Behavioral Methods to Study Learning and Memory in Rats

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(v21 - may 24, 2006)

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1. INTRODUCTION

"The only proof of there being retention is that recall actually takes place"
William James, 1872

This century-old observation is still valid today, despite everything we have learned about the mammal nervous system, specially in the area of neurobiology of learning and memory. After "training" an experimental animal, such as a rat or a mouse, the only way to be sure that a "memory" was formed is by evoking it back, i.e., by recalling it in a "test" session: this "memory" is expressed by a behavior that differs from that one emitted in the training session. Until proof to the contrary, the best explanation for this *new response to the same context* is that some kind of internal "record" mediates it in the animal: this is what we call "memory". Everything else is consequence: if recalling depends upon the established memory trace intensity, it will be a function of the experience intensity during the acquisition - "training" - session, and so on.

"Memory" is a slipery concept because we still have not produced a complete, consensual notion about the physical nature of its trace. Notwithstanding the fact that we may be getting closer to this aim, the only sure way to *grab at* such phenomenon is *by measuring behaviors and their modifications*, i.e., quantifying it in an *indirect* fashion. Such approach is called "phenomenological", and opposes itself to the so-called "mechanistic" vision, that departs from previously existent knowledge about the intrinsic machinery operating behind the phenomenon. We all know that modern science is the business of examining - and refuting, if unfit - the best mechanistic explanations for nature facts, but Neurobiology of Learning and Memory is one of those frontier areas where complexity forces us to begin with phenomenological description and gradually move to mechanistic explanation. This chapter aims to briefly describe the behavioral approach to the study of learning and memory.

2. TAXONOMY OF MEMORY TYPES

"What need the bridge much broader than the flood?"
William Shakespeare
'Much Ado About Nothing', 1599

Memories can be classified according to many different criteria: function (e.g., Working vs. Reference M.), content (e.g., Declarative/Explicit vs. Procedural/Implicit M. – see Squire & Cohen, 1984), duration (e.g., Immediate or Short-Term vs. Long-Term or Remote M.), nature (Associative vs. Nonassociative M.), or motivation (Appetitive/Reward vs. Aversive M.). In this chapter we will sketch a simplified taxonomy of memory types, gathering most of the above categories in one classificatory tree.

Presently there is great conceptual diversity in this area and the need for such conceptual organization is a matter of debate among memory specialists, but we believe it still has a role, at least for educational reasons. Figure 1 synthesizes our classificatory attempt, with some illustrative examples of behavioral tasks (described below). This taxonomy sketch does not try to suggest the existence of a fundamental organization of the natural world that is more "rigid" than the different, overlapping experimental approaches employed by different labs around the world.

Notice that Figure 1 does not make reference to time; despite being obviously applicable to long-term memories (LTM), it also subserves short-term (STM) and even working memories (WM). In fact,

Working Memory differs a lot from STM and LTM due to its essentially "executive" function, since it basically stores information for a very short period of time (seconds to minutes) in order to compare it with previous records, and, based upon this, decide which behavior to express; it is also distinguishable from other types because it leaves no long-lasting record of any kind, possible relying upon reverberating electrical activity, and mainly settled in the prefrontal cortex (see Baddeley, 1997 and Goldman-Rakic et al., 1996). To know more about these types of memory, see, e.g., Izquierdo (2002).

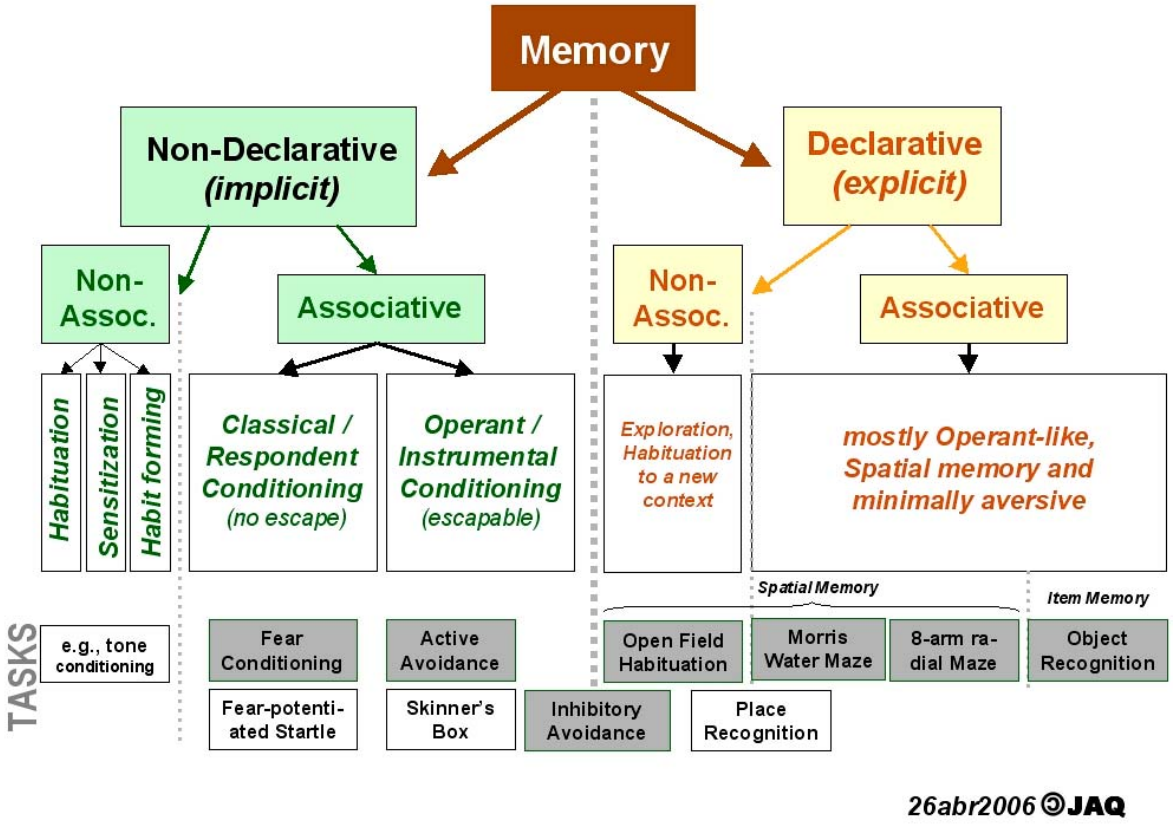

Figure 1 – A possible Taxonomy for Memory types and some Behavioral Tasks that may have access to them. Please, DO NOT USE THIS TABLE WITHOUT READING THE COMPANION TEXT BELOW, since those tasks only serve as example according to the specific protocol employed (as described in section 4).

Some concepts in Figure 1 deserve some further explanation:

1st Level – Declarative vs. Procedural: Most of these memory classes are derived from human experience, with obvious difficulties in being extended to animal models [1]. *Declarative* M., for instance, concerns with facts / knowledge ("semantic" M.) or events ("episodic" M.), while *Nondeclarative* M., previously known as *Procedural* M., refers to motor or sensory abilities, or "habits". Human examples of both classes include reminding dates, names, faces or places, as well as autobiographic episodes (Declarative M.), and driving a bicycle, butterfly swimming, declaiming the multiplication table or spelling a word (Nondeclarative M.). To avoid anthropomorphization problems, it has been suggested to rename these types of memory, respectively, as *Explicit* and *Implicit* M. (Schacter, 1987), terminology that we will use here.

[1] By the other hand, when interpreting behavioral results we must avoid to *anthropomorphize* them; remember that human memory has at least two very different, "nontranslatable" aspects (to animal models), symbolic language and conscience.

Implicit M., due to its robustness, is a category in itself: as soon as it is established, it (a) tends to last for the whole life, (b) are difficult be extinguished, and (c) are less vulnerable to emotional modulation (Izquierdo, 2002). Compared to it, Explicit M. may be (a') short or long-lasting [2], and (b') undergo a complex *consolidation* process, involving different receptors, enzimes, signalling cascades and brain structures (see Izquierdo et al., 1998, 1999, 2002; Izquierdo, 2002). Before consolidation, during at least some hours, memory traces are *labile / unstable* [3], and may suffer weakening or reinforcing disturbances (by head trauma, drugs, different situations, etc) that modify the original record in the first hours after acquisition (e.g., McGaugh, 1966, 2000; Izquierdo, 2002). Short-term M. (STM) last a few hours, and usually express itself *inside* the time window necessary for the Long-term M. (LTM) consolidation. STM is not the "initial" phase of LTM, and these two processes take place in parallel and are quite independent one from the other, sharing the same neuroanatomical substrates but with different subcellular, neurochemical and/or electrophysiological mechanisms (Izquierdo et al., 1998, 1999, 2002). The loose of explicit M. is usually denominated *amnesia*.

2nd Level – Associative v.s Nonassociative: behavioral tasks that promote associations between stimuli and responses, or between two stimuli, are known as *Associative*. Through them animals learn how to predict future events in order to express a proper, anticipatory behavior. The two main categories of non-declarative (implicit) associative M. are (a) *Classical* o*r Respondent* (*Pavlovian*) *Conditioning* and (b) *Instrumental* or *Operant Conditioning*.In the first kind, the contingencies between stimuli and responses are arranjed and controlled only by the experimenter (Pavlov, 1927), and the associative experience is somewhat unavoidable from the animal's point of view. In the second type, the environment is arranjed in order to permit that certain response from the animal be necessary in order to obtain some result, such as avoid a painful stimulus or receive food (Skinner, 1937), i.e., the "escape" or avoidance is an option available to an animal that could learn and perform it. Skinner himself coined the expressions *elicited behavior* to describe respondent conditioning, and *emitted behavior* to describe operant conditioning. Thus, in the classical Pavlovian example, a dog was trained to associate an initially neutral stimulus, such as a bell (that will be the *conditioned stimulus*, CS), to a *unconditioned response* (UR), such as salivation (concomitantly provoked by a US - *unconditioned stimulus* such as showing a juicy beef), and obtain, in the end, a *conditioned response* (CR), i.e., "salivate to the bell", a nonnatural response not previously recorded. Both experimental frameworks have a decisive role in the history of behavioral psychobiology, but intrumental conditioning is more flexible, general and spontaneous than respondent conditioning, once this last one is based upon a limited set of innate responses natural to the animal (Sanger & Blackman, 1989; Beninger, 1989; for a careful characterization of the typical tasks in each of these categories see Hölscher & O'Hara, 1997).

3rd Level – Other categories: Subdivisions of the previous level may include the two types of associative conditionings described above, the dichotomy between aversive (punishing) and appetitive (reward) behaviors, the stronger or weaker spatial nature of the task, or its motivational "drive" (e.g., reactive vs. exploratory vs. decision taking tasks).

This classification is as inaccurate as any classification built for didatic reasons, but our intention was to favour a field vision about which (and why) behavioral methodologies may be used to study learning and, in particular, memory. We have previously mentioned that active researchers may not agree, in different degrees, with this classificatory attempt. The existence of so many and slightly different behavioral tasks in the psychobiological litterature is due to the fact that every author approaches a limited set of problems and try to adapt the available tools to them. It is the old tenet that says that "there is no methodology without a theory behind". In the limit, there would be no possibility of classification ever since every experiment would imply in a particular classification, essentially nontranslatable to other situations.

As an additional disclaimer, we should point two problems: first, the fact that the tasks described ahead as "typical" instances in each category may be somewhat deceiving, and second, the frustrating fact that the "frontiers" drawn to divide categories are not clearcut and rigid.

The tasks described as examples in each category must not be taken as sole, exclusive instances of that memory type, since slight protocol changes may be enough for the task to be used for other finalities, moving it to another category. Some of these protocol modifications will be described for each task, but we may mention two cases: slight adaptations in the *8-Arm Radial Maze* protocol may turn this

[2] Some authors mention another type of long-lasting memory, dubbed *Remote* M., that may endure several decades in humans but still is not well characterized .

[3] Hence, the term "consolidation", created by Miller and Pilzecker in 1900 (McGaugh, 2000).

explicit/associative/spatial/decision-taking task into an implicit/associative/habit-forming one (see Packard, Hirsh &White, 1989); *Inhibitory Avoidance*, not a pure "spatial" task itself, may be easily adapted to study some forms of spatial memory (e.g., Cimadella et al., 2000). Since there are so many possible variations it is hard to follow all of them in the scientific litterature, **although** there is a positive side on this: there are broad margins of manoeuvre for creating new task variations, and even yourself may advance new suggestions (its methodological validation will just depend upon ingeniousness and determination).

The second problem is a consequence of the logical limitations of the suggested classification, unable to bear the whole richness of possibilities inherent to real-life situations. For instance, *inhibitory avoidance* classification (according to the protocol described in Section 4) faces lots of controversy (see, e.g., Xavier, 1982), as illustrated by its position in the diagram of Figure 1, irresolute between the implicit/associative/instrumental and explicit/nonassociative/exploratory classes (this task probably contains something of each type in its nature).

To favor intelligibility, Figure 1 omits several important subclasses of memory types and behavioral tasks, as, for instance, *Imprinting* - a type of nondeclarative/nonassociative learning characteristic of birds, and *Priming* - a clue-evoked nondeclarative/associative learning.

With the cumulative neurobiological knowledge, some of us feel tempted to substitute much of these inaccurate characterizations for new ones focused, for instance, in the neuroanatomical bases of each behavioral task, or the involved neurotransmitter(s)/modulator(s), or the electrophysiological patterns exhibited, etc. Despite being possible, this may be somewhat hasten, and be no more useful than the previously existing classifications.

3. THE LOGIC OF EXPERIMENTAL DESIGN

"The purpose of models is not to fit the data but to sharpen the questions"
Samuel Karlin

3.1. MEMORY PHASES

The Memory Consolidation Theory, proposed by Müller and Pilzecker in 1900, is a fundamental paradigm in the psychobiology and neuropharmacology of memory (McGaugh, 2000; Dudai, 2000). One consequence of the *Consolidation Paradigm* is that we can plan our experimental intervention to take place in two or three different moments around memory *Formation* and *Recall*. Since the formation is not an instantaneous process, we may distinguish two formation phases, *Acquisition,* better known as *learning*, and *Consolidation*, the labile phase during which the memory trace will be physically stored; *Recall*, also known as memory *retrieval, elicitation* or *expression*, takes place during the reexposition to the learning context, with or without previously delivered stimuli, and, as we have said before, is the only way to prove that a memory was really formed and stored.

Figure 2 – Phases of Memory Formation (acquisition / consolidation) and Retrieval (recall)

Acquisition can take place in one or several "learning" trials, when the animals are exposed to a context with several controlled variables; immediately after this, and during some hours, the memory trace will undertake a labile phase when the animal is susceptible to disturbances that may modify the original memory record (e.g., McGaugh, 1966, 2000; Izquierdo et al., 1989, 1999, 2002; Dudai, 2000).

Recall test may be done with different training-test intervals, and LTM is agreed to take place after a minimum period of, say, 6 hours; usually it is performed with a 24h interval, but larger time spans are

possible, according to the memory attributes under scrutiny (memory intensity, forgetting, extinction, reconsolidation), going from several days, to months and even years. In rats this interval is limited by its average lifetime of 24-48 months (an 18 month old rat may be considered senile, and is a useful model of memory senescence – Krinke, 2000). Studying recall with just a few hours training-test interval is considered to be a STM (Izquierdo et al., 1998) and if we reduce the interval to some minutes (say, 1-3min) we may be dealing with *Working* M., at least according to some authors (Barros et al., 2002).

A consolidated M. may also suffer new modifications, for instance, be weakened by the repetition of the training context *without* the aversive US (e.g., a shock) – an *extinction* -, or, when this US *is being repeated*, the memory may be strenghtened – a *reinforcement* (Izquierdo, 2002). Some authors suggest that the already consolidated memory trace may be already become labile and, after that, be *reconsolidated* with different information (Dudai, 2000; Frenkel et al., 2005; Bustos et al., 2006).

According to this sequence of events, any experimental intervention that have consequences upon memory processing (e.g., a pharmacological treatment – the preferred procedure explored in this chapter from now on) depends on *when* it is applied:

Pretraining: any intervention done may affect both acquisition and/or consolidation; if training-test interval is too short, it may affect recall also (this is less probable with a typical 24h interval, but pay attention to the few, very slowly metabolizing drugs – such as benzodiazepines – that may still be active after this period);

Posttraining: since acquisition already took place, only consolidation may be affected [4] (see above); as said above, recall is hard to be affected if training-test interval is large (e.g., 24h); different times after-training may give access to different stages of the neurochemical/physiological processes behind consolidation (e.g, Quillfeldt et al., 1996; Izquierdo et al., 1997); since some drugs take some time to act, they may not be suited for posttraining treatment, and pretraining administration, despite its flaws, will remain as the only option if you are looking for an actual "immediate" posttraining action: the "price" to pay is a lot of additional, complementary experiments in the case of finding an affect;

Pretest: intervention only affects recall simply because time only moves forward (e.g., Jerusalinsky et al., 1994; Izquierdo et al., 1997); also, depending on the time between treatment and the test session, some procedures may not work well (some may be done a few minutes before, others should be applied 20-30min or more before).

3.2. CONTROLS

Any scientific experiment aims to test for some hypotheses, and in order to make this possible, two conditions must be fulfilled first: (a) there must be *control experimental groups*, and (b) experimental setup must be made *simple and invariant* during multiple sessions/assays of the complete experiment.

The only way to be sure that a drug (or treatment) was *the real cause* of some observed effect (as seen in the *treated* group) is by repeating the exact same experimental procedure just without the main substance (or intervention) under study (the *control* group); in a behavioral pharmacology experiment with a drug, control groups must receive the administration of the exact same amount of the drug's vehicle (be it a buffered solution, be it saline solution [5]), under the same protocol (time of infusion, etc). Some procedures / treatments, such as surgical procedures, may be controlled by a *sham group* in which the whole procedure is repeated – anaesthesia included - except for the specific, last step under study (e.g., a surgical removal of a

[4] This treatment must be applied in the first few minutes, preferrably in less that one minute, in order to still act upon its targets while possible.

[5] In intraparenchimal (intracerebral injections) and intracerebroventricular administrations, special attention must be paid to the vehicle's chemical characteristics in order to assure it is as functionally "neutral" as possible: phosphate buffered saline solution (PBS - a buffered isosmotic 0,9g% NaCl solution) of pH 7.4 are strongly recommended. If the drug is somewhat lipophylic, trouble may be avoided if the substance is first dissolved in a hydrophobic medium such as ethanol or DMSO, and then suspended in PBS to a reasonable percent: control groups in this case must be exactly like the drug's solvent, just without the drug. Systemic administration (endovenous, intraperitoneal, intramuscular or intradermic) must at least avoid osmotic effects, i.e., plain, distilled water is *never* an acceptable vehicle!

brain structure, a blood vessel clamping, etc). The comparison among the performances of treated and control groups will allow us to decide if there is an effect or not.

Since both control and treated groups involve several animals, *statistical tools* are always necessary in order to analyse these data [6]. In behavioral experiments the N per group should never be smaller than 6-8, and, according to the nature / difficulties of the treatment and/or the task, this may extent to 25-30 animals per group.

Simplification of the experimental context is the second necessary condition once behavior is already a complex enough variable to analyse: if one leaves these additional variables free to change, how could the results be interpreted ? For example, it would be really hard to interpret a behavioral experiment made in different times of the day, temperatures, stimuli intensity, etc: most, if not all basic variables must be made *as constant as possible* to warrant a nice experimental design.

Drug administrations can be designed to access both *acute* (one administration) or *chronic effect*, but be aware that some acute treatments may result in chonic changes (e.g., as in the pilocarpine model of temporal lobe epilepsia or the MPTP model of Parkisonism). It is always elegant to determine the *range of effectiveness* of a drug (or treatment): an assay with several different doses allow the preparation of a *dose-response curve* [7] (some treatments may cause responses arranjed in degrees according to its intensity, and an *intensity-response* curve is desirable). In terms of pharmacological studies,choosing the best possible dose [8] is an art to be mastered by practice, but there are no general rules out of the do-it-and-check-for-yourself [9]. This curve is not necessary, but still recommended, if it was previously published (specially if published by your own group), and it is a good support to discuss the observed (or not observed) effects in terms of drug specificity, selectivity, competitivity, and so on. Finding effective ranges different from the literature does not necessarily mean your data are wrong, since animals, even from the same strain, may be quite different in varied lab conditions.

Finally, a nice additional check may be performed in the case of pretest treatments. Since most drugs diffuse and/or metabolize after some time, animals can be *retested*, say, 90-120min after the original test session: if an observed pretest effect disappears, it may have been caused specifically by the drug; if it doesn't, the "effect" may have other cause(s).

3.3. SEPARATING MEMORY FROM THE REST

Depending on the treatment and on the type of behavioral task employed, the observed behavioral change could be interpreted otherwise. For instance, in the inhibitory avoidance task, a "good" memory is shown by a larger latency to step down from a platform into a previously electrified bronze-bars grid (see section 5), and any drug (or treatment) that changes this latency could be taken as amnestic (if decreasing latency) or facilitatory (if increasing latency). In order to prove the effect as genuinely mnemonic, one must be sure that that drug, in that dose (or that treatment, in that intensity) causes no such behavioral change by itself, e.g., affecting motor performance. This test must be performed in the appropriate behavioral tasks, according to the behavior one wants to check for, such as, for instance, a free exploration box (an open field would do the job) for gross motor effects, an elevated plus maze for anxiety effects, or a discrimination task for sensory effects. This must be considered a third control "layer" for behavioral experiments, after the fundamental control (vehicle-injected) groups and the simplicity/constancy of the experimental setup, as described in the previous section.

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[6] On this subject, we recommend two introductory books, Norman and Streiner (1994) and Callegari-Jacques (2003) – this last, in portuguese; for advanced information, one of the best manuals in biostatistical analysis is Zar (1999). Finally, since nonparametric statistics is frequently necessary to analyse behavioral data, one excellent reference is Siegel and Castellan (1988).

[7] This must be done both for systemic and/or intraparenchimal injections, and every targeted structure into the brain may display its own dose-response curve due to histological particularities.

[8] Even when this (choosen) dose is known, it is recommended to produce a dose-response curve centered in this value.

[9] Intracerebral doses may sometimes be defined taking concentrations effective in *in vitro* experiments and administering a volume containing the substance in a 10 to 20 times larger concentration: this supposes a reasonable diffusion volume in the quite compact brain parenchima (but this may vary in different regions, in the presence of nerve fibers, etc).

Since the burden of the proof is ours, we must provide results of other behavioral tasks done in parallel with memory tasks themselves in order to eliminate the possibility of a false positive (or false negative). Drugs (or other treatments) under study could always be acting upon unanticipated neural substrates that cause observed behaviour that, if unnoticed, may "mask" for a memory effect. Hormonal state, for instance, may affect one or several of these behavioral manifestations, so it must be controlled by additional serum and/or tissue measurements (regular changes resulting from the estrous cycle phase in females are easy to check, for instance).

The list of possible false positive/negative factors is usually finite and not too large, and may include one of those shown in figure 3.

Non-mnemonic Factors	Typical Behavioral Task / measured parameters
Motor performance	Deambulation characteristics in an OF (open field) Rotarod test Pole or Chord climbing
Anxiety	Elevated Plus-maze Light-dark transition task Time in central region of the maze vs. tygmotaxia
Pain sensitivity	Tail flick test Hot Plate and/or Paw Pressure test
Sensory perception	Sensory discrimination tasks
Attention	Discrimination/reaction tasks
Arousal level	

Table I – Possible factors to be excluded by complementary behavioral tasks.

If a certain drug (or treatment) have an effect upon the memory task *and* upon one or more of the non-mnemonic tasks, it is *not* recommended to advance a strong interpretation based on mnemonic mechanisms... A rule of thumb: **the "cleaner" the results, the easier to interpret them**. But do not forget that neither mammal nervous systems are simple structures, nor behaviors show always a clearcut, straightforward causation. It may be the case that a drug (or treatment) affects both memory neural substrates *and* other, non-mnemonic mechanisms, and we may not be able to separate them just on a base of behavioral tasks, being also necessary to collect a broad range of additional data (neurochemical, histological, electrophysiological, etc), and, as usual, to lay hold of great creativity to solve the puzzle. It is never simpler than that.

Summarizing the experimental controls studied in sections 3.2 and 3.3, we may take three control "layers" into account in order to perform a good experimental design:

(1) **Treatment-specific controls** - vehicle, sham, etc vs. treated groups; retest;
(2) **Reproducibility controls -** simple and invariant experimental setup;
(3) **Behavior-specific controls -** additional non-mnemonic behavioral control tasks.

3.4. McGAUGH'S PARADIGM OF POSTTRAINING PROTOCOL

"(...) Consequently the posttrial injection studies are difficult
to interpret in terms of motivational of perceptual effects"
James L. McGaugh (1966)

In the past, researchers in the area of neuropharmacology of learning and memory have been strongly criticized by other specialists based on the above-mentioned difficulties in proving that a (supposed) mnemonic phenomenon was, in fact, *mnemonic*. Even well-designed experiments with adequate controls, invariant setup, and complementary non-mnemonic behavioral tasks, could not overcome a final, quite philosophical objection probably inspired by the belief that complex brain functions (such as memory, attention and anxiety) may not be separable at last. This objection, however, was eliminated by a seminal conceptual observation first advanced by James L. McGaugh in 1966 (reproduced above): since posttraining treatments do not affect acquisition (i.e., learning) - just consolidation - at least those experimental designs may be considered totally *clean* in terms of non-mnemonic "contaminating" para-effects, and any observed effect can be clearly taken as an effect upon memory *consolidation*. This seems so obvious for us now, but we may remember that at that time, forty years ago, most of the learning and memory studies were performed - by "default" - with pretraining treatments. Science advancement comes usually with simple yet powerful ideas.

This does not mean that we cannot study memory under pretraining treatments. Since they are less cleaner, they demand additional behavioral tests in the case of an effect being detected.

Finally, recall studies may be considered even "cleaner" than posttraining ones, since they only may affect the memory retrieval process. This completely separates it from any consolidational process, and there are extensive work done showing that these two phases of memory differ in some respects (Izquierdo, 2002).

3.5. POSSIBLE EXPERIMENTAL INTERVENTIONS

Several *in vivo* treatments / interventions, of different categories, are possible:

(1) Reversible Chemical / Cryolesions or Local, Selective, Reversible Pharmacological Actions;
(2) Non-lesioning electrostimulation;
(3) Behavioral manipulation (task, immobilization, stress, fear, defense, etc);
(4) Mechanical (surgical), Electrical or Chemical Irreversible Lesions;
(5) Transgenic Animal Models;
(6) Concomitant Electrophysiological / Imaging recording;

Interventions 1 and 2, usually delivered in an acute fashion, since being short-lasting, less invasive and even reversible, are suited for application in any of the three *moments* above (pretraining, posttraining and pretest).

Intervention 3 may be suited for all moments if they it is acutely applied; chronic procedures, since they take a long time to be completed (e.g., chronic stress by restraint), should be mostly pretraining.

Interventions 4 and 5, since they tend to be irreversible, are more suited for pretraining manipulation protocols with its inherent limitations. Slowly diffusing or late acting drugs are also not adequate for the so-called *immediately posttraining* [10] treatment, and must be applied before the training session, fostering the need for the non-mnemonic behavioral tasks (mentioned in section 3.3 above) in order to help the interpretation of the experimental data

Intervention 6 is mentioned only to emphasize that most *measurements* are always somewhat invasive, and tend to interfere with normal behavior. By the other hand, be aware that concomitant recordings may only come in support to *correlations*, not *causations*.

[10] By *immediately posttraining* we usually understand a treatment delivered (such as drug infused) in less than 1min; despite the resistance of some authors, since this procedure *starts to take place* well between 0 and 59sec (and frequently ends in less than 2-3 min), we may term it as a "0 min" treatment.

Null-mutated *transgenetic* (knockout) animal models demands such a great amount of time to be developed that can only be studied under the pretraining protocol, with all the necessary additional non-mnemonic behavioral tasks and other assays in order to interpret its data. In the case of the fashionable, ultrareductionistic transgenetic models, except for some particularly well-designed cases (as in Tsien et al., 1996), this intrinsic experimental limitation for behavioral studies seems not to be the only burden, because they frequently loose track of most of the possible modified parameters with consequent impact in its reproducibility (see, e.g., Routtenberg, 1996).

3.6. A CLOSER LOOK INTO THE REAL EFFECTS: STATE DEPENDENCY

Difficulties in the interpretation of results may arise not only from possible non-mnemonic effects, but also from the (otherwise) plain fact that in several common experimental protocols, animals are *trained in one state* – say, under the action of a drug, or in a certain hormonal state – and *tested in another state* – for instance, without the drug or not in that hormonal state. The difference in the performance between the training and the test sessions (be it amnestic or facilitatory, it doesn't matter) could be attributed to the simple fact that each session was done with the animals' brain in a different neurochemical/neurohumoral state! Specially *when the same response is observed with the same state* being promoted in the training *and* in the test session: in this case we have a phenomenon called *State Dependency*

State dependency can be promoted by the exogenous administration of drugs, or by the stimulus to generate a certain internal neurochemical/neurohumoral state, situation which is called as *endogenous state dependency*. When this type of phenomenon is proved, the interpretation of the effects as purely mnemonic *may* loose strenght, but some authors sustain that, if there are no non-mnemonic effects, it could still be the case that memory mechanisms are being affected because the engram could bear an additional "tag" that records for the concomitant neurochemical/neurohumoral state in which the memory trace was formed (Izquierdo, 1984). Figure 4 below illustrates the the classical experimental design necessary to disclose state-dependent learning.

STATE-DEPENDENT LEARNING		TRAINING	
		No drug	Drug
TEST	No drug	Good recall	*Less-effective recall*
	Drug	*Less-effective recall*	Good recall

Table II –Experimental design to prove State Dependent Learning – in this case, an instance of memory facilitation (adapted from Meyer & Quenzer, 2004)

3.7. WHAT ARE WE OBSERVING?

The effects of the intervention, in any of the above-mentioned *three moments* – pretraining, posttraining or pretest -, may produce only three kinds of results concerned with *memory* [11]:

(1) *amnesia*, i.e., memory reduction or blocking /deficit;

[11] Supposing we are sure they are specifically mnemonic effects, and not motor, sensory, attentional or emotional *memory-masking* effects (see item 3.3).

(2) *facilitation*, i.e., memory improvement;
(3) *no measurable effect*.

Responses (1) and (2) may admit *degrees of intensity*, usually being more robust /marked when stimuli (both appetitive or aversive) are more intense (*one-trial* training) or repeatedly presented (*multi-trial*, repetitive training). The observed modifications / effects in responses (1) or (2) may also be detected in different temporal directions in relation to memory:

(a) *Retrograde* effect – acts upon recently-formed memories;
(b) *Anterograde* effect – acts upon new memories still to be formed, after the treatment;
(c) *Ambigrade* effect – when it acts in both directions.

Result (3) deserve some additional comments, rarely mentioned in typical course books. First, the absence of an effect is commonly interpreted as a frustrating "no result at all". This, however, is a mistake, since in a well-designed and executed experiment, finding no effect at all *is, also, a result*! As a scientific piece of evidence, it may be as - or even more - important than finding an amnestic or facilitatory effect, depending on the hypotheses under scrutiny. It may, anyway, help to prove (or disprove) the hypotheses. Second, even if an expected result is not attained, it still remains valid the old motto that says that "absence of evidence is not proof of absence", and we may still keep looking for new evidence under more refined/modified versions of the same or different experimental designs.

It is quite healthy to remember that nature tends to be much more complex than our limited, reductionist empirical investigation models. When we fail, it is always our move next.

3.8. THE METHODOLOGICAL TRIANGLE AND OTHER LIMITS

Another fundamental aspect is that there are always limits to what can be extracted as *conclusions* from one experiment, notwithstanding the fact that sometimes in history there are some key experiments that really solve questions of great conceptual importance (and who doesn't want to really *do* one of these few!). Of course, most of what is done in regular science (our work usually included) belongs to the first category – something Thomas Kuhn called "ordinary science" in its classic book, "The Structure of Scientific revolutions".

Specifically when doing behavioral neuropharmacology experiments such as those described in this chapter, we must impose clear limits to the reaching of the derived conclusions. Although there can be several limiting factors, we cannot ever escape from three (illustrated in Figure 5, below):

(a) the specific **CNS structure** being targeted (delimited, when accessed through stereotaxically-implanted canullae; or more wide-embracing, with intracerebroventrocular or systemic treatments);

(b) the specific **neurochemical/neurohumoral system** being targeted (according to the employed drugs, their selectivity, doses, administration pathway, etc);

(c) the specific **behavioral neural substrate** / **type of memory** being targeted (according to the specific behavioral task employed – the reason for this is evident in Figure 1, that shows different types of memory that actually rely upon different neuroanatomical/neurochemical substrates).

So, observing an effect in one behavioral task of a specific drug applied into certain brain structure does not warrant that other behavioral tasks would be equally affected, or that other neurochemical / neurohumoral target would cause the same effects, or that the same would be observed targeting other brain structures. For instance, it is common that drugs effective upon aversive tasks (such as, inhibitory avoidance) show no effect at all upon non-aversive, exploratory tasks (such as open field habituation), and drugs causing some effect when administered into the hippocampus not necessarily cause the same effect into the amygdala (they may actually differ in a radical way).

By the other hand, more diffused, wide-embracing treatments such as, for instance, systemic drug infusion, may be harder to interpret. Since this treatment may simultaneously target very different CNS

structures, it is quite difficult to identify the main neural substrate upon which it would be acting upon, even in the case that *no effect* is detected (brain targets that individually respond in *opposite* fashion may be neutralizing each other's action). This is why systemic treatments, although useful in providing valuable, preliminary information concerning memory, has limited reaching in terms of memory mechanisms identification. Notice that this comment is valid only in relation to the study of the neural substrates of memory, and does not mean any criticism to other systemic treatment models.

The situation with systemic treatments may get even more complicated when we use a drug that *crosses* the blood-brain barrier (BBB), that may target not only central structures but also peripheral neural substrates. One example of systemic treatment in which these peripheral effects can be simultaneously blocked by another, non-BBB-crossing drug acting upon the same target-receptors, is the pilocarpine epilepsy-inducing treatment, where methyl-scopolamine is (previously) infused in order to block undesired peripheral effects (Cavalheiro et al.,1991). Of course, drugs that do not cross the BBB are useful to investigate peripheral neural effects without any central action.

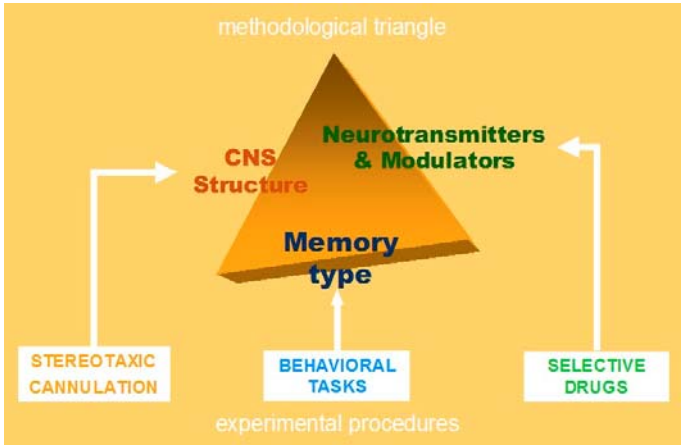

Figure 3 – The Triangle of Methodological Limitations of the Neuropsychopharmacology of Learning and Memory: each one of the three dimensions demands a specific experimental approach. Conclusions must not extrapolate these limits.

One last limitation affects how scientific conclusions obtained from experimental animals can be extrapolated to human clinical cases. Laboratory animals allow us to perform experimental manoeuvres that (a) would never be possible in humans, and, beyond that, (b) allow large scale (large N - number of animals used) in order to attain results with good statistical reliability. The biological relevance of these results derives from the scientific fact that all of us, humans, monkeys, rats and mice, are the product of the same *Darwinian Natural Selection Evolutionary Process*, sharing common ancestors in different points of the phylogeny, and, due to this, sharing several fundamental characteristics in terms of metabolism, brain organization and even behavioral strategies. This kinship can be extendend much farther, to other vertebrates and even invertebrates, according to the level of complexity of the shared characteristic. The main consequence of this *biological continuity principle* is that results obtained in animal models *can* be identical in humans, but this demands an independent demonstration.

All of this does not mean that *everything* is allowed in terms of experimental procedures, and there are ever-growing concern inside and outside scientific community in order to implement better *ethical principles* to guide laboratory animals' use (see specific chapter in this book about this matter). The biggest human ethical imperative is still to endeavor whatever possible effort to help our fellow conspecifics, but this is being progressively refined and deepened with the complementary animal use ethical legislation (and the same should happen with environmental questions). Researchers in the behavioral sciences must be fully committed to the continuous implementation and upgrade of Russell and Burch's 1959 *three Rs principle* – to replace, to reduce, to refine – not only for obvious humane reasons, but because science will still need to use animal models for a long, long time.

4. TYPES OF BEHAVIORAL TASKS

"Frustra fit per plura quod
fieri potest per pauciora"
(It is vain to do with more
what can be done with less)

As proposed in section 2, here we will describe some examples of behavioral tasks that cover most of the fundamental types of memory (see Figure 1), although some of them are quite hard to fit in just one category... To know more about behavioral tasks, including those *not* studied here (operant conditioning, sensitization, etc), please, refer to the literature suggested in the end of this chapter (in particular the books of Anisman & Bignami, 1978; Boulton et al., 1989, and Izquierdo, 2002, and the reviews of Hölscher, & O'Mara, 1997 and Stecker et al., 1998).

In most (but maybe not all) of these experimental protocols, it helps a lot if the animal is previously *habituated to the manipulation* in order to avoid behavioral interference of an enormous list of nonspecific aspects of the whole procedure: transporting to and from the experimental room, removing from / returning to home cages, handling, weighing, etc. These procedures should be applied dayly, for at least some days.

For practical reasons, the presentation order for the example behavioral tasks below is not exactly the same as shown in Figure 1.

4.1. OPEN FIELD HABITUATION

Open Field Habituation (OF) consists of exposing an animal to an open arena, a new environment without any clearly aversive or appetitive stimuli[12], and let explore it freely for a fixed amount of time. In this sense, it is the classical non-aversive and non-associative task.

Session duration may range from 2 to 10 min, specially during daytime experimentation; 2-3 min may be the minimum time to assure it *habituates* to the context, and more than 10 minutes seems to be useless, because the animal will start grooming and/or resting, even sleeping, since there seems to be no novelties / risks around).

The Open Field may have any geometric form, but circular and rectangular arenas are more common. Both types of arenas must display lines subdividing the floor [13] in regular sectors, be it rectangles (in the rectangular arena) or circular sections (in the circular arena) – see Figure 6a below. Rectangular arenas may be observed from above or through a frontal glass wall; circular arenas are usually observed from above. The box may be built in any washable material, such as metal, plastic or plywood, and a typical size (for rats) is 50 cm high, 40 X 60 cm, for the rectangular arena, or a 40-60 cm radius, for the circular one.

Figure 4 – (a) Two possible geometries for the Open Field Habituation arenas - rectangular and circular - displaying visible lines that subdivide the floor in regular sectors; (b) typical *rearing* exploratory posture of a rat.

During the first exposure – **the "training" session (TR)** – some variables may be quantified in order to measure exploratory behavior. The two most important indexes are (1) the number of *rearings* (REAR)

[12] This may be considered a "neutral" environment, but it is well-known that even the novelty of a new environmental may be stressful for the animal, with intensities that vary according to its intrinsic sensibility.

[13] Linoleum is recommended, because it is easy to clean up: a 70% alcohol solution is ideal, since it is still somewhat volative, and yet it does not smells too intensely. Some authors employ different floor textures in order to create subtle context modification.

exhibited by the animal, and (2) the number of *crossings* (CROSS) over the lines separating floor sectors. *Rearings* are innate exploratory postures of small rodents, and the newer the environment, the more rearings the animal will exhibit. *Crossings* also express exploration, of course, and have the advantage to measure basal motricity also (some drugs or treatments may affect this function and, in doing so, mask the mnemonic effect if there is one. This task may be automated with a grid of infrared photocells than measures crossings and may record rearings with some confidence (the reliable identification of a rearing posture is somewhat complex).

In order to test for *memory* in this behavioral task, **the "test" session (TT)** is performed after some interval (24h for long-term memory, less than 6h for short-term memory, up to 3min for working memory) putting the same animal in the same arena under the exact same environmental conditions and measuring again the same variables descrived above.

The rationale of this task is as follows:

- normal memory retention (as should be displayed by control animals) is indicated by a *reduction,* between training and test sessions, of the number of rearings *and/or* crossings;
- this reduction means that the animal has *learned* correctly the task; the difference between training and test sessions, in the number of rearings (or of crossings), is the measure (or score) of retention of the habituation to the open field;
- a behavioral experiment to study memory is valid only if the control group learns adequately the task, otherwise the whole data analysis may not be performed and the experiment should be disconsidered;
- a treatment is *amnestic* when, between training and test sessions, there is no significant difference in both variables (robust amnesia), or in the number of rearings only (partial amnesia [14]);
- a treatment is *facilitatory* when (a) animals *learn* (i.e., their number of rearings *and/or* crossings decreases between training and test sessions), and, moreover, (b) test measurements are *lower* than the control-group test values;
- the observed differences among variable measurements, be it a decrease or an increase, must be *statistically significant* in order to be considered (choose carefully the statistical tests and post hoc tests).

Results may be classified according to its *degree of robustness*: considering just one of the indexes, we may talk about *partial* or *full* effects; for instance, if the number of rearings does not change between training and test sessions (being not significantly different in statistical terms), we may call it a robust, full amnesia; if the number of rearings in the test session is significantly *higher* than its control-group counterpart, but still significantly different from its training value (i.e., it still learns, so memory is present), we may talk about a partial amnesia [15].

Important to notice is the attitude of the experimenter: both in the training and in the test session, the animals should be gently placed facing one of the bottom corners and allowed to explore the arena for the specified time. Between training and test sessions, it is recommended to repeat the illumination pattern, temperature, noise level and even the basic odor – remember rodents are hyperosmic small mammals, and this is why it is recommended that the same experimenter be present in both sessions, with similar clothings (odors).

Other variables may be considered, with different meanings: (3) *time to leave the first quadrant*, (TLFQ) usually relates to the anxiety level (despite there being better ways to quantify this – see the chapter on anxiety measurement - a too large leaving time may indicate an abnormal behavior of the animal, possibly *freezing*, and, if this is not related with the experimental desing, this situation prompts for the discard of the experimental subject to avoid biased data); (4) *grooming frequency/duration*, a disputable variable indicating both familiarity with the environment and lower levels of anxiety; (5) *defecation boli*, a more controvertable anxiety indicator, for which no agreement is achieved to this point.

The geometry of the arena is mostly a matter of taste: Open Field Habituation may be performed in the circular or the rectangular arenas with similar results. The circular version makes simpler the observation and quantification of *thigmotaxic* vs. *centrophobic* behavior: in the beginning of the exploration session, the animal walks nearer to the walls, guided by tactile / proximal information ("thigmotaxis"), avoiding exposing

[14] By partial amnesia we understand a situation in which memory was formed, but its trace is less prominent (so, measureed memory indexes display lower values).

[15] An even stronger amnesia is the one observed when these two variables does not change their values and are taken together.

itself to the open space, probably an evolutionary-selected adaptation; later, after assuring its safety, normal animals explores more the center of the open field (this may change according to the experimental design).

OTHER USES: Since this task permits the measurement of the number of crossings, it may always be used as *a control for the possible motor and general performance effects of the drug* previously administered; in this case, one session would suffice. Another interesting modification is the *Water Licking Task*: the only modification in the protocol described above is the introduction of one small extra detail in the arena, a dropping spout from a water bottle - the animal explores the new environment *and* records the position of the spout; after returning to the home cage, it is water-deprived for 24h; in the test session, we measure rearings, crossings, and the latency to find the spout and lick the water; this behavior is called *Latent Learning*.

4.2. ONE-WAY STEP-DOWN INHIBITORY ("PASSIVE") AVOIDANCE

Inhibitory Avoidance (IA) involves learning to inhibit a response in order to avoid an aversive stimulus, and the learning (training) session may be one-trial or multi-trial. Since there is punishment to the natural exploratory drive of a rodent with a non-letal, pulsating electric footshock, this is clearly an *aversive* task. IA is hard to classify according to criteria discussed in section 2 (see also Figure 1, above), because it involves both an explicit, associative component (to the context), and an operant-like conditioning [16] component (to the shock), this last being considered a type of implicit memory, specially in the one-trial version of IA.

There are two different approaches to the IA behavior, the step-down IA, here described in more detail, and the step-through IA (see, e.g., Bermúdez-Rattoni et al., 1997). The typical step-down IA apparatus is an automatically operated, brightly illuminated box with dimensions around 40.0 X 25.0 X 25.0cm [17] and a frontal glass wall; the floor consists of a grid of parallel 0.1cm caliber bronze (or steel) bars spaced 1.0cm apart; the left extremity of the grid is covered by a 7.0-to-10.0cm [18] wide, 5.0cm high formica-covered non-conductive platform. There may be a sliding door separating two halves of the box (as in the step-through IA) and each side may also be painted in different color (e.g., one black, the other, white).

In the one-trial, step-down IA task **training session**, animals are gently held by the body and lowered onto the platform with their noses pointing to the bottom corner, and a chronometer is started. Immediately upon stepping down with their four paws on the grid (when the chronometer is stopped), animals receive a 3.0s scrambled footshock of 0.2-1.0mA, according to the experimental design: the stronger the shock, the better the memory retention (and its duration) [19].

The pulsating shock may be delivered during some seconts (3-5s), and, after that, the animal is removed from the apparatus. Some authors preconize holding the shock until the animal climbs back onto the platform, but this is very stressful if kept for more than 10s, and could lead the animal to a freezing reaction, and no climbing up at all.

Since the training session is a quick procedure, this task is not simple to be automated.

In the **test session** [20] the animal is put in the same apparatus, under the exact same environmental conditions, except that no footshock is delivered. A ceiling of 180s up to 300s is imposed to the step-down latency, i.e., latencies larger than, say, 300s, will be counted as 300s. Notice that if more than one test is made with the same animal, the first test session may involve some degree of memory extinction that would modify the performance in the next one.

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[16] The operant factors are described as follows: "In one-trial inhibitory avoidance (IA), a fear-motivated learning task (Gold, 1986), rats associate a conditioned stimulus (CS; an elevated platform present in a given context) with an unconditioned stimulus (US; a shock given to the foot when they step down from that platform)" (Cammarota et al., 2003)

[17] Small variations around these values are possible.

[18] The variation may be larger than this: usually the platform should cover ¼ (or 1/3) of the grid-floor, but depending on the experimental design, it can be larger (e.g., to measure animal activity, Netto and Izquierdo, 1985, have used a platform that covered ½ of the floor).

[19] Visible signs of reaction to the shock may include piloerection, back-arching, eyeball-protrusion and even jumping and squeaking, according to the intensity of the shock and/or the sensibility of the animal; extreme reactions such as freezing should be avoided.

[20] As described for the OF habituation, test session may be done after an interval of 24h (for long-term memory), less than 6h (for short-term memory), or up to 3min (for working memory).

The rationale of this task is as follows:

- normal memory retention (as should be displayed by control animals) is indicated by an *increase,* between training and test sessions, of the latency to step-down from the platform;
- this increase means that the animal has *learned* correctly the task; both the difference between training and test session latencies, or the test session latency can be used as retention scores;
- a behavioral experiment to study memory is valid only if the control group learns adequately the task, otherwise the whole data analysis may not be performed and the experiment should be disconsidered;
- a treatment is *amnestic* when, between training and test sessions, there is no significant difference at all in the latency to step-down from the platform between sessions (robust amnesia), or this latency is significantly smaller than the controls one, but still higher that its training value (partial amnesia);
- a treatment is *facilitatory* when (a) animals *learn* (i.e., their latency to step-down from the platform increases between training and test sessions), and, moreover, (b) test measurements are *higher* than the control-group test values; facilitatory drugs / treatments are easier to be detected with lower shock intensities, since higher shocks tend to promote ceiling values in the test latency, and no further increase would be observable;
- the observed differences among variable measurements, be it a decrease or an increase, must be *statistically significant* in order to be considered (choose carefully the statistical tests and post hoc tests).

Figure 5 – (a) Step-down Inhibitory Avoidance apparatus showing the elevated platform and electrified grid floor; (b) in the test session, the animal recalls the aversive experience of the training session - having received a footshock after stepping-down into the grid: the better the retention, the larger the latency to descend from the platform.

The same basic attitude mentioned in 4.1 may be observed by the experimenter, both in the training and the test sessions: between training and test sessions, it is recommended to repeat the illumination pattern, temperature, noise level and the basic odors in the room.

Despite the fact that this task is called *inhibitory avoidance* by some (Izquierdo and Dias, 1983) and *passive avoidance* by others (e.g., Anisman, 1978), both terms does not have the same meaning: “passive” suggests inactivity, and inhibition refers to a more active restraint. Since it was shown that the retrieval of this task involves a fair amount of activity that was not related to retrieval scores under different shock intensities (Netto and Izquierdo, 1985), we understand it is not adequate to call it “passive”.

Step-through IA (e.g., Bermudez-Rattoni *et al*., 1997) employs a trough-shaped alley divided into two compartments separated by a guillotine door that retracts into the floor: a “safe” compartment is illuminated by a fluorescent lamp from above, and is separated from a darkened compartment where animals received the shock (possible dimensions: 90 cm long, 20 cm wide at the top, 6.5 cm wide at the floor, 15 cm deep; safe X dark compartment length proportion is 1:2, i.e., safe compartment would be of 30cm). Animals are placed in the dark compartment facing away from the door leading to the illuminated compartment and when they turn toward the door, it is opened and a timer is started; a footshock (variable intensities from 0.2 to 1.0mA) is administered until the animal escapes into the illuminated compartment. The rat is then, retained in the illuminated compartment with the door closed for 60 s. After this, according to the experimental protocol, the animal returns to its home cage (one-trial protocol) or is removed from the lighted compartment and placed back into the dark compartment where the same procedure is followed for the remaining trials (multi-trial protocol). In the test session, the animal is placed in the lighted compartment and the latency to step-through is measured.

4.3. CONTEXTUAL FEAR CONDITIONING

In Fear Conditioning (FC) the animal learns that certain environmental stimuli predict aversive events. Since there is no possibility to to escape from he aversive stimulus, this task is an example of a classical, Pavlovian (respondent) conditioning, and represents a defensive behavior selected by evolution in all animals (Maren, 2001). The recent interest in this model derives from the fact that this task provides an interface between memory and emotion (LeDoux, 2000).

Here we describe the **Contextual Fear Conditioning** protocol, arguably the simplest version of FC once there are only the context (the CS) and the aversive stimulus (the shock, or the US) to be paired. Refer to the literature if you are interested in the more Pavlovian-like auditory FC (Wilensky et al., 2000) or the amygdala-dependent fear-potentiated startle memory (Walter and Davis, 2000).

Two conditioning boxes are necessary: they should be placed in different, acoustically isolated separate rooms, and maintained at constant temperature (e.g., 25^{o}C). The first one (the paired context) has a floor that consists of an electrified grid of bronze (or steel) bars. Despite being similar to the one employed in the IA task, its dimensions are smaller (say, around 20 X 25 X 20cm) [21]. Internal illumination, provided by a 2.5W white light bulb, and background noise (ventilation fans, air conditioning, etc) should be kept constant in both sessions. The second box (the unpaired context) differ from the first in its size, color, illumination, floor texture, and wall properties to warrant a context as different as possible from the original one (used in training) in order to maximize the possibility of different levels of memory expression. Both chambers (paired and unpaired) should be cleaned (with, e.g., 70% aqueous ethanol solution) before and after each session.

The measured variable is the time the animal spent *freezing*, taken as an index of fear in rats (Blanchard and Blanchard, 1969; Bolles and Collier, 1976): an animal is considered to be freezing when crouching, without any visible body movement of the body and head, except for breathing.

The contextual FC training session is made as follows: on the day of conditioning, animals are transported from the housing room and individually placed in the paired context. A 3min (preshock) habituation (acclimation) period is followed by at least three unasigned scrambled footshocks; to assure a strong aversive learning, a shock of 0.7-1.0mA is recommended (3s of duration each and 30s intershock interval – for this intense protocol randomness among shocks is not necessary). Animals remain in the chamber for anoher 2 min (postshock period), and then removed back to their home cages (and housing room).

Testing for contextual fear conditioning is assessed 24h after training: animals are randomly assigned to two subgroups, half of them being reintroduced in the paired context for a 5-10min period (without shocks), and the other half, exposed for the same period of time to the unpaired context. Freezing is observed (and/or video recorded) during the exposure period, minute by minute (or in consecutive 5min periods), both in the paired and the unpaired contexts. Total time spent freezing in each period, in each context, is quantified in seconds with a stopwatch [22].

Figure 6– Fear Conditioning may be performed in the inhibitory avoidance apparatus (using just half of the grid) or in (a) a dedicated box (the loudspeaker is used in the auditory FC protocol, not in the contextual FC); after some footshocks, animals exhibit (b) a *freezing* reaction, a long-lasting absence of movements - except for respiration (also observable is piloerection, back-arching, and, sometimes, eyeball protrusion)

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[21] Actually, the same IA apparatus may be used for this task, provided only that a dividing wall is positioned in order use only one side of the box (without a platform).

[22] It is highly recommended to measure freezing behavior at the end of the experiment, preferably by a person who was blind in relation to the treatment applied to each animal (and videotaping is important).

The rationale of this task is as follows:

- normal memory retention (as should be displayed by control animals) is indicated by a larger percent time in freezing in the test session, compared to the training, or no freezing at all in the training session;
- this increase means that the animal has *learned* correctly the task; memory is expressed as the percentage of time the animals spent in this defensive behavior; it can be used as retention scores; the better the memory, the more the animal spends in freezing behavior;
- a behavioral experiment to study memory is valid only if the control group learns adequately the task, otherwise the whole data analysis may not be performed and the experiment should be disconsidered;
- a treatment is *amnestic* when, between training and test sessions, there is no significant difference at all in the percent time spent in freezing (robust amnesia, expressed by no freezing at all), or this percent time is significantly smaller than the controls one, but still higher that its training value (partial amnesia);
- a treatment is *facilitatory* when (a) animals *learn* (i.e., their percent time spent in freezing increases between training and test sessions), and, moreover, (b) test values are *higher* than the control-group test values;
- the observed differences among variable measurements, be it a decrease or an increase, must be *statistically significant* in order to be considered (choose carefully the statistical tests and and post hoc tests).

4.4. TWO-WAY ACTIVE (OR SHUTTLE) AVOIDANCE

In the **Two-Way Active Avoidance** (AA) the animal learns that a random stimulus (a tone, the CS) is a reliable predictor for a coming aversive experience (a shock, the US), and can prompt an evasive action in order to avoid it, i.e., it moves to the other side of the shuttle box (the CR) when the stimuli predict aversive events. Since there is the possibility to learn how to escape, this task may be clasified as an operant (or instrumental) conditioning, i.e., the animal must learn the relation between CS (sound) and US (shock) in order to anticipate US with a CR (escape) and avoid it. This task is also called **Shuttle Avoidance**, in a reference to the strategy the animal must learn and perform.

The shuttle box apparatus (approximate dimensions 60 X 20 X 30cm), is similar to the IA box (see above), only it (a) has no platform, and (b) the floor grid is visibly divided at the middle by a 1-cm high acrylic (or similar) hurdle [23].

Both, the **training** and the **test sessions** have an identical protocol that consist of a fixed number of tone-footshock pairing trials (30 is a good number), in which the CS is a 5s, 70 dB, 1 kHz tone [24] emitted by a loudspeaker attached in the midline position of the rear wall of the shuttle-box. As soon as the 5s tone ceases, a 0.5mA [25] footshock (US) is delivered until the animal crosses the midline; if the animal crosses to the other side of the box [26] *during* the tone (avoidance CR), the shock is interrupted: this must act as a reward.

The shuttle-box should be a fully automated apparatus where it does not matter in which side of the grid the animal is, when the tone comes up, it must move to the other side, without any prefered direction [27] - so, the "two-way" characteristic.

Each session starts with a 3-5 min [28] free exploration of the environment, without any stimuli, and the intertrial interval must vary at random between 10 and 50s: intertrial interval randomness and the two-way

[23] In some cases, the delimitation is made by a wall with an opening (say, 7 X 10cm) situated on the grid-floor level, and each side is independently illuminated by a 5W lamp inside the compartment.

[24] Some variation may be introduced in these values without problems, according to the experimental design.

[25] Higher footshock values may be used, up to 1.0 mA.

[26] Delimited by the hurdle. In the automated shuttl-box IR photocells constantly monitor the side the animal is.

[27] The alternative would be the (less employed) One-Way Active Avoidance in which the tone-shock pairing is done in just one and the same side (and the task would be much easier to learn).

[28] Some authors use an elaborate protocol in order to habituate the animals to the situational cues of the apparatus: the pre-habituation may last up to 10 min and be repeated for two consecutive days (Savonenko et al., 2003); in this case, the training session takes place in the following day and the tone-footshock pairings start sooner, after 20s. Another modification consists of limiting the shock duration up to 30s.

protocol are essential to assure that the only established association is done between the tone and the shock, without other predictive elements such as tone-delivery regularity and/or side of the grid. The shuttle-box apparatus should be placed in a soundproof, dim lighted room.

The automated box should record the total number of crossings, the number of escapes (crossing during the tone) and the number of mistakes; sometimes is useful to record the time receiving shock in each mistaken trial. Usually, the animal makes more mistakes in the beginning, and then starts to perform better; the learning will be expressed in the test session with a lower number of mistakes. Larger training sessions (with more trials) should improve performance, but the stress involved may be a problem due to fatigue or even freezing response (that is why 30 is a good number of trials).

Figure 7 – (a) The Active Avoidance apparatus, also known as the Shuttle-box, resembles an IA box, only without the platform; each half of the grid is separated by a plastic hurdle and is independently electrified in order to deliver (b) the aversive stimulus - a footshock - according to the tone and the side in which the animal stands (two-way AA protocol).

The rationale of this task is as follows:

- normal memory retention (as should be displayed by control animals) is indicated by higher avoidance responses (or lower number of mistakes) in the test session;
- this increase means that the animal has *learned* correctly the task (and the better the memory, the less mistakes the animal makes); the difference in the avoidance responses between the test and the training sessions is a measure of the memory retention;
- a behavioral experiment to study memory is valid only if the control group learns adequately the task, otherwise the whole data analysis may not be performed and the experiment should be disconsidered;
- a treatment is *amnestic* when, between training and test sessions, there is no significant difference at all in the number of avoidance responses (robust amnesia), or this number is significantly smaller than the controls one, but still higher that its training value (partial amnesia);
- a treatment is *facilitatory* when (a) animals *learn* (i.e., their number of avoidance responses increases between training and test sessions), and, moreover, (b) test measurements are *higher* than the control-group test values;
- the observed differences among variable measurements, be it a decrease or an increase, must be *statistically significant* in order to be considered (choose carefully the statistical tests and and post hoc tests).

Additional variables may be recorded during sessions with the assistance of a video camera (or additional shuttle-box gadgets): so, besides the number of avoidance responses, it is possible to measure, e.g., the number of reactions on CS (rearing, turning, freezing, flinching, moving across compartment, vocalization etc.), the latencies of reaction on CS or US [29], and the intertrial crossings (ITC); the overt behavior during US presentation may be visually discriminated into directional (needs a wall with opening) or nondirectional escape response (Savonenko et al., 2003). The type and number of reactions to CS, besides visually controlled may be divided into three groups according to the definition of these authors: (1) freezing reactions, defined as the lack of any movement except that related to respiration; (2) preparatory responses during CS presentation, i.e. turning of the body and orienting of the head toward the opening during CS presentation, excluding the cases when preparatory response is followed by avoidance reaction, and (3) attention reaction to the CS, i.e., any change in ongoing behavior observed during the first seconds of CS presentation, such as initiation of preparatory response, dissipation of freezing, or the interruption of any previous activity. It is recommended to measure these visually-classified behaviors at the end of the experiment, preferably by a person who was blind in relation to the treatment applied to each animal.

[29] Escape latency in the shuttle box may be affected by the modality of the CS, be it a tone or the illumination level.

4.5. MORRIS WATER MAZE

In the beginning of an OF, walking rats navigate mostly based on proximal information obtained with their vibrissae – hence, the "thigmotaxic" behavior (from the greek *thigma,* to touch). Small rodents are also noticeable for their spatial learning abilities, supposedly dependent upon visual information. Water Maze and Radial Maze have been widely accepted as major spatial learning paradigms (Hölscher and O'Mara, 1997). Several variants of each of these tasks may be used in order to obtain abundant behavioral indexes of contextual/spatial habituation, cue-driven navigation, operant-like navigation responses learning, and/or decision-taking.

In the **Morris Water Maze** (MWM or simply WM) task, the animal learns to swim in a water tank, guided by external cues, and find (and climb up to) a submerged platform (Morris, 1984). Based upon spatial information, this animal learns how to escape to a platform, so this task may be classified as explicit, associative memory with operant-like spatial learning (see Figure 1) [30].

The water maze is a black-painted [31] circular pool of 120-200cm diameter, 50 cm high, filled with water to a depth of 25-30cm. Water temperature is a critical factor (optimum is $26\pm2^{o}C$ [32]) as much as the room "decoration": it must be rich in consistently positioned spatial cues, such as the room's door, furniture, noticeable posters (in one or more walls); even the position of the experimenter must be kept constant. The only escape from the water is a platform, with minimum diameter of 10cm and submerged 0.5-1cm below the surface. This platform must be invisible to the animal (from its point of view), located in the middle of one of the quadrants (equidistant from the wall and the pool center), and kept in the same quadrant on every trial during the training session.

Rats and mice are natural swimmers, but in this task they just want to get out of the water; swimming for short periods of time does not distress them [33]. Two advantages of Morris water maze over others mazes are that (a) it is a self-driven task (rats want to get out, so it actively searches), and (b) water environment is devoid of local cues, such as scent trails (except for the tank walls).

Training sessions consist of repeating a number of trials, several days in a row (4-8 trials a day, for 2 to 5 days - or more, when training to a criterion). In each trial, the animal is released from one different starting position randomly selected from eight possible "geographic" points around the perimeter of the pool (Figure 8a). It is important that, during each learning trial, the experimenter is not visible to the animal [34]. The only relevant variable measured in the training trials is escape latency.

A trial begins by placing the animal in the water, usually facing the pool side (to minimize bias), and timing the latency to reach platform and climb it (escape response). Care must be taken when putting the animal in the water to avoid stress that has a disruptive effect upon learning: gently place it with the tail-end lower, so the head does not dip under water (dropping them in head-first is stressful). Notice that rats may be cheaters, and instead of learning where the platform is, they can learn search strategies such as swim around at some distance from the side, or make a series of sweeps trying to guess platform position.

Trial duration is usually of 60s (but may take up to 2min). If the animal fails to climb the platform (escape) within this time window, it will be gently conducted to it by the experimenter. In any case, when on the platform, it is allowed to stay there for 10-30s to orientate: then, the animal rears and looks around. It is recommended at least three extra-maze visual cues (e.g., posters in the wall, room's door or some furniture). After some swim trials, animals will go directly to the platform.

After each trial, animals are gently lift off, dried [35], and returned to their home cages until the next trial; at the end of the day (or the training session), home cages are returned to the housing room. For a good

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[30] A lot of practical information about water maze techniques can be find at http://www.hvsimage.com/documents/watermaze_tips.pdf

[31] If you use albino or white strains, a black pool maximizes visual contrast for video recording; for dark-haired animals, use white-painted pool. In this last case, some authors, including Morris himself whitens the water with skim powder milk or titanium dioxide in order to increase animal-background contrast and prevent animal from seeing thru the water. However, this may be an overcare, since shuttling the animal from homecage to pool goes by swiftly, and, when on water, the animal cannot easily see anything under waterline; in our experience, it suffices to have a transparent platform.

[32] Although colder water would encourage activity, it may induce hypothermia, known to impair learning; warmer water would favor animal relaxation and decrease exploration.

[33] Swimming for more than 12-15min without finding any escape is, otherwise, stressful; actually, this is a classic stress model called "forced swimming"

[34] To avoid recue expectation from the animal.

[35] Dr. Morris' tips (note 23 above) suggests that it is much better to put the animal in a litter of tissues, so it can dry itself.

retention, an inter-trial interval (ITI) of between a minimum of 10min and a maximum of 20min is recommended. All movements (including the path course) are recorded from a camera attached to the ceiling, and either videotaped, or digitally stored in a computer (depending on the automatic setup you have at your disposal [36]) for posterior analysis.

Test session (*probe trial*) takes place 24h after the last training session: the platform is *not present* and the animal swims only one trial of 60s (sometimes of 120s, but data may still be recorded minute by minute), and then the animal is rescued [37]. Measured variables include (a) *latency* (sec) *to reach* the original position of the platform for the first time, (b) number of *crossings* in that exact place, and (c) the *time* (sec) *spent in the target quadrant* (TQT) compared to the opposite one (OQT).

Additionally, other variables can be measured: (d) *path length* (in cm), (e) *mean swimming speed* (cm/s), (f) *departure angle* in relation to the target position, and (f) *time spent in the peripheral ring* vs. central spot (thigmotaxis). Manual record of these variables is pretty hard, but most automated systems (such as HVS) provide them without difficulties. If an altered swim speed is detected, any measured difference between group latencies cannot be clearly interpreted; this is when path length measurements can be helpful: if this variable differs between groups, animais may be experiencing both motor *and* leaming impairments; if they are approximately equal across groups, then these latency differences, if detected, may be due to motor, not learning impairments (Hölscher and O'Mara, 1997):

Figure 8 - (a) the Morris Water Maze setup, showing the external cues in the walls, quadrants of the tank (with submersed platform), and eight different starting positions; (b) a black-painted tank is best for white animals, but room illumination must be adjusted to avoid reflections that disturb videotaping or automatic data acquisition.

The rationale of this task is as follows:

- normal memory retention (as should be displayed by control animals) is indicated by (a) small test session *escape latency* (less than 10s), and (b) significantly higher *mean time spent in the target quadrant* compared to the opposite one; it is also desirable that it crosses the exact place where the platform was more than once;
- the small escape latency (and higher TQT vs. OQT) means that the animal has *learned* correctly the task; the difference in the test session escape latency compared to the first training trial latency is a measure of the memory retention; higher TQT than OQT is another measure, although less robust if taken alone;
- a behavioral experiment to study memory is valid only if the control group learns adequately the task, otherwise the whole data analysis may not be performed and the experiment should be disconsidered;
- a treatment is *amnestic* when, between the first training and the test sessions, there is no significant difference at all in the escape latency (robust amnesia), or when this number is significantly higher than the control one, but still smaller that its training value (partial amnesia);
- a treatment is *facilitatory* when (a) animals *learn* (i.e., their escape latency decreases between training and test sessions, and/or TQT > OQT), and, moreover, (b) test latency is even smaller (or TQT higher) than the control-group test value.
- the observed differences among variable measurements, be it a decrease or an increase, must be *statistically significant* in order to be considered (choose carefully the statistical tests and and post hoc tests).

Interesting alternative protocols are possible, such as the Reversal task and the Transfer task (see Hölscher and O'Mara, 1997). In the *Reversal task*, platform is moved from one quadrant to the next from trial to trial, and the task consists of learning the new location "overriding" the knowledge about the previous

[36] For instance, the HVS tracking system for water maze, from Dr. Morris' lab (www.hvsimage.com).

[37] Since rodents are good at spatial learning, do this only on trained animals, and not too often: when done at the start, it tests for spatial bias; when done after trainings, it tests for spatial learning.

location; this learning is a sensitive way to test animals that have difficulties in learning to "switch" from one procedure to a new one, what is known as *perseverative behavior* (a hippocampus-dependent trait).

Nonspatial WM tasks, such as the Visible Platform and the Visual discrimination tests, not only provide interesting behavioral information, but may also be powerful control tasks. In the *Visible Platform test,* the platform is visible above the water; spatial elements may be removed from the task by randomly moving the platforrn around in each trial, or simply by wrapping up the WM tank with a curtain that obliterates distal visual cues. This nonspatial visual discrimination task tests if the animals can see the target and move normally to it, but is may also involve a striatum-dependent type of procedural memory, different fro the spatial task, that relies on the hippocampus [38] (Packard and Teather, 1997): if there is any sensory, motivational or motor impairment, there will be a diference in the latency/distance between groups. In the *Visual discrimination tasks*, animals are trained to to recognize and discriminate between visual cues: one protocol used two different visible platforms, one stable and the other, floating (but anchored). Since the last one does not support the animal's weight, it must learn to choose which platfrom to mount in orderto escape water; this task controls for general motor skills, visual discrimination, and learning ability (though it does not depend on hippocampus – see, e.g., Bannerman et al., 1994).

4.6. 8-ARMS RADIAL MAZE

The **8-Ams Radial Maze** (8ARM) apparatus must be elevated from the ground (a minimum of 60cm is recommended) and may be made of wood or plastic. Dark surface colors are interesting, since rodents are somewhat photophobic, and it will be useful in the case of videotaping and/or automatic tracking due to contrast (at least for white animals) [39]. Arms have dimensions of 60cm long X 10cm (up to 20cm) wide, and may or may not have elevated walls - height goes from 2 to 30cm. Guillotine doors are useful to set different contexts. Central platform size may vary from the regular octagon defined by each of the eight arms width to a somewhat larger arena, i.e., its diameter goes from 25 to 45cm. Good illumination may be provided from above the maze (in the case of open arms) or independently inside each arm (in the case of walled arms), e.g., with 6W light bulbs controlled by a switchboard.

Food cups (wells) are drilled into the floor at the end of each arm, to place the baits (food pellets [40]) in order to avoid visibility from the central platform; rebaiting process may be performed both manually or automatically, depending on the available setup and the experimental design. In order to obtain the "drive" to execute this task, animals are first reduced to 85% of their ad lib feeding weights; after this, **training sessions** may begin, be it once or more times a day, in consecutive or every other day, for a limited number of days or for an unpredictable number of days (e.g., when training to a criterion).

Radial maze is one of the most versatile and adaptable behavioral tasks, thus, to summarize all the possible experimental designs, is actually impossible. In here we pick two or three simple versions of 8ARM task. Also, if this apparatus is built with detachable arms it will be of great advantage, since (a) it will be apropriate for storage, and (b) may easily be reconfigured into, say, a T-maze, or Y-maze, or a 4-arm "plus" maze.

It is important to begin allowing the animal to freely explore the maze (habituation) in the first 1-2 days, with no food available, for 5-10min each trial. Only in their home cages they will be introduced to the reward (baits), in a limited number (e.g., no more than 8-10 small pellets).

Food trials begin on the following day, and the simplest **training** procedure to study spatial memory consists of baiting only one arm and training the animal to find it, for several trials (this task can be called *spatial delayed matching*). The experimental room setup is similar to the one employed for the WM task (above), with extra-maze visual cues in the walls (posters, door, etc) and, preferably, without the presence of the experimenter during trial. An arm entry takes place when four paws cross into it. The entry of the animal

[38] These two variants of the WM task have demonstrated a double dissociation of the mnemonic functions of the hippocampus (with the Spatial WM task) and the dorsal striatum (with the Cued WM task), a phenomenon also observed with the win-shift and win-stay radial-maze tasks (Packard et al., 1989), and, to some extent, with the allocentric vs. egocentric maze tasks (Kesner et al., 1993).

[39] Tracking programs may assist in measuring animal's running speed, useful to evaluate nonspecific effects upon motor performance.

[40] These may consist of palatable pellets such as peanuts, *Froot loops* (Kellogg's sweet pellets of wheat and corn starch and sucrose) or even special brands, such as Noyes Formula A pellets.

into an arm previously entered in the same session may also be considered an error (clearly, a working memory deficit), but some protocols tolerate at least two entries during training.

Animals, then, return to their home cages for a delay interval that may vary from 5s to 24h or more (depending on which kind of memory is under study). After this, they are put back into the maze for the **retention test**, in which all eight arms are open and only those arms that had not been blocked before the delay contain food. Animals are removed from the maze after all baited arms have been chosen. The entered arms and the order of entry are recorded, including errors.

Considering that healthy rodents explore actively new environments and they naturally tend to (a) alternate between arms and (b) not to visit the same arms twice, a more complicate version of the task may be implemented involving, say, 2, 3 or 4 baited arms - be it in a simple spatial arranjement (e.g., arms 1, 3, 5[41]), be it in a more complex pattern (such as 1, 4, 5). Different authors use different strategies to reinforce learning such as turning lights on and off in baited arms, use floors with distinct textures, or close guillotine doors when animal enters to add an internal delay period (and check for working memnory effects). As usual, testing for long-term memory depends only on the duration of the training-test interval (e.g., more than 6 hours).

Between trials, and specially, between diferent subjects, the maze must be cleared of feces and urine vestiges (wipe out and clean with a low% alcohol solution to remove scents).

In another version of the 8ARM, the *spatial delayed nonmatching* or *delayed spatial win-shift task* (Melo et al., 2005), animals already trained make a last (pre-delay) visit to the learned baited arms - nonbaited arms are kept closed, and, then, are removed to home cage for the delay interval (see above). Thus, the animal is returned to the center of the radial maze, with all eight arms open: the difference is that now only the previously closed/unbaited arms contains the reward, and animals are allowed to complete all choices, i.e., find all the baits (the post-delay recall test). They can be trained until reaching a stable response (e.g., no more than one error in at least three consecutive sessions) and then submitted to a new "challenge" again.. This procedure has some similarity with memory extinction, and tests for the ability of changing memory referentials (resistent animals are said to be *perseverant*).

Finally, a nonspatial visual discrimination version of this task (the so called *win–stay* protocol) is possible by wrapping up the maze with opaque curtains, and dimly illuminating it from above; rebaiting must be made by an unobstrusive overhead tubing system, and the animal is observed through a slanted overhead mirror from outside the curtain. Maze and pellet habituation sessions (2 days) are similar to those described above. On each food trial (training session), four randomly selected maze arms are illuminated *and* baited with a single food pellet in the food cup after eating it and returning to the center platform, the arm remains lit and is rebaited; after the consumption of the second pellet in that arm, light is turned off and no rebaiting takes place in that arm. Each daily training session ends when animals eat eight pellets (within a trial) or 10 min have passed. Entered arms are recorded and visits to the unlit/unbaited arms are scored as errors; food trials are run once a day for 6 days. This task may involve habit formation and some degree of associative memory.

Figure 9 - (a) 8-Arm Radial Maze schematics showing each aisle (arm) with food cups carved in its extremity; (b) the apparatus must be elevated from the floor and external cues, similar to those used in the Water Maze, must be showing in the walls.

The spatial delayed matching and the win-shift versions of the 8ARM (see above) are good examples of explicit, associative memory tasks, only with an appetitively-motivated (rewarded) spatial learning component (see Figure 1). A comparison between the MWM and the 8ARM is shown in Table III.

[41] Regular spacing favours search strategies that do not involve long-lasting forms of memory (only, of course, working memory), such as, "entering every other arm to the left"

	Water Maze	8-Arm Radial Maze
Advantages	• No motivational problems – problem cases (e.g. aged or uncooperative animais) are easily tested. • Olfactory cues are not present. • Animais learn readily and quickly.	• Distinguishes beeween motor impairment and spatial learning - if the animal has motor problems it can still choose an arm. • Different memory types can be tested: working memory, long-term (reference) memory, motor (egocentric) memory. • A tracking program is not essential. • Cheap, simple set-up, which can be dismantled and easily stored after use.
Disadvantages	• Differences in platform finding latency are the product of learning *and* motor performance; it is not possible to subtract one from another; if the test group displays motor impairments it is impossible to separately assess spatial learning impairments. • Working memory cannot be tested independently. • A videotracking system is essential for evaluating the swim tracks of animais. • A fairly large pool has to be installed in a dedicated room.	• Animais must be well-handled and motivated to perform the tasks. • Appetitive task: the animais must be mildly food-deprived to be motivated to look for baited arms; may be a problem in some cases (e.g. in aged rats which need full food supplies to stay healthy). • Training the animals usually takes much more time than MWM.

Table III – Advantages and disadvantages of the two major paradigms of spatial learning (adapted with permission from Hölscher and O'Mara, 1997)

4.7. OBJECT RECOGNITION TASK

Recognition is defined as the process by which a subject is aware that a stimulus has been previously experienced. It is strongly dependent on memory, since it requires a series of cognitive operations (such as perception, discrimination, identification and comparisons) that rely on previously known information in order to "match" the observed event against a memory of the previously experienced ones.

Recognition tasks may also be classified in two major paradigms, *object recognition* (item memory) and *place recognition* (spatial memory). Since this last one has obvious relations both with exploratory behaviour and some elements discussed for the OF habituation task (see Figure 1, for instance), and may involve even spatial elements, it will not be discussed here. But this does not mean it is less important, its just a matter of personal choice. A very important type of place recognition task, for instance, is the *Spontaneous Alternation Behavior* (SAB), that employs the classical "T" (ot "Y") Maze: about this task, please, refer to the excelent review of Hughes (2004).

Since recognition memory is based in the general principle of "matching", experiments consist of three clearly defined phases: a sample phase, a delay phase (or retention interval) and a choice (or comparison) phase. These phases are analogous to the scheme we have been using till now, with a training session, a training-test interval, and a test session. In this framework, two rules of response are possible to be learned, *matching* and *non-matching*, so the great variety of experimental designs, such as DMS, DNMS, etc (a complete discussion is in Steckler et el., 1998).

Object Recognition (OR) may be performed in any simple box, with or without a transparent wall (if it is the case, the animal is to be observed from above). A typical apparatus has a 50cm high, 40 x 60cm box made of wood (or plastic) with a frontal glass wall, the inside of which is painted with clear colors. Usually the recognition objects are made of plastic or metal to allow easy cleaning between sessions with different animals.

Before starting the trainings, all animals have at lest two free exploration sessions for contextual habituation, with no objects inside the box. **Training sessions** (sample phase) consist of allowing the animal to explore two different objects during a certain fixed amount of time (e.g., 5min). It is important that (a) the

objects have a "neutral" shape [42] in terms of its significance to the animal, (b) be devoid of any marked characteristics, such as odor and movement, for instance, and (c) both must be positioned more centrallky in the box, at least 10cm from the side wall, to avoid accidental touching during the initial, thigmotaxic exporation.

The total time spent exploring the two objects is recorded by the experimenter with the assistance of two stop watches: "object exploration" is defined as directing the nose and vibrissae to the object at a distance of less than 2 cm, as if "smelling" it with caution; bumping, turning around or sitting upon the object are not considered exploratory behaviours. At the end of the training trial, the animal is removed from the box and returned to its home cage.

After an interval (the retention delay) that may be of 15min, the animal is reintroduced into the box for another trial, the **test session** (choice phase), now with a different set of objects - one familiar (identical to, but not the same one previously explored object), and the other, a new / unexplored object - both placed in the same position as the sample stimuli. Due to its particular characteristics, object recognition is usually more employed to investigate short-term memory [43].

According to the phase analysed, different indexes can be used: a *discrimination index*, the difference in time spent exploring each of the two objects in the *choice phase* (i.e., time with B minus time with A), and the *discrimination ratio*, which is the difference in exploration time, expressed as the ratio of total exploration time with *both* objects in the *choice phase* (this ratio allows to adjust for individual or group differences in the total amount of exploration time).

Recognition memory raises some complex conceptual questions: (1) pure *recall* may sometimes mask an effective *recognition* procedure; (2) at least two types of very different recognition memories can be distinguished, *familiarity/novelty* and *recency*; and (3) different cognitive strategies, from the simplest stimulus-response association to more intrincate concept formation, may be involved to a varying extent (for a detailed discussion of these topics, see Steckler et el., 1998).

Figure 10 - (a) Object recognition task setup: two different plastic (washable) objects, A and B, are explored by the animal in the sample phase (training session); in the choice phase (test session) they are substituted by A' (identical to A) and C (totally unfamiliar); (b) object exploration is defined as directing the nose and vibrissae to the object at a distance of less than 2 cm.

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[42] As far as it could be ascertained, the objects should have no natural significance for the rats, to avoid being associated with a reinforcer (or an aversive stimulus).

[43] By the other hand, place recognition tasks are more versatile and may allow the investigation of long-term memory processes (with "delays" of more than 6 hours).

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Behavioral Methods to Study Learning and Memory in Rats

Figures

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May of 2006

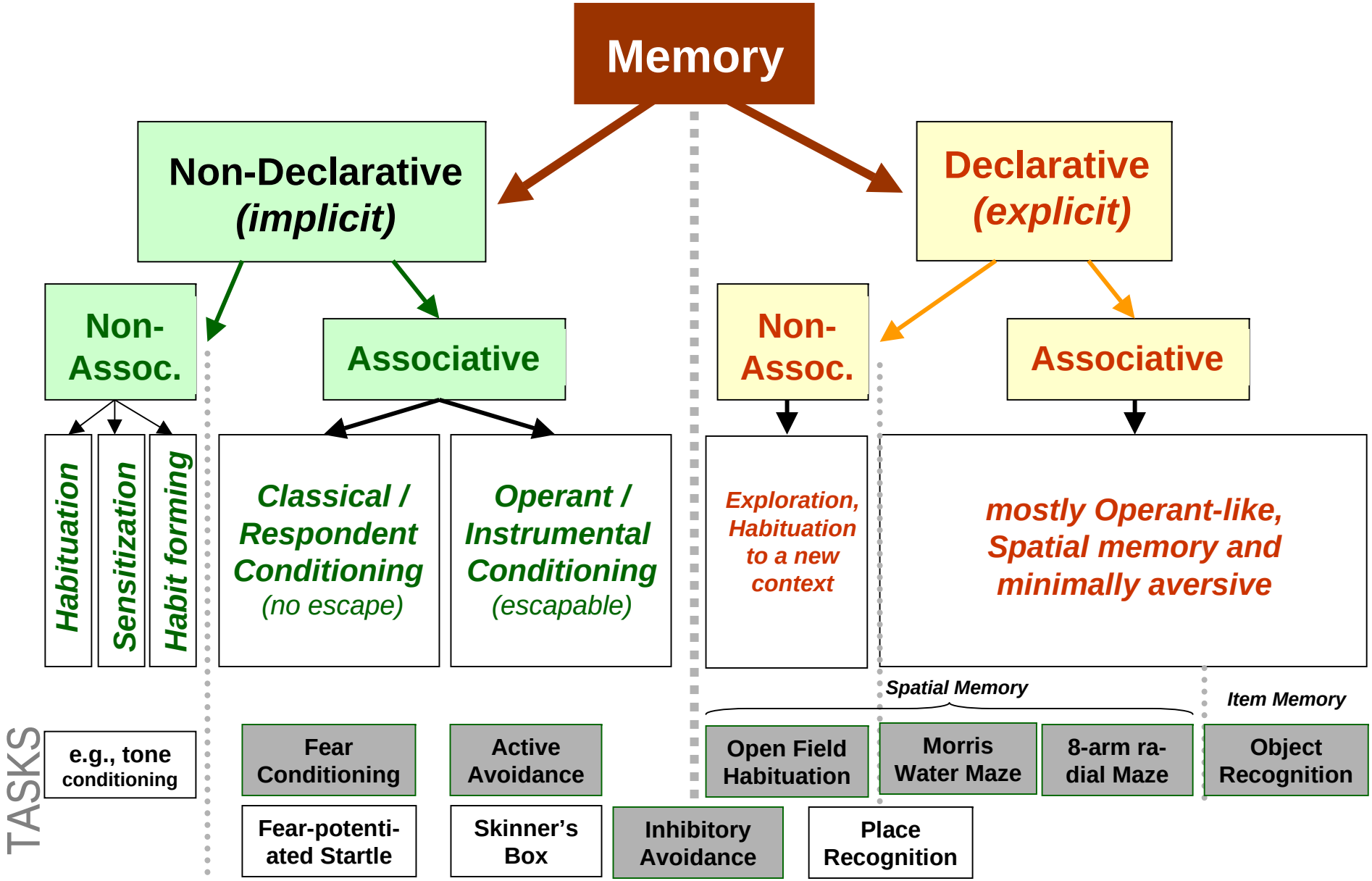

Figure 1 – A possible Taxonomy for Memory types and some Behavioral Tasks that may have access to them. Please, DO NOT USE THIS TABLE WITHOUT READING THE COMPANION TEXT BELOW, since these tasks only serve as examples according to the specific protocol employed (as described in section 4).

Figure 2 – Phases of Memory Formation (acquisition / consolidation) and Retrieval (recall).

Non-mnemonic Factors	Typical Behavioral Task / measured parameters
Motor performance	Deambulation characteristics in an OF (open field) Rotarod test Pole or Chord climbing
Anxiety	Elevated Plus-maze Light-dark transition task Time in central region of the maze vs. tygmotaxia
Pain sensitivity	Tail flick test Hot Plate and/or Paw Pressure test
Sensory perception	Sensory discrimination tasks
Attention	Discrimination/reaction tasks
Arousal level	

Table I – Possible factors to be excluded by complementary behavioral tasks.

STATE-DEPENDENT LEARNING		TRAINING	
		No drug	Drug
TEST	No drug	Good recall	*Less-effective recall*
	Drug	*Less-effective recall*	Good recall

Table II –Experimental design to prove State Dependent Learning – in this case an instance of memory facilitation (adapted from Meyer & Quenzer, 2004)

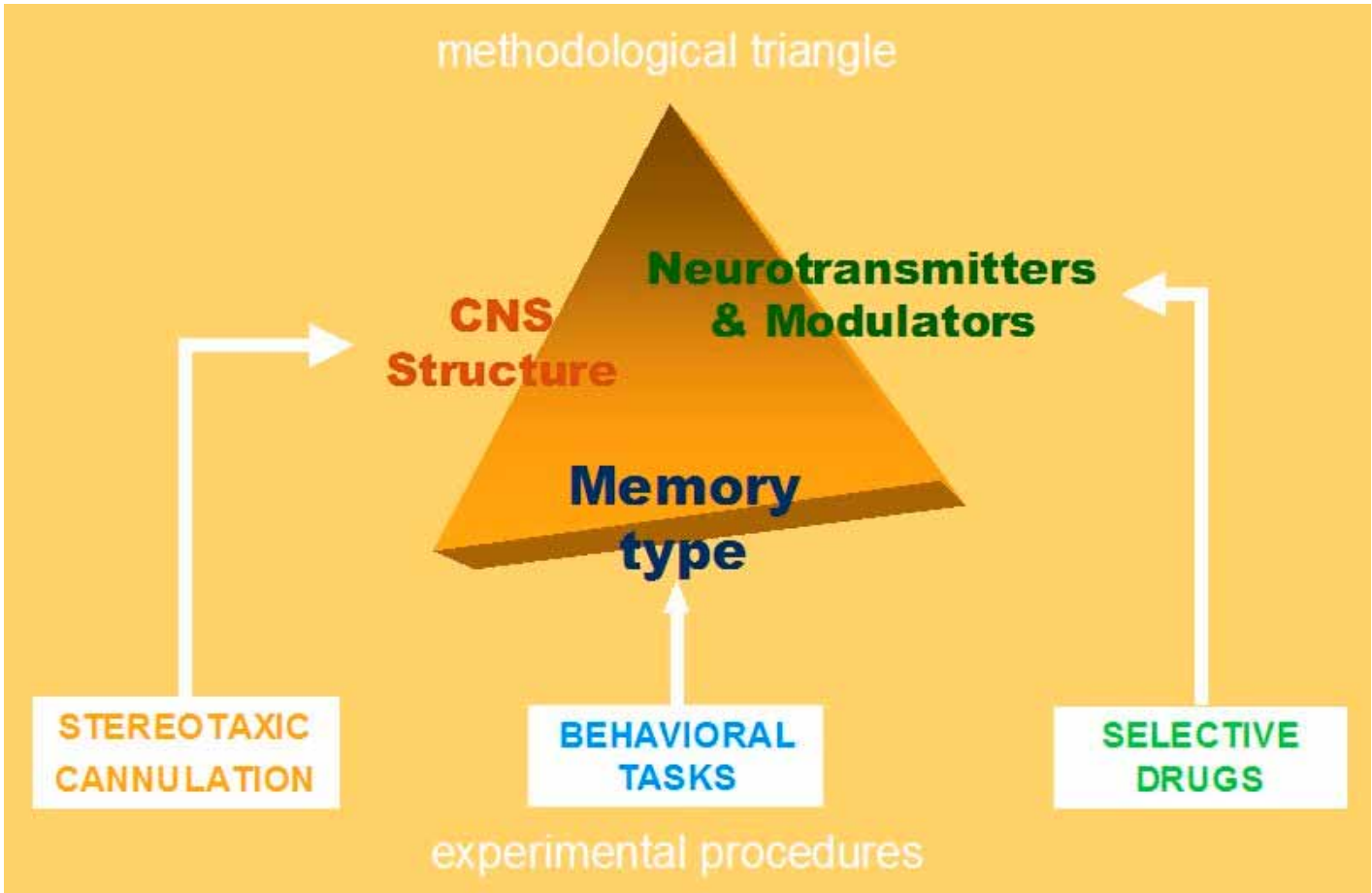

Figure 3 – The Triangle of Methodological Limitations of the Neuropsychopharmacology of Learning and Memory: each one of the three dimensions demands a specific experimental approach. Conclusions must not extrapolate these limits.

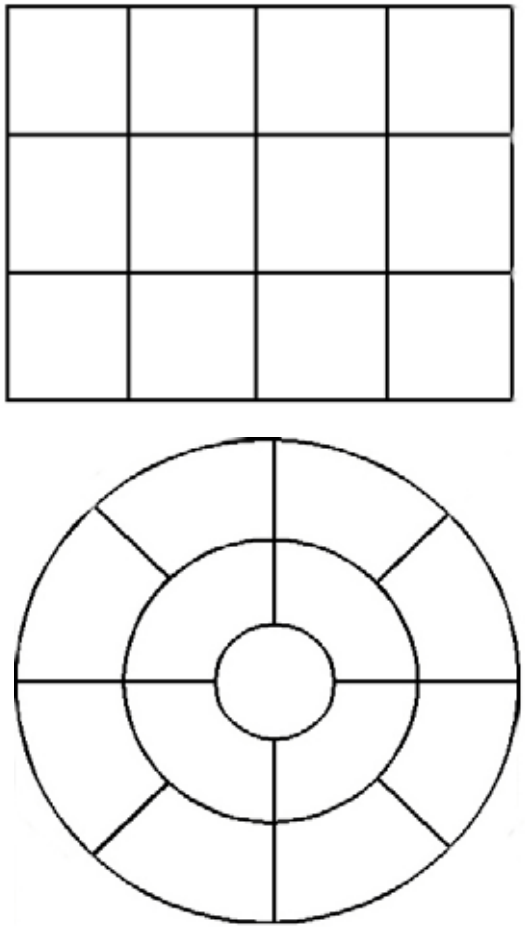

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Figure 4 – (a) Two possible geometries for the Open Field Habituation arenas - rectangular and circular - displaying visible lines that subdivide the floor in regular sectors; (b) typical *rearing* exploratory posture of a rat.

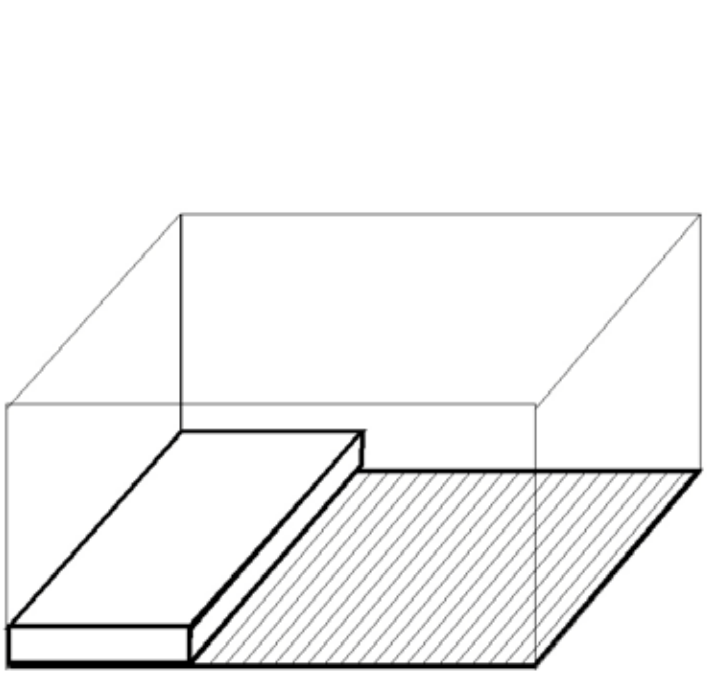

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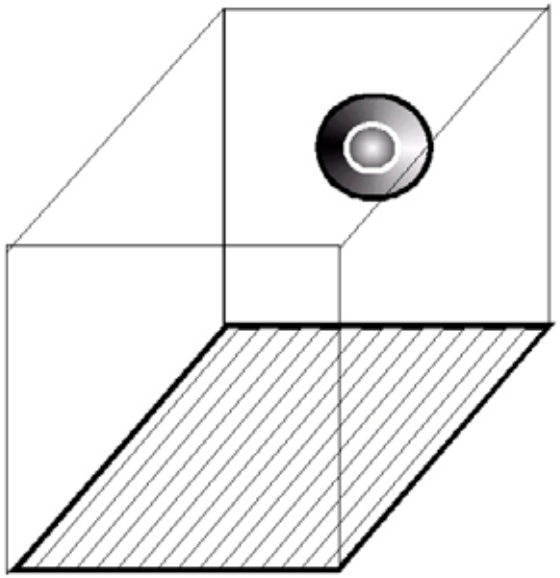
Figure 5– (a) Step-down Inhibitory Avoidance apparatus showing the elevated platform and electrified grid floor; (b) in the test session, the animal recalls the aversive experience of the training session - having received a footshock after stepping-down into the grid: the better the retention, the larger the latency to descend from the platform.



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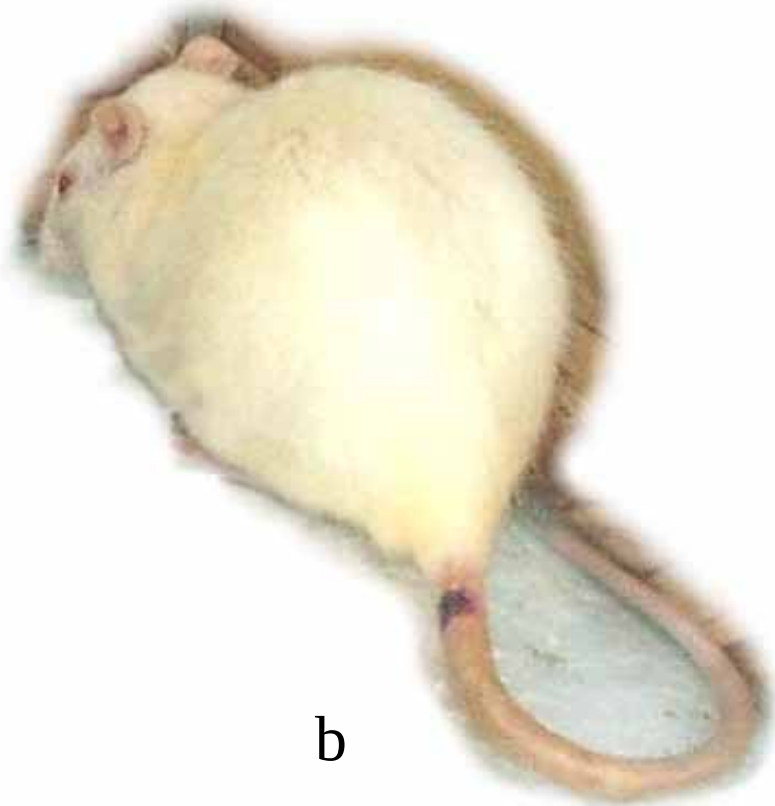

b

Figure 6 – Fear Conditioning may be performed in the inhibitory avoidance apparatus (using just half of the grid) or in (a) a dedicated box (the loudspeaker is used in the auditory FC protocol, not in the contextual FC); after some footshocks, animals exhibit (b) a *freezing* reaction, a long-lasting absence of movements - except for respiration (also observable is piloerection, back-arching, and, sometimes, eyeball protrusion)

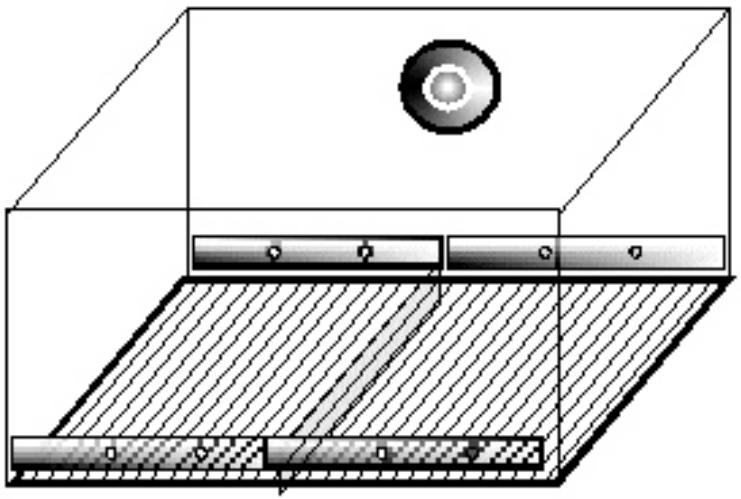

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Figure 7 – (a) The Active Avoidance apparatus, also known as the Shuttle-box, resembles an IA box, only without the platform; each half of the grid is separated by a plastic hurdle and is independently electrified in order to deliver (b) the aversive stimulus - a footshock - according to the tone and the side in which the animal stands (two-way AA protocol).

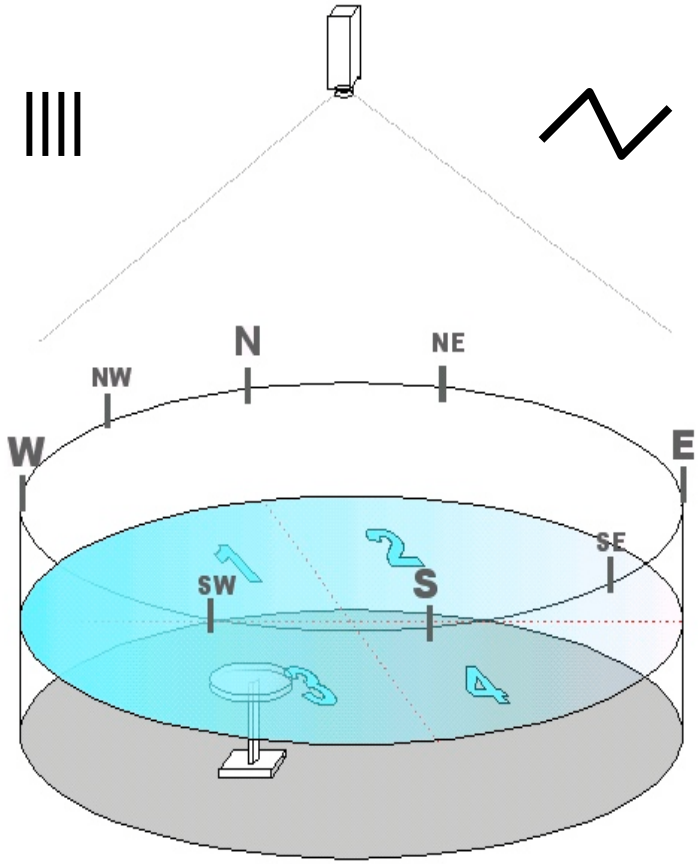

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Figure 8– (a) the Morris Water Maze setup, showing the external cues in the walls, quadrants of the tank (with submersed platform), and eight different starting positions; (b) a black-painted tank is best for white animals, but room illumination must be adjusted to avoid reflections that disturb videotaping or automatic data acquisition.

	Water Maze	8-Arm Radial Maze
Advantages	• No motivational problems – problem cases (e.g. aged or uncooperative animais) are easily tested. • Olfactory cues are not present. • Animais learn readily and quickly.	• Distinguishes beeween motor impairment and spatial learning - if the animal has motor problems it can still choose an arm. • Different memory types can be tested: working memory, long-term (reference) memory, motor (egocentric) memory. • A tracking program is not essential. • Cheap, simple set-up, which can be dismantled and easily stored after use.
Disadvantages	• Differences in platform finding latency are the product of learning *and* motor performance; it is not possible to subtract one from another; if the test group displays motor impairments it is impossible to separately assess spatial learning impairments. • Working memory cannot be tested independently. • A videotracking system is essential for evaluating the swim tracks of animais. • A fairly large pool has to be installed in a dedicated room.	• Animais must be well-handled and motivated to perform the tasks. • Appetitive task: the animais must be mildly food-deprived to be motivated to look for baited arms; may be a problem in some cases (e.g. in aged rats which need full food supplies to stay healthy). • Training the animals usually takes much more time than MWM.

Table III – Advantages and disadvantages of the two major paradigms of spatial learning (adapted with permission from Hölscher and O'Mara, 1997)

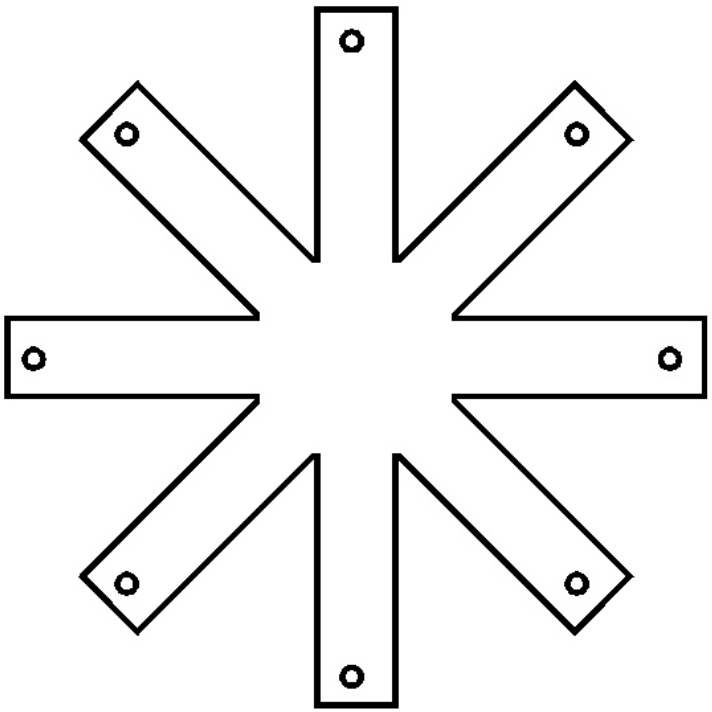

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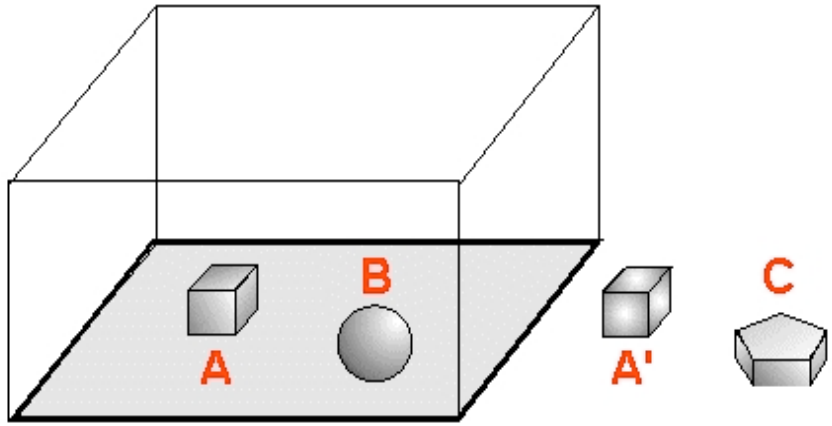
Figure 9– (a) 8-Arm Radial Maze schematics showing each aisle (arm) with food cups carved in its extremity; (b) the apparatus must be elevated from the floor and external cues, similar to those used in the Water Maze, must be showing in the walls.



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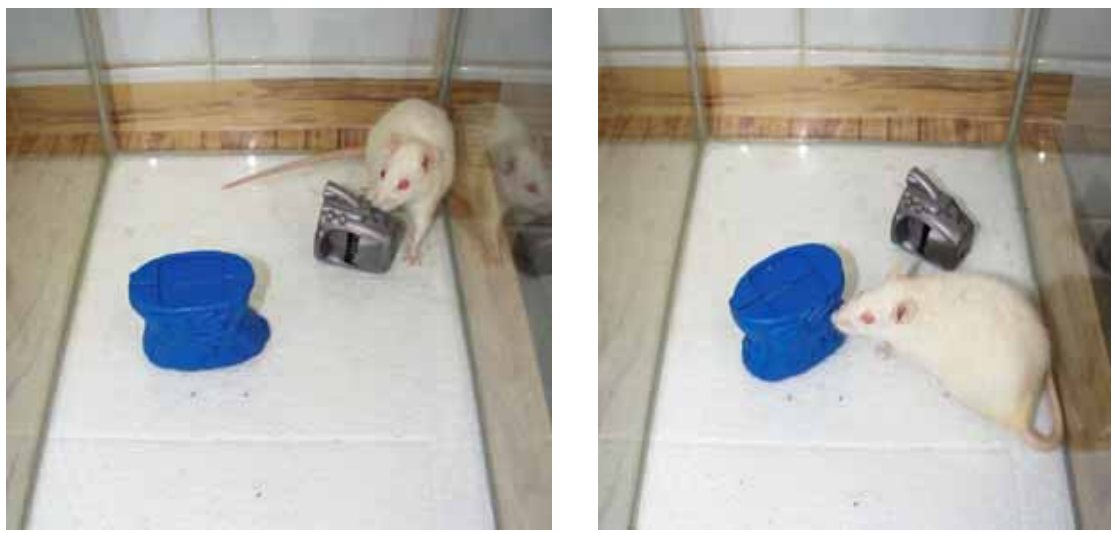

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Figure 10 – (a) Object recognition task setup: two different plastic (washable) objects, A and B, are explored by the animal in the sample phase (training session); in the choice phase (test session) they are substituted by A' (identical to A) and C (totally unfamiliar); (b) object exploration is defined as directing the nose and vibrissae to the object at a distance of less than 2 cm.