time spent in dark compartment between strains [$\chi^2(2) = 30.367$, $p < 0.001$]. Post hoc analysis with Mann-Whitney U test was conducted with a Bonferroni correction applied, resulting in a significance threshold set at $p < 0.017$. We found WI rats (Mdn = 830.993) spend significantly more time in dark compartment during Day 3 compared to both LE (Mdn = 658.631) [U = 15.000, $p = 0.002$ (2-tailed)] and SD rats (Mdn = 570.333) [U = 24.000, $p = 0.001$ (2-tailed)]. There was no statistically significant difference in values of time spent in dark compartment between LE and SD rat strains [U = 90.000, $p = 0.119$ (2-tailed)]. Average time spent in dark compartment by WI, SD and LE rats during habituation sessions is displayed in Graph 2.



Graph 1 | Average number of transfers between light and dark compartment during habituation sessions. WI rats showed the least amount of transfers between two compartments of the three rat strains on each habituation day. The average number of transfers of WI rats was 3.5 ± 0.64 on Day 1 and 5.76 ± 0.64 on Day 3. The average numbers of transfers of WI and SD rats were significantly lower than that of LE rats during Day 3 [$\chi^{2(2)} = 13.119$, $p = 0.01$]. The error bars represent the standard error of the mean (SEM), * denote a significant difference between groups at $p < 0.017$.



Graph 2 | Average percentage of time spent in the dark compartment during habituation sessions. WI rats spent the most amount of time in the dark compartment on each day. The average time spent in dark compartment by WI rats was 841 ± 5.5 s on Day 1 and 821 ± 5.5 s on Day 3. Moreover, the average time spent in dark compartment of WI rats was significantly higher than that of LE and SD rats during Day 3 [$\chi^{2(2)} = 30.367$, $p < 0.001$]. The error bars represent SEM, * denote a significant difference between groups at $p < 0.017$.

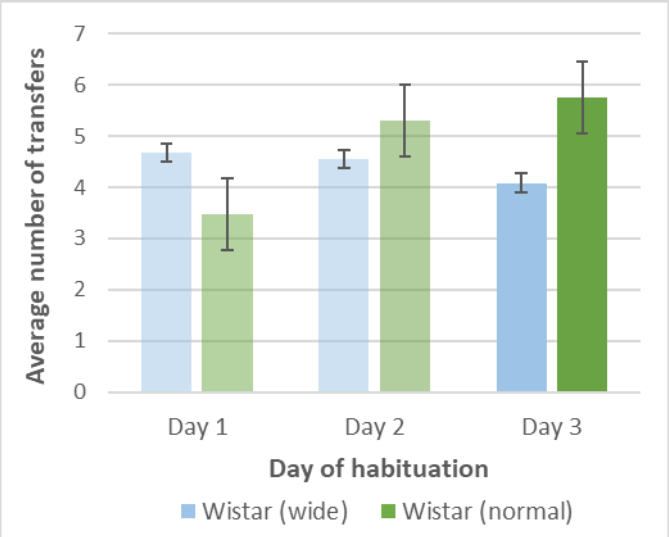
8.1.2 Effect of "wide doors"

Second, our aim was to assess the effect of wide doors on the establishment of invariable behaviour of Wistar rats in OTTAT experiment. Since we previously observed rats to explore specifically visually inaccessible areas behind doors of light compartment, we hypothesized that wide opening in door divider might increase field of view of rats and hence decrease their motivation to physically explore light compartment. This would, in turn, lead to fewer transitions between compartments and all increase of preference of the dark compartment. Mann-Witney U test (non-parametric) or one-way ANOVA (parametric) were used to compare the number of transfers between compartments and time spent in dark compartment between Wistar rats habituated to apparatus with wide (WIW group) and normal doors (WIN group) during 3rd habituation sessions, respectively.

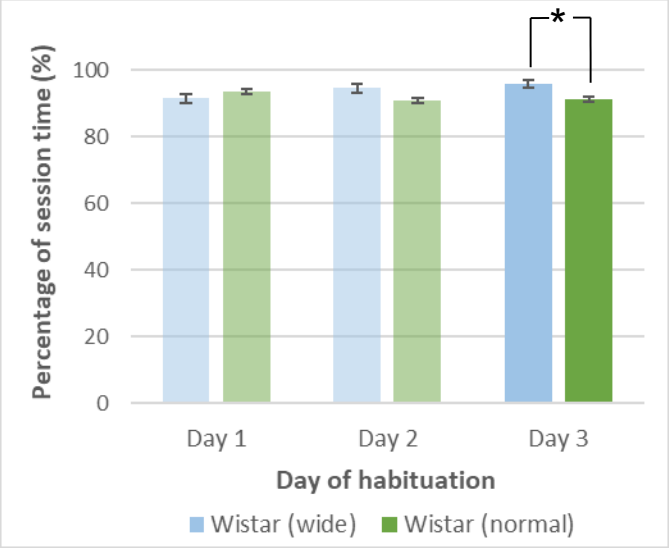
The number of **transfers** between compartments during Day 3 was not normally distributed either WIW or WIN group (Shapiro-Wilk test; all $p < 0.05$). Since data did not meet parametric assumption of ANOVA, Mann-Whitney U test was conducted and found no statistically significant difference in number of transfers between WIW (Mdn = 4.0) and WIN group (Mdn = 5.0) [U = 218.500,

p = 0.156 (2-tailed)]. Average number of transfers between compartments per door type is shown in Graph 3.

The values of **time spent in dark** compartment during Day 3 were not normally distributed in WIW group (Shapiro-Wilk test; p = 0.014) but were normally distributed in WIN group (Shapiro-Wilk test; p = 0.081). Despite one group did not meet parametric assumptions, we proceeded to parametric test, risking type 2 error. One-way ANOVA found significant increase in time spent in dark compartment in WIW group compared to WIN group [F (1, 49) = 0.9797, p = 0.003]. Average time spent in dark compartment divided by door type and rat strain is shown in Graph 4.

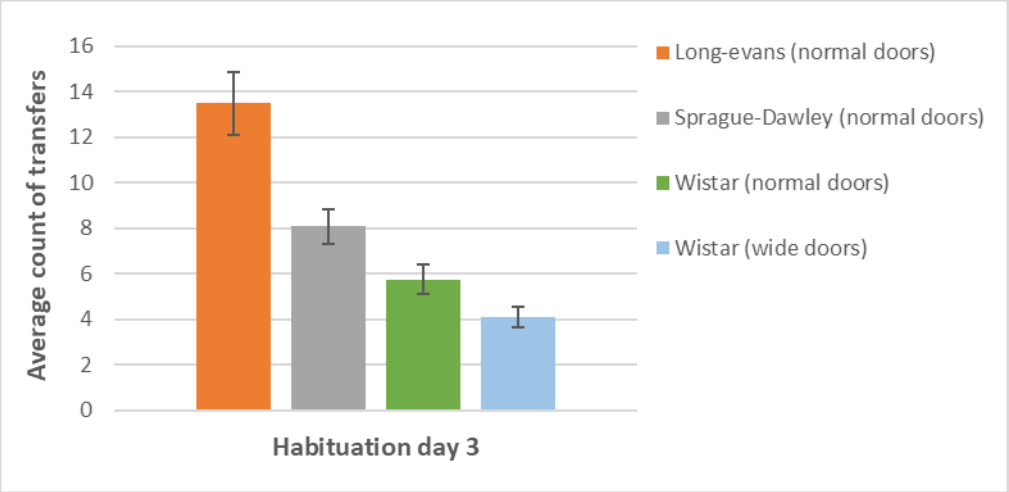


Graph 3 | Average number of transfers between compartments during habituation sessions. Average number of transfers on Day 3 in WI with wide doors was 4 ± 0.44 in comparison to 5.76 ± 0,64 transfers by WI rats with normal doors. The error bars represent SEM.

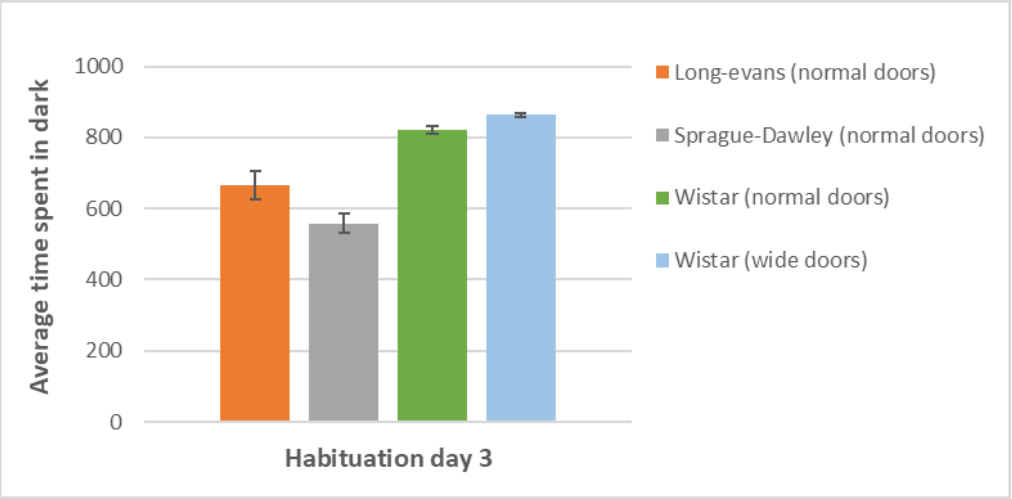


Graph 4 | Average percentage of time spent in dark compartment during habituation sessions. WI rats habituated with wide doors spent in average more time in dark compartment on Day 3 than WI rats habituated with normal doors [F (1, 49) = 0.9797, p = 0.003]. The error bars represent SEM, * denote a significant difference between groups at $p < 0.05$.

Finally, average number of transfers and average time spent in dark compartment on the Day 3 for all assessed measures in experiment 1 and 2 are depicted in Graph 5 and Graph 6 respectively.



Graph 5 | Comparison of average number of transfers between compartments of individual rat groups during final habituation session. WI rats habituated with wide doors made the fewest transitions between compartments of all examined measures (on average 4 ± 0.44 transfers compared to 13.5 ± 1.38 for LE rats, 8.1 ± 0.76 for SD rats and 5.76 ± 0.64 for WI rats habituated with normal doors).

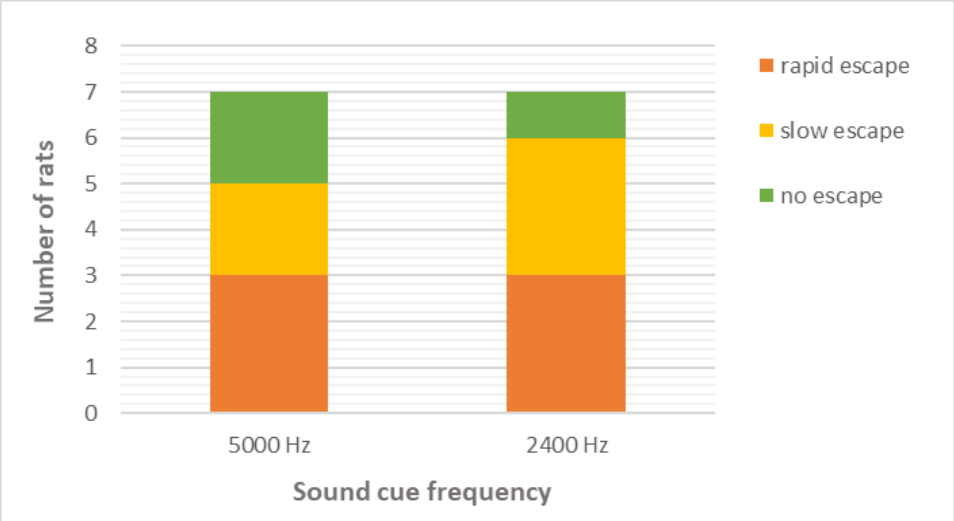


Graph 6 | Comparison of average time spent in dark compartment of individual rat groups during final habituation session. Wistar rats habituated with wide doors spend the most time in dark compartment of all examined measures (on average 863.2 ± 5.5 s compared to 666.0 ± 39 s for LE rats, 558.7 ± 28.6 s for SD rats and 821.2 ± 10.9 s for WI rats habituated with normal doors).

8.2 Specificity of conditioned reaction to sound cue

To determine if conditioned reaction ("rapid escape" to the light compartment) is specific to the sound cue paired to the foot-shock during pairing session or is more generalized, we assessed number of rats with **"rapid escape"** (transfer to the light compartment within 2 seconds of sound cue termination) following administration of either un-paired (5000 Hz) or paired (2400 Hz) acoustic cue of same duration and intensity during testing session of OTTAT.

All animals (n = 16) escaped to the light compartment following CS-US pairing session but one. This animal was excluded from testing session, as it could not form a memory of successful avoidance. Additionally, one animal stayed in light compartment for the duration of testing session. This animal was excluded from testing session, since for the test of memory recall an animal must have been present in the dark compartment. Altogether, the reaction of 14 animals to the sound cue 5000 Hz or 2400 Hz during OTTAT testing session was observed. We found no difference in number of rats with "rapid escape" to the light compartment following 5000 Hz sound cue administration (42,9 %) in comparison to the control group (2400 Hz) (42,9 %). Results showing number of rats with "rapid escape" as well as other obtained responses for both sound cues are illustrated in Graph 7.



Graph 7 | Reactions of rats to sound frequency of 5000 Hz in comparison to frequency of 2400 Hz (control group) during testing session of OTTAT. The frequency 2400 Hz was used during pairing session for both groups of animals. In case of both sound frequencies, 3 rats (42.9 %) escaped in accordance with "rapid escape" criterion (transfer to the light compartment within 2 seconds of acoustic cue termination).

9 DISCUSSION

In our laboratory, we recently developed OTTAT, a novel episodic-like memory task in rats to study associations of temporally distinct stimuli presented only once (Radostova et al., unpublished).

To further improve protocol of OTTAT and enhance the reliability of subsequently obtained results, we examined which of the selected variables promote the establishment of invariable behaviour of animals during habituation to the largest extent. As a selection criterion we sought measures that would lead to highest values of time spent in dark compartment and the least number of transfers between two compartments. Fulfilling these criteria will result in very low possibility of movement of animals to the light compartment due to exploratory behaviour. Thus, transfer to the light compartment will be limited to avoidance behaviour in response to foot-shock (pairing session) and acoustic stimulus (testing session).

Based on our observations, we consider Wistar rat strain to be the most suitable rat strain for OTTAT procedure. In overall, Wistar rats spent the most time in dark compartment (on average 91.2 % of time during 3rd habituation session), which was significantly more than Long-Evans (74.0 %) and Sprague-Dawley rats (62.1 %). Moreover, Wistar rats showed the lowest amount of transfers between dark and light compartments (on average 6 transfers during 3rd habituation session) which was significantly more than Long-Evans rats. These results are in accordance with previously observed anxiety-like phenotype and emotional reactivity of Wistar rats as they tend to spend more time in closed and dark portions of elevated plus maze and light-dark box than other rat strains (Van Der Staay & Blokland, 1996). It was previously proposed high amount of time spent in the most protected areas of apparatus is reflective of high emotional reactivity (Aulich, 1976). Moreover, it is possible that photosensitivity of Wistar rats contributed to their preference of dark compartment as it was previously observed male Wistar rats display increased anxiety-like behaviour when tested under bright white light (Roman & Arborelius, 2009). The intensity of white light in the light compartment in our apparatus was increased to 1090 lx, almost twice the light intensity used in the study of Roman and Arborelius (2009), possibly causing the exploration of light compartment uncomfortable for Wistar rats. The high number of transfers between compartments and relatively low amount of time spent in dark compartment of Long-Evans rats

across days is in concordance with previous studies reporting high activity, high exploration and low anxiety-like behaviour of Long-Evans rats (Turner & Burne, 2014). Interestingly, Sprague-Dawley rats were, similarly as Wistar rats, previously shown to express high level of anxiety-like behaviour (Rex et al., 2004). Our results are only partly in accordance with this observation. Despite number of transfers between compartments were only slightly above those of Wistar rats, Sprague Dawley rats spent the least amount of time in dark compartment of all three strains during the 3rd habituation day. A possible explanation observation emanates from the finding that Sprague-Dawley rats display wide array of behavioural and physiological intrastrain variability depending on what vendor or breed colony they originate from (Pecoraro et al., 2006). Pecoraro et al. (2006) shown Sprague-Dawley rats obtained from different rat vendors significantly differ in their hypothalamic-pituitary-adrenal (HPA) axis responses to stress. Another study shown high-responder and low-responder Sprague-Dawley rats with distinct HPA axis responses to habituation in circular corridor and elevated plus maze (Dellu et al., 1996). Although we did not assess HPA axis responses of Sprague-Dawley rats to habituation in multi-conditioning shuttle in our experiment, we assume Sprague-Dawley rats used in our experiment might possibly be less responsive to stress than those used in study of Rex et al. (2004).

Next, our results demonstrate habituation with "wide doors" (compartment divider with wider opening) is more beneficial for the establishment of invariable behaviour of rats than habituation with "normal doors". In Wistar rats, wide doors usage resulted in significant increase in time spent in dark compartment (on average 95.9 % compared to 91.2 % of session time for wide and normal, respectively) and in non-significant decrease in number of transfers between compartments (on average 4 transfers compared to 5.8 transfers for wide and normal doors, respectively) during the 3rd habituation session. In previous experiments, we noticed animals often explored areas of light compartment not visible from the dark compartment when standard doors were in place. We assume that the reason for decreased exploration of light compartment when wide doors were present was that they allowed animals to visually check the light compartment without need to physically enter it. This assumption is supported by the observed trend in reduction in number of transfers and the increase in time spent in dark compartment.

To summarize the outcomes of experiment 1 and 2, we consider the combination of Wistar strain and wide door divider to be the most appropriate combination leading to profound establishment of very high preference of dark compartment in rats during habituation. When animals are 'by default' in the dark compartment during all sessions, we can be confident that escape during testing sessions in OTTAT is a deliberate response to sound cue triggered by previous experience (sound followed by a foot shock) and not the result of random exploratory activity. Furthermore, we noticed number of transfers to the light compartment remained low in 10 minutes habituation in testing session in most rats (although this analysis is not a part of this work), therefore we assume majority of rats acquired no contextual conditioning. The lack of contextual association could have been aided by inclusion of three habituation sessions prior to pairing session as habituation to the environment reduces the salience of the context as previously reported by Czerniawski et al., (2011).

Moreover, we show that rats responded in similar manner to both novel and foot-shock-paired sound. We found no difference in frequencies of rats with "rapid escape" following novel (5000 Hz) and shock-paired (2400 Hz, control) sound cue as in both cases 42.9 % of rats transferred to the light compartment within 2 seconds of sound cue termination. This suggests generalization of acoustic signal or lack of discrimination between different acoustic frequencies. However, rats' ability to discriminate acoustic frequencies was demonstrated by Pascoe and Knapp (1985) using a differential fear-conditioning paradigm. Pascoe and Kapp (1985) reported that neurons of central nucleus of amygdala exhibit learning-related changes during fear conditioning which result in selective increases in single unit activity to paired tone which signalled US but not to a different (non-paired) tone that did not signal the US, resulting in selective reaction of animal to the paired tone. In this approach, however, animals heard the non-paired sound before testing session and therefore already knew that this tone, unlike the novel tone in our experiment, does not precede shock administration. There are more possible explanations for the outcomes of our experiment. First, rats successfully acquired sound-shock association but were physiologically unable to distinguish between novel and shock-paired tone frequency. This explanation is, however, unlikely as Wistar rats are sensitive to pure tones of frequency ranging from 250 Hz to 80 kHz (Kelly & Masterton, 1977) with mean

Weber ratio (frequency difference limen/frequency) of 3.06 ± 0.44 % in the frequency range 5-32 kHz (Talwar & Gerstein, 1998). Considering this, the difference of 2.6 kHz in tone-sensitive range of rats should be sufficient to distinguish between two different pure-tone sound cues. Another explanation is the rat successfully forms the association between CS and US, but the memory trace formed during single CS-US pairing is not accurate or detailed enough to distinguish between previously heard and novel sound of chosen characteristics. According to this hypothesis, the rat, being naïve and previously never harmed during habituation sessions, hears an unfamiliar sound but as the rat does not expect danger it pays little attention to it. Suddenly, a painful shock is administered which lasts until the rat transfers to the less favoured light compartment. On the next day, the rat is returned to the multi-conditioning shuttle and an acoustic stimulus is presented after some time. Was it the same tone the rat heard the previous day? Despite differing in frequency, the two sound cues had the same duration, sound intensity (80 dB) and similar tone colour, which could make the distinction between two tones challenging if rat did not pay much attention to the acoustic stimulus played during pairing session. The rat therefore flees to the light compartment "just in case," fearing it would receive foot-shock otherwise. Such generalization of acoustic stimuli does not deter from validity of episodic-like nature of OTTAT, as single-experience episodic memories in humans, for example eyewitness identification and testimony, are often only partly accurate (N. Brewer & Wells, 2006; Kunimoto et al., 2001). Moreover, this explanation would further support the assumption of incidental encoding of tone-shock association. Nonetheless, this hypothesis needs to be further experimentally tested.

In overall, when compared to other episodic-like memory tasks, the newly developed OTTAT represents several important advantages. First, the presence of dichotomous outcome (escape/no escape) is advantageous as it can be used to readily distinguish learners and non-learners. Importantly, all-or-nothing nature of episodic memories is stressed when characteristics of episodic recollection are described (W. F. Brewer & Dupree, 1983; Schacter & Addis, 2007). This is superior in comparison to widely used TFC and CFC paradigms, as the outcome of TFC and CFC is a measurement of time spent freezing (Lugo et al., 2014), NOR task where the outcome is the time spent exploring the novel object (Silvers et al., 2007) or OTPM task, where the outcome is a measure of time spent searching for food (Bast et al.,

2005). Second, the association of CS-US in OTTAT is rapidly established, available after single CS-US paring. In comparison, other tasks which include trace interval between CS and US require multiple presentations of CS-US pairings (e.g. more than 100 CS-US pairings in TEC and usually 5 pairings in TFC), which is not descriptive of naturally occurring situations and increases the probability of rule-learning in animals. Third, OTTAT procedure requires no pretraining or shaping of animals, as is required in OTPM task, DAID imitation task or OLSL task (for detailed procedures see Chapter 4). Fourth, the encoding of trace association is incidental as both sound cue and foot shock were administered unexpectedly, only once and without previous warning. Fifth, the recall of previously experienced event is induced unexpectedly and in unprepared animal since the experimental question is asked only once (i.e. only one testing session is conducted). Sixth, one-trial nature of this task is advantageous since it is, in fact, the most natural way to test the formation of temporal associations in a framework of declarative episodic memory as outside of experimental conditions. In nature, episodic events are reconstructed from components presented only once. Seventh, as both slow and rapid escape can be considered [7]learners, the combined rate of all animals which escaped ('slow' + 'rapid' escapes; on average 73 %) during testing session is similar to success rates in human episodic-memory tests. For example, Cheke and Clayton (2013) reported success rate of 73 % in human analogues of What-Where-When task and success rate of 61 % in Unexpected question task.

Regarding the limitations of this study, the main limitation of OTTAT task is, similarly as in all learning tasks, that it currently does not allow us to distinguish animals who did not escape during testing sessions because they simply did not learned the association from those who learnt the association but did not escape.

In future, we intend to explore the neurobiological substrate of OTTAT by examining the involvement of hippocampus. Since presence of trace interval is associated with the recruitment of hippocampus in procedurally similar TFC (McEchron et al., 1998), we speculate the presence of trace interval is connected to the recruitment of hippocampus in OTTAT as well. This would, in fact, greatly

[7] Traditionally, avoidance have been defined as CS-elicited 'flight' response to imminent danger (Bolles, 1970). On the other hand, some argue avoidance have more complex form depending on the context and could resemble an 'active wandering' (= 'slow-escape' in our study) in some animals (LeDoux et al., 2017). Arguably, both groups can be therefore considered as learners of CS-US association.

support the constructive validity of OTTAT as a model of episodic-like memory since hippocampus is critical for establishment of episodic memories in human (Kramer et al., 2007). Next, should we confirm the involvement of hippocampus in OTTAT, we would determine the involvement of hippocampal subregions during successful memory retrieval following sound cue presentation in trial session to closely determine the role of individual hippocampal subregions in retrieval of trace associations. Additionally, we intend to more broadly address the accuracy of CS-US associations by assessing the reaction of rats to sound cues of differing characteristics, such sounds of much higher frequency than used here (e.g. 10 kHz), lower sound intensity, white noise, variable sound length or pulsating sounds. Moreover, in future we would like to address one of the limitations mentioned above – that is, our inability to differentiate which of rat who did not escape to the light compartment upon hearing shock-paired sound actually did not learn the association. We plan to use heart-rate changes (measured by radio-telemetry) and freeze responses as indicators of learning as we hypothesize non-learners would display no changes in heart rate and no freezing following sound cue administration. However, animals in our task never display freezing behaviour in testing session (visual observation). In follow up, we plan to measure corticosterone level in animals following their performance in testing session. Even though only mild shock is used during pairing session, we speculate some animals could have been highly stressed by this experience. It has been proposed stress can cause shift away from more controlled processing dependent on hippocampus towards more reflexive and cognitively less demanding amygdala-based processing (van Marle et al., 2009). Such shift might be mediated by activation of mineralocorticoid receptor by elevated levels of cortisol during stress event (Schwabe et al., 2013; Vogel et al., 2015). The shift from hippocampal to amygdala-based learning might be more prominent in individuals more sensitive to cortisol (Vogel et al., 2015) and hence could explain the behaviour of animals who did not escape following sound cue yet remember the association.

10 CONCLUSIONS

Animal models represent an important component of episodic-like memory research. Yet, majority of currently available episodic-like memory tasks do not reflect episodic-like memory or encompass important methodological caveats. This thesis deals with the improvement of the newly devised OTTAT, a one-trial episodic-like memory task in rodents. To improve reliability and replicability of results obtained from OTAT, we sought measures most optimally promoting the establishment of invariable behaviour of rats during OTTAT. Additionally, we explored specificity of conditioned response ("rapid escape") to the sound cue frequency previously paired with foot-shock administration in OTTAT.

Based on our findings we conclude following:

- Wistar albino rats are the most optimal rat strain for use in OTTAT since they display the fewest transfers between compartments and spend the most time in dark compartment during last habituation session.
- The habituation of Wistar rats with wide doors produce reduction in number of transfers and increase in time spent in dark compartment in comparison to Wistar rats habituated with normal doors.
- The combination of Wistar rats and wide doors is the most suitable combination of OTTAT, most effectively leading to the establishment of invariable behaviour of rats in OTTAT.
- During memory recollection in OTTAT, rats are not able to distinguish between novel and foot-shock-paired acoustic stimulus of the same duration and intensity differing by 2.6 kHz.

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