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Behavioral assays with mouse models of Alzheimer's disease: practical considerations and guidelines

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Abstract

In Alzheimer's disease (AD) basic research and drug discovery, mouse models are essential resources for uncovering biological mechanisms, validating molecular targets and screening potential compounds. Both transgenic and non-genetically modified mouse models enable access to different types of AD-like pathology *in vivo*. Although there is a wealth of genetic and biochemical studies on proposed AD pathogenic pathways, as a disease that centrally features cognitive failure, the ultimate readout for any interventions should be measures of learning and memory. This is particularly important given the lack of knowledge on disease etiology – assessment by cognitive assays offers the advantage of targeting relevant memory systems without requiring assumptions about pathogenesis. A multitude of behavioral assays are available for assessing cognitive functioning in mouse models, including ones specific for hippocampal-dependent learning and memory. Here we review the basics of available transgenic and non-transgenic AD mouse models and detail three well-established behavioral tasks commonly used for testing hippocampal-dependent cognition in mice – contextual fear conditioning, radial arm water maze and Morris water maze. In particular, we discuss the practical considerations, requirements and caveats of these behavioral testing paradigms.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized clinically by progressive cognitive decline (reviewed in [1]). Currently, AD is the most common type of dementia worldwide; and since age is the biggest risk factor, the prevalence is expected to greatly increase over the next few decades with aging population structures. Unfortunately, despite decades of research, the etiology of AD is unknown, and many fundamental

questions remain unanswered. Continuing research into the basic underlying biology of AD as well as renewed efforts in developing disease-modifying drugs are necessary to address this problem. In both the basic research and translational arenas, animal models of the disease are critical. In particular, genetic and non-genetic mouse models of AD pathology have become key research tools for discovering disease pathways and targets as well as testing new therapeutic approaches (reviewed in [2, 3]).

Ultimately, as a disease of synaptic and cognitive failure (reviewed in [4, 5]), both preclinical hypotheses and translational developments in AD research need to address the crucial therapeutic endpoint – amelioration and/or prevention of cognitive dysfunction. Indeed, the most striking characteristic of Alzheimer's is the progressive decline of cognitive functioning that is caused by massive loss of neurons and synapses. Most importantly, focusing on the behavioral phenotype offers the advantage of avoiding assumptions on the etiopathogenesis of the disease, ones which may be disproved by future studies. The currently-approved medications for AD, which include acetylcholinesterase inhibitors and a N-methyl-D-aspartate receptor (NMDAR) antagonist, offer only minimal temporary improvements in this regard. In testing new therapeutic targets and compounds, mouse models are key resources for providing access to AD-type pathology *in vivo* concurrently with behavioral testing options. The mouse models offer the ability to validate molecular targets and screen potential compounds on the translational pathway leading to clinical testing.

Several cognitive assays are available for assessing mice, particularly in their hippocampal-dependent learning and memory abilities. Given that AD pathology initiates and is most severe in the hippocampus and entorhinal cortex of the medial temporal lobe (reviewed in [6]), these murine cognitive assays that are hippocampal-dependent are ideally-suited for AD research. The use of these cognitive assays versus other readouts maximizes the likelihood of selecting a target or compound that is relevant for memory systems *in vivo*.

Among the cognitive assays that test murine learning and memory, we will highlight and discuss three behavioral tasks that are commonly used to examine associative memory and spatial memory: fear conditioning (FC), radial arm water maze (RAWM), and Morris water maze (MWM). Among their advantages, these tasks are straightforward in implementation and allow for the relatively fast assessment of several batches of mice in a short period of time. However, key parameters need to be well-controlled in order to minimize variability in the results and maximize reproducibility between experiments. In this review, we focus on the practical considerations of these assays – the protocols, guidelines and caveats based on our experience with various AD mouse models.

2. Alzheimer's disease pathology

In Alzheimer's disease, there are two primary histopathological features evident upon post-mortem examination of brain tissue – amyloid plaques and neurofibrillary tangles (NFTs) (reviewed in [1, 7]). The plaques consist of insoluble extracellular deposits of the amyloid-β (Aβ) peptide and can be observed throughout the cortex. Neurofibrillary tangles consist of aggregates of hyperphosphorylated tau, a microtubule-binding protein. Evident with both the

plaques and NFTs, the misfolding and aberrant aggregation of the constituent protein exemplifies a key pathogenic feature of the disease.

Although amyloid plaques were observed histopathologically in AD brains since Alois Alzheimer's early descriptions [8], the composition of the plaques remained unknown for decades. In 1985, researchers were finally successful in purifying Aβ and identifying it as the predominant constituent of the plaques [9]. A vast amount of subsequent research implicated Aβ as the main molecular culprit in AD pathogenesis, in what is classically known as the "amyloid cascade hypothesis". Isolating the peptide then led to the identification and sequencing of the amyloid precursor protein (APP), from which Aβ is produced [10].

APP is a type I transmembrane glycoprotein that is abundantly expressed in the brain, particularly by neurons. APP can undergo a series of cleavages by secretase enzymes, one pathway of which results in the production of Aβ peptides. APP contains α-, β- and γ-secretase cleavage sites. Processing at the α-secretase site releases a large portion of the ectodomain and precludes the formation of Aβ since the cleavage occurs within the Aβ sequence. Alternatively, APP can be cleaved at the β-secretase site, which together with an intramembrane γ-secretase cleavage, produces Aβ peptides. In neurons, the sole β-secretase is β-site APP cleaving enzyme 1 (BACE1), a transmembrane aspartyl protease that generates the N terminus of Aβ. The γ-secretase complex is comprised of four subunits: presenilin 1 or 2 (PS1/PS2), nicastrin, presenilin enhancer 2 (PEN-2) and anterior pharynx-defective 1 (APH-1). There are multiple γ-cleavage site possibilities, which result in the production of Aβ peptides with varying lengths (usually 37–43 amino acids). Approximately 90% of secreted Aβ is 40 amino acids long ($A\beta_{40}$). However, there is also a smaller proportion of 42 residue-long Aβ peptides ($A\beta_{42}$) that make up <10% of the total Aβ pool. In a series of studies, $A\beta_{42}$ was found to have a much higher propensity for aggregation compared to the shorter peptides, leading to a focus on $A\beta_{42}$ as the main amyloidogenic species in AD [11–14].

A strong body of evidence indicates that soluble oligomers of Aβ, consisting of 2 to 12+ peptides, are a primary neurotoxic culprit in AD pathogenesis (reviewed in [5]). In contrast to amyloid plaques, which do not correlate well with cognitive decline, soluble Aβ species are significantly correlated with disease symptoms and severity [15, 16]. These aggregates can be formed from synthetic or natural Aβ peptides, including those secreted by cells or directly isolated from the brain tissue of AD patients and transgenic mouse models. Many studies have established that Aβ oligomers can exert detrimental effects on neuronal physiology and synaptic transmission. For example, Aβ oligomers appear to preferentially bind to or cluster at synapses, with one study observing that >90% of Aβ oligomer binding in neurons occurs at dendritic spines at sites positive for PSD-95, a marker for post-synaptic compartments [17]. On a structural level, Aβ oligomers have been shown to cause changes in spine morphology and decreases in spine density [18, 19]. This loss of synapses is highly relevant for underlying the cognitive impairments of AD, and indeed, it has been found to be a major structural correlate of dementia [20].

Although the receptor(s)/binding partner(s) and downstream signaling mechanisms induced by Aβ oligomers are not fully elucidated, the functional effects on cognition are well-established. High concentrations of Aβ (> nanomolar) – whether chronically or acutely present, synthetic or naturally-derived – can markedly impair neuronal physiology and synaptic plasticity [e.g. long-term potentiation (LTP)] [21–29], an electrophysiological correlate of memory. More importantly, pathological Aβ exposure can strongly impact behavior, including performance in learning and memory tasks [30–34]. This has been thoroughly demonstrated in various AD mouse models, including both genetic (e.g. transgenic) and non-genetic (e.g. acute exposure) models.

Although the predominant focus in AD research and drug discovery has been on Aβ, accumulating evidence indicates that the other major protein that undergoes misfolding and accumulation – tau – plays a key mediating role in the pathogenesis of AD as well as other tauopathies. Tau is a natively unfolded microtubule-associated protein that functions in regulating microtubule stability and therefore, axonal development and transport [35]. As a result of alternative splicing, there are multiple tau isoforms. A primary differentiating feature of these isoforms is the number of microtubule-binding repeats in the C-terminal region, with three (3R) and four (4R) repeat-containing tau as the predominant isoforms in neurons. Both 3R- and 4R-tau are included in the NFTs of AD [36]. However, similar to Aβ, there may be isoform-specific characteristics in the aggregation and fibril-forming capacity of tau [37]. In general, with AD and other tauopathies, the dysregulation of tau phosphorylation and its subsequent aggregation into NFT structures impairs neuronal functioning and ultimately leads to cell death. In fact, recent research points to tau as a requisite mechanistic factor in Aβ-induced pathology – lowering of tau can prevent neurotoxicity in Aβ-treated cultures and AD mouse models [38–41].

2.1 Genetically modified mouse models of AD

Serving as the foundational rationale for many genetically modified AD mouse models as well as strengthening support for the amyloid hypothesis, a large number of mutations in the genes for APP or PS1/2 have been discovered in families with early onset familial AD (FAD) [42–46]. All of these mutations were found to increase total Aβ production, enhance aggregate formation or increase the ratio of $A\beta_{42}$:$A\beta_{40}$, resulting in a higher proportion of aggregation-prone Aβ [47–52]. So far, over 30 APP mutations and almost 200 PS1/PS2 mutations have been identified and linked with FAD [53]. Although FAD accounts for < 5% of all AD cases, there is a very high degree of phenotypic similarity between FAD and sporadic late-onset AD (LOAD), suggesting that mechanistic information obtained about FAD would also be directly relevant for LOAD [54].

Following upon the discoveries of the various FAD mutations, the creation of transgenic mouse models which express the mutated genes further emphasized the pathogenic link between Aβ and AD (Table 1; reviewed in [2, 3]). These mouse models include ones which overexpress mutant human APP, PS1 and/or microtubule-associated protein tau (MAPT). The first such model to be developed was the PDAPP transgenic mouse that overexpresses APP with a FAD-associated mutation (V171F) [55]. Located at the γ-secretase cleavage site, this mutation shifts processing to longer Aβ peptide lengths, thereby increasing the ratio

of $A\beta_{42}$:$A\beta_{40}$. Another widely-used model is the Tg2576 mouse line, which overexpresses human APP with two point mutations (K670N, M671L) that were originally identified in a Swedish family with FAD [43, 56]. These mutations are located by the β-secretase site and bias APP processing towards the amyloidogenic pathway, leading to higher overall levels of Aβ.

Transgenic mouse models have demonstrated that overexpression of APP and/or Aβ is sufficient to recapitulate many important characteristics of AD. In general, the mutant APP-overexpressing mice develop amyloid plaque pathology, loss of synapses, impaired synaptic plasticity and cognitive deficits. Co-expression of a mutant PS1 transgene accelerates the pathology to a significantly younger age, as evidenced by the double transgenic APP/PS1 model [66, 67]. A caveat with some of the AD mouse models is that there is minimal widespread neurofibrillary tangles and neurodegeneration, a defining characteristic of AD. However, a triple transgenic mouse model (3xTgAPP) that expresses mutant tau along with the APP and PS1 transgenes has been reported exhibit significant neurodegeneration along with neurofibrillary tangles [70]. In addition, the removal of nitric oxide synthase (NOS) 2 in mice expressing mutated APP results in the development of a much larger spectrum of AD-like pathology, including amyloid plaques, tau pathology, neuronal loss and behavioral impairments [76, 77].

In AD research efforts, transgenic mouse models have been valuable tools in elucidating disease mechanisms as well as testing potential therapeutic strategies. In particular, behavioral testing with cognitive assays are a primary component of their utility and value. The transgenic APP-overexpressing mouse models develop cognitive dysfunction at ages ranging from 2 to 12 months, depending on the specific transgenes. In the PDAPP mouse model, significant memory impairments are evident after 6 months of age in several behavioral tasks including FC, RAWM and MWM. In the Tg2576 mouse model, the onset of the performance impairments in these tasks varies depending upon the task, from approximately 4 months with FC [78–82] to more than 6 months with the RAWM and MWM [83, 84]. In general, deficits at the earlier ages tend to be more subtle but usually progress to marked impairments at older ages. Significantly, behavioral impairments can start prior to the accumulation of Aβ plaques, a fact that highlights the central importance of soluble Aβ species in AD pathogenesis.

Mutations in the MAPT gene are associated with familial fronto-temporal dementia (FTD) and parkinsonism linked to chromosome 17 (FTDP-17). These mutations generally decrease tau binding to microtubules or increase the proportion of 4R-tau, which enhances susceptibility to aggregation. Various transgenic mouse models expressing mutant human MAPT have been developed and do recapitulate tau pathology characterized by hyperphosphorylation and accumulation into NFTs [64, 65]. However, due to significant pathology in the brainstem and spinal cord in many of these strains, there can be age-dependent motor defects that prevent their use in behavioral tasks such as FC, RAWM and MWM. However, newer models with inducible tau transgene expression [63, 85] as well as the 3xTgAPP model [70] are reported to exhibit less motor-related pathology and have been characterized in behavioral cognitive assays.

Although no current model exhibits all of the signs, symptoms and anatomopathological hallmarks of AD, the use of mouse models has greatly contributed to a better understanding of the pathophysiology of the disease and enabled the development of novel therapeutic strategies. In general, in picking an AD mouse model, the mice should present an increase in Aβ levels and/or hyperphosphorylated tau along with synaptic and memory deficits as well as synaptic and neuronal loss (i.e. a human AD-like phenotype).

2.2 Non-genetic mouse models of AD

Although genetically modified models of AD have been very valuable resources for investigating the mechanisms underlying disease pathogenesis and progression, the overexpression of AD-related genes might mislead the interpretation of the findings derived from these studies. For instance, the APP and PS1 transgenes could affect neuronal function through a variety of mechanisms that are not necessarily related to Aβ and/or AD-like pathology [86, 87]. Indeed, the trafficking and signaling properties of the full-length form of APP and its natural cleavage products, which include Aβ, soluble APPα (sAPPα), sAPPβ and amyloid precursor protein intracellular domain (AICD), are likely different in non-disease physiology and could therefore impact CNS development, synaptic function and memory with overexpression. To separate Aβ-specific effects from the other effects of APP and PS1 overexpression, we and others have often used models of the disease in which Aβ *per se* would be responsible for observed deficits (Table 2). Given that natural oligomers of human Aβ as well as synthetically produced human Aβ oligomers impair LTP [21–29], oligomeric Aβ directly infused into the ventricles or hippocampi of adult animals provides an acute Aβ pathology model. The Aβ can be infused via pre-implanted cannulas, which facilitate direct delivery and help avoid issues related to the blood brain barrier [30–34]. These experiments have shown that Aβ *per se* is capable of impairing different types of memory including associative and reference memory, suggesting that the peptide plays a direct role in memory impairment in general.

Another advantage of using the Aβ-infusion model is the possibility of using animals other than mice. For instance, intracerebroventricular infusion of Aβ in rats induced an impairment of MWM performance [88–93], an effect that was worsened by a concomitant cerebral hypoxia [94]. Additional advantages of using this model include the low costs and significantly less labor/time requirements compared with developing a transgenic mouse colony. However, disadvantages of using the Aβ infusion model are: i) it does not reproduce the characteristic hallmarks of AD represented by senile plaques, neurofibrillary tangles and neuronal loss; ii) it requires a very careful methodological standardization of Aβ preparation and efficacy testing in interleaved experiments; iii) it does not allow testing of a treatment on disease progression; iv) the animals are cannulated, which can be an issue for behavioral studies since cannulas can fall out, and multiple injections can stress animals, making it difficult to follow animals over time.

Following upon the discovery of extracellular soluble tau in the cerebrospinal fluid (CSF) of both healthy individuals and, in higher amounts, in AD patients [95–100], our group first tested the effect of tau oligomers on synaptic function and demonstrated that, similar to Aβ, tau oligomers are capable of impairing LTP [101, 102]. These experiments led to

investigations on whether tau oligomers infused onto the dorsal hippocampi are capable of affecting memory. Consistent with the LTP results, it was found that memory was impaired in oligomeric-tau infused mice [71, 103, 104]. Taken all together, these studies demonstrate the potential of using alternative, acute exposure models for investigating AD pathogenesis and drug testing (Table 2).

3. Behavioral Studies

The medial temporal lobe (MTL) has been recognized as a critical region for explicit or declarative memory since the famous case studies of memory impairment resulting from hippocampal damage in H.M. and other patients [105]. The MTL belongs to the limbic system, which supports a variety of functions such as learning, memory and emotional behavior via the integration of multiple structures including the hippocampus and the regions surrounding it (perirhinal, parahippocampal, and entorhinal cortex), the amygdala, the cingulate gyrus, the fornix, the mammillary body, the septal area, the piriform cortex, the anterior thalamic nuclei, the hypothalamus, the epithalamus, and other structures. The hippocampus, in particular, has been recognized to be critical for spatial memory and is therefore the focus of this review [106–108]. Because of its vulnerability to damage at the earliest stages of AD, the hippocampus is considered fundamental for understanding the disease pathophysiology [8, 109–114]. Indeed, most of the behavioral protocols aim to measure hippocampal-dependent memory, and many efforts have been made to develop models of human memory in monkeys and rodents [115, 116]. However, differences across species should be considered; for example, the hippocampus codes spatial and episodic verbal memory in humans [117–119], whereas in rodents it is mainly associated with spatial navigation and a highly represented olfactory behavior [120–125].

Among the various types of behavioral paradigms, we will mainly consider behavioral tasks that might reflect defects in humans affected by AD. For instance, the FC task studies associative memory that is affected in AD patients. The RAWM task studies short-term memory (STM), a type of memory that is affected early in the disease [126]. The MWM task studies long-term memory (LTM) which is affected at late stages of the disease. Regardless of the experimental paradigm, it is crucial that the behavioral task is designed and conducted appropriately by taking into account the species-specific behavioral characteristics, the correct interpretation of the results, and the biological mechanisms underlying the type of memory studied, other than strain, age, sex, and the peculiar features of genetic modified animal models.

3.1 Fear Conditioning (FC)

Memory can be distinguished as explicit and implicit; the latter can be further differentiated as non-associative (habituation and sensitization) and associative learning (classical and operant conditioning). FC is a form of associative learning based on the classical or Pavlovian conditioning, which involves the learning of relationships between two stimuli: a neutral stimulus, named conditioned stimulus (CS), causing a weak responses that typically has no relation with the task to be learned (e.g. light or sound); and a stimulus able to evoke a typical behavior response (e.g. salivation) known as unconditioned stimulus (US). The Russian physiologist Ivan Pavlov (Nobel Prize for Physiology or Medicine in 1904) was the

first to demonstrate the phenomenon of classical conditioning. Having noticed that the mere sight of the plate with food induced salivation in dogs (unconditioned response - UR), he decided to investigate whether salivation could be induced in response to a neutral stimulus, such as the sound of a bell, associated with the meal presentation. While the sound of the bell itself did not evoke any response, after a few trials in which the sound was coupled to the food, dogs began to salivate before receiving the food in response to the simple sound of the bell (conditioned response - CR). This kind of conditioning was called “classical reward conditioning”, or appetitive conditioning since the US was followed by a pleasant event such as food. In contrast, aversive or defensive FC occurs when the US was followed by an unpleasant event (such as an electrical shock) triggering a series of automatic responses e.g. freezing. The important variables in the phenomenon of Pavlovian conditioning, and thus of FC, are: i) the CS should be administered before the US; ii) the interval between CS and US is critical for most of the examples of conditioning; iii) CS and US should be contiguous; iv) if the CS is repetitively presented in the absence of the US, the CR will decrease in intensity until it disappears according to the phenomenon of extinction, which also involves the learning of new information and is therefore not an equivalent of forgetting. Classical conditioning has the advantage of being long-lasting and quickly-acquired, thus allowing the additional testing of memory persistence [127]. FC can be investigated both in animal models and humans, e.g. as a model for post-traumatic stress disorder [128]. It is a useful tool for investigating both emotional and contextual memory, and by extension, the integrity of the main brain structures involved: the amygdala and the hippocampus, respectively. Furthermore, FC has the advantage of being much faster than other behavioral tasks since it requires only one day of training. Finally, it offers the advantage of precisely confining to a very short time (we use 2 seconds) the moment at which contextual learning occurs (an advantage that turns very useful for biochemical experiments).

3.1.1 Methodology—FC has been deeply investigated by LeDoux’s and Fanselow’s research groups (for reviews see [129–132]. In the most commonly used procedures, a one-trial learning of the CS, represented by a light or a sound, is paired to an electric foot shock (aversive US). Rodents will learn to associate: i) the light or the sound with the aversive stimulus, which is evident when the CS is presented again to stimulate remembrance of the foot shock (Cued FC); ii) the place or context where they received the aversive stimulus, which is demonstrated when they are later placed in the same context (e.g. the conditioning chamber) to stimulate memory of the foot shock (Contextual FC). Following an aversive stimulus, the CR will be represented by fear [133], which is physiologically accompanied by a series of behavioral responses evolved for escaping from the source of danger [134]. The fight-or-flight sympathetic response [135] involves an increase of catecholamine, ACTH and cortisol secretion along with a consequent increase of function in cardiovascular, respiratory systems, and an inhibition of parasympathetic functions such as digestion. In rodents, several studies have demonstrated that the defensive behavior associated with fear is represented by freezing [136]: a total absence of movements except for those necessary for breathing. This is thought to be an innate defense mechanism aimed at feigning death in front of a predator.

Although several protocols have been proposed [see for example [137], here we will describe a procedure that has been successfully used in our laboratories to investigate both contextual and cued conditioning in animal models of AD [33, 34, 138–144] (see Fig. 1 for a picture of the apparatus). Mice are tested individually in a conditioning chamber that is located in a sound-attenuating box with a clear Plexiglas window that allows video recording of the experiment. The conditioning chamber has a 36-bar insulated shock grid floor. The floor is removable, and after each experimental subject, it is cleaned with 75% ethanol and then again with water. To provide background white noise (72 dB), a single computer fan is installed in one side of the sound-attenuating chamber. Mice are handled once a day for 3 days before behavioral experiments. Only one animal at a time is present in the experimentation room. It is placed in the conditioning chamber for 2 minutes, following which a discrete tone (CS) at 2800 Hz and 85 dB is delivered for 30 sec. In the last 2 sec of the tone, the mouse receives a foot shock (US) through the bars of the floor (0.50–0.80 mA for 2 sec). The experimenter might regulate the intensity of foot shock; for example it is possible to elicit stronger memory by using a 0.75 mA foot shock. After the CS/US pairing, the mice are left in the conditioning chamber for another 30 sec and are then placed back in their home cages. Freezing behavior can be scored by using dedicated software. In our personal experience, double-checking by manually scoring freezing time has been very useful. Twenty-four hours after training, the mouse is placed back in the conditioning chamber. This allows the evaluation of contextual fear learning, since a mouse with intact contextual memory should be able to recognize the context where it received the foot shock the day before. Freezing is measured for 5 consecutive minutes. To evaluate cued fear learning, we replace the mouse in the conditioning chamber 36 hours after training. In this case, we create a novel context by inserting a triangular cage with smooth flat floor and with vanilla odorant. After 2 min (pre-CS test), the mouse is exposed to a tone with the same characteristic of the tone used during training for 3 min (CS test), and freezing is measured. To obtain a sample appropriate for statistical analyses, we usually use 15–20 animals per condition.

An important control that needs to be performed during testing of fear memory is the sensory threshold assessment. Changes in the sensory perception of the shock might be due to the experimental manipulation leading to a false attribution to memory of the observed phenotype. A sequence of single foot shocks is delivered to animals placed on the same electrified grid used for FC. Initially, a 0.1 mA shock is delivered for 1 sec, and the animal's behavior is evaluated for flinching, jumping, and vocalization. At 30 sec intervals, the shock intensity is increased by 0.1 mA to 0.7 mA and then returned to 0 mA in 0.1 mA increments at 30 sec intervals. Threshold to vocalization, flinching, and then jumping is quantified for each animal by averaging the shock intensity at which each animal manifests a behavioral response to the foot shock.

3.1.2 Brain regions involved in FC—Whereas the amygdala is the main brain structure in the fear circuit that is involved in the formation and storage of emotional memories, the hippocampus participates in learning the context that triggers fear [129–132, 145–150]. Lesion studies performed in rodents have demonstrated that the amygdala is involved in the fear responses triggered by both the cue and the context; when the hippocampus is damaged,

both pre- and post-training, only contextual conditioning is impaired [146, 151, 152]. Thus, the amygdala has a primary associative role in the processing of fear, whereas the hippocampus communicates the sensorial representation of the context that informs the amygdala of the environment of the dangerous event. It acts as a sensory relay for visuo-spatial, auditory, olfactory, or other sensory input, thus requiring the integrity of thalamus [153] (reviewed in [131]), and as a motor relay, since hippocampal lesions can also result in increased locomotor activity that affects freezing [151]. Contextual conditioning occurs also in the absence of the cue -the impairment of contextual conditioning by lesions of the hippocampus has been related with loss of a conjunctive representation of the features of the context [154]. Once the hippocampus has recognized and consolidated these features, contextual information would presumably be stored in the neocortex.

At a molecular level, contextual FC is based on a form of synaptic plasticity sharing mechanisms with NMDA-dependent LTP. Actually, administration of an NMDA antagonist into the dorsal or ventral hippocampus selectively impairs contextual FC [151, 155]. Also NMDA modulation in other brain areas may affect contextual memory. For example, at the dorsolateral periaqueductal grey, inhibition of NMDA receptors, neuronal NOS, and soluble guanylyl cyclase attenuate contextual FC in rats [156], whereas activation of NMDA receptors in the medial prefrontal cortex has been found to be needed for the acquisition of both trace and contextual fear memories [157, 158]. The involvement of cAMP/PKA and nitric oxide/cGMP/PKG pathways in contextual fear learning and consolidation has been widely demonstrated by pharmacological and genetic approaches in hippocampus, amygdala and prefrontal cortex [159–165], and has the role of the transcription factor c-AMP-Responsive-Element binding [CREB; [166–168]. Cholinergic, serotoninergic and GABAergic signals have been also demonstrated to be crucial in contextual FC [160, 169–179].

3.1.3 FC in AD models—Conditioned fear responses have been demonstrated to be impaired in patients with mild to moderate AD [180, 181]. Consistent with these findings, the Tg2576 mouse model showed an impairment of contextual fear learning at 4–6 months [78–82] when plaques are not yet detectable, but the Aβ42:Aβ40 ratio is modified [80]. This defect worsens with age, [78, 182] and becomes profound at 12–14 months [183, 184]. It should be also noted that the same animals have provided different results in different studies assessing cued learning which was found to be either normal [183, 184] or altered [185].

Double transgenic APP/PS1 mice present an impairment of contextual FC starting at about 3 months, whereas cued FC is normal, indicating that the learning deficits is based upon the hippocampal dysfunction [78, 138, 140, 186]. In these mice the behavioral changes are concomitant with the increase of Aβ and early plaque deposition. Some authors have shown a reduction of auditory FC in aged APP/PS1 mice at 12–14 months, notwithstanding a very low presence of plaques in the amygdala [187].

The APPswe/PS1dE9 mouse, another double transgenic model of AD, shows early contextual memory impairment [188, 189], even if some authors have detected it at older age [14-months; [190], and others have found normal spatial learning and memory, FC, and

sensorimotor gating at 7 months, with abnormal social recognition memory [191]. Additional transgenic models that present abnormal contextual fear memory before the onset of amyloid plaques are the PS1M146V knockin mice [192], the APP-SL 7-5 transgenic mice overexpressing human APP695 harboring the double Swedish and London mutations [193], the APP J20 carrying the APP Indiana (V717F) and Swedish (K670M) mutations [194], the 5XFAD mice (Tg6799 line) that co-overexpress FAD mutant forms of human APP (the Swedish mutation: K670N, M671L; the Florida mutation: I716V; the London mutation: V717I) and presenilin 1 (PS1) (M146L; L286V) [195]. Finally, it has been demonstrated that contextual memory is impaired in acute models of AD induced by i.c.v. or hippocampal [33] injections of oligomeric Aβ42 [34, 196].

3.2 Radial Arm Water Maze (RAWM)

The RAWM was designed to measure spatial learning and memory performances in rodents [197–199], as a combination of dry radial arm mazes (RAM; [121, 200] and MWM [201]. Dry RAM consists of a platform equipped with a variable number of spaced arms radiating from the center. Rodents should enter one of the arms, using spatial cues in the room, to reach food or water. This kind of task requires both working memory (WM) when retaining information for a very short time and reference memory when retaining memory for longer times. The limitations of RAM include the deprivation of food or water and the presence of confounding olfactory signals. Moreover, dry RAM is less sensitive in detection of search strategies than MWM, because once the rodent has chosen an arm, it goes to the end of it expecting the reward. Thus, RAM is more useful for investigating changes of error patterns than searching patterns. Another difference between RAM and MWM entails the acquisition time. Indeed, spatial learning can be achieved in about 6–10 trials in MWM, whereas for RAM acquisition rate depends upon the protocol used ranging from 2 days to 3–4 weeks [for a review on RAM and MWM comparison, see [202]].

Thus, the advantages of RAWM result from the ability: i) to combine a complex spatial environment with an easy way of measuring animal performances by counting errors, which does not require a video tracking system or computers; ii) to give animals a strong motivation (to escape from water) without requiring food deprivation or foot shock; iii) to possibly test both WM and reference memory.

3.2.1 Methodology—Several RAWM protocols have been used, with differences in the apparatus and animal acquisition phase [83, 198, 199, 203, 204]. Here, we will describe two different protocols that have been used in our laboratories, one requiring three weeks, mostly testing WM [83, 138, 140, 205], and another one requiring two days, testing reference memory [33, 34, 204], respectively.

The RAWM apparatus is the same described for MWM (see below), except for the addition of arms [from 4 to 16, depending on the pool size and the chosen procedure; [206]], that are equidistant and radiating from the center. Arms can be made of aluminum, Plexiglas or plastic. In our apparatus, we have used a 110 cm plastic large circular pool in which we inserted 6 arms (height 50 cm) made of white-glazed aluminum and fixed one to each other to form a 60° angle at the center between two adjacent arms (see Fig. 2). Each arm is 35 cm

long, and the central free area has a diameter of 40 cm. Each swim alley is 25 cm wide. We label each alley with a number (1–6) by placing a label on the pool edge where mouse cannot see it (it should not be a cue). The pool is filled until 25 cm of the arms are submerged by water and 25 cm are outside. We add non-toxic paint to increase water opacity.

As in MWM, rodents are tested individually and the goal of the mouse is to escape from water by finding a submerged platform positioned at the end of one of six arms. In the three week “long” protocol, the platform remains in the same location for each day of trial; this location is changed randomly day by day. On each day, the mouse undergoes 4 consecutive acquisition trials (named A1–A4). For each trial it starts the test from a different randomly chosen arm, so that it has to rely on its STM of the platform location based on spatial cues present in the room, and not on its LTM of the platform location from previous days. For each trial (lasting up to 60 seconds), the experimenter counts the errors made by the mouse each time it enters the wrong arm or when the animal needs more than 15 seconds to make a decision. Indeed, as in MWM, thigmotaxis or passivity can occur, whereby a mouse floats or swims in the central area without choosing an arm. After each error, the mouse is gently pulled back to the starting arm for that trial. After four consecutive acquisition trials, the mouse is placed in its home cage for 30 minutes and then returned to the maze and administered a fifth retention trial (R), which is an indicator of STM. Animals are trained for 3 weeks, i.e. until the wild type (WT) control mice reach asymptotic performance (an asymptotic level of one to three errors on A4 and R). The scores for each mouse on the last 3 days of testing are averaged and used for statistical analysis.

In the “short” 2-days RAWM version of the task, the goal arm does not vary from day by day but is kept constant for all trials, with a different starting arm on successive trials. This protocol allows reaching of the learning criterion in only 2 days. During the first day, the mouse is trained in 15 trials (T1–T15) to identify the platform location in a goal arm by alternating between a visible and a hidden platform from T1 to T12 (beginning with the visible platform in the assigned arm). In the last four trials (T13–T15) only a hidden platform is utilized. During the second day the same procedure is repeated by using only the hidden platform from T1 to T15. An entrance into an arm with no platform, or failure to select an arm after 15 sec is counted as an error and the mouse is returned to the start arm. Each trial lasts up to 60 sec, and at the end of each trial, the mouse is kept on the platform for 15 sec. The number of errors and time to complete the trial is recorded. The average of errors made in 3 consecutive trials for each mouse is calculated and used for statistical analyses.

We have found this procedure highly suitable for rapidly testing STM. A confounding factor could be the physical fatigue that results from 15 consecutive trials, which may interfere with the learning process. For this reason, spaced practice training might be established by running the mice in cohorts of 4 and alternating different cohorts through the 15 training trials over 3 hour testing periods each day. The goal platform location will be different for each mouse. After all of the mice in cohort 1 have performed a trial to locate a visible platform, this is switched from visible to hidden. After each mouse from cohort 1 completes six alternating visible-hidden trials, they are left to rest under a heating source, and mice

from cohort 2 are tested in the same way. When all the cohorts have performed the T1–T6 trials, all of the cohorts are tested again to complete the T7–T12 trials in a similar alternating fashion. At this point, all mice have to perform the last 3 hidden platform trials (T13–T15).

In conclusion, in the "long" RAWM protocol, mice are required to learn that the platform is maintained in the same position on each trial within a day, but it is moved to a different arm each day; in contrast, in the "short" RAWM protocol, the goal arm does not change day by day.

As for all experiments, one must be assured of the validity of the controls. Thus, if a large number of WT mice have not reached the learning criterion in the final three trials, more days of training can be added.

In both versions of the RAWM, on the day after the last test, the mice undergo a visible test to control for possible motivational, visual and motor deficits. This is very important especially when using genetically modified animals; for example, certain strains can inherit a retinal degeneration mutation leading to blindness [207, 208]. Following testing with the RAWM, animals undergo two daily sessions (each consisting of three 1 min trials) for two days, and the time taken to reach a visible platform (marked with a green flag) randomly positioned in a different place each time is measured. Moreover, both during the hidden and the visible test it is possible to measure swimming speed and distance travelled to reach the platform in order to analyze possible motor effects on the performance. Indeed, an animal can reach the platform faster or slower, not because of an alteration of its spatial memory skills but because of potential effects on its swimming or sensory abilities. An impairment of the RAWM test with normal performance on the visible test indicates that the mouse has a memory impairment that is not related to motivational, visual and motor skills.

Finally, when assessing performance with the RAWM, the following factors should also be taken into account: i) differences between rats and mice, especially in correlation with the different strains of mice (see below); ii) differences between males and females, even if the literature is contradictory regarding better WM performances in males [209] or females [206, 210]; for this, we suggest performing behavioral tests in sex-balanced groups of mice; iii) methodological factors such as size of the pool, lightening, procedures; iv) animal handling. Another essential factor is the age of the animals, which will be discussed in detail in the next section.

3.2.2 RAWM and Working memory—The definition of WM is still an issue of debate in the neuroscience community. Another issue is the difference between rodent and human WM. Memory can be classified by a temporal or qualitative criterion: the first takes into account the time that the memory of the information remains effective, whereas the second distinguishes memory according to the type of information retained. Thus, depending of the "length" of memory, we can distinguish: i) iconic memory and echoic memory, two forms of very short sensory memory for visual or auditory stimuli; ii) STM, which is able to hold information only for few seconds if it is not transferred to iii) LTM, which is a form of permanent memory theoretically able to store an indefinite amount of information for a life-time. Conversely, memory classification according to a quality criterion differentiates

declarative or explicit memory and non-declarative or implicit or procedural memory. Declarative memory refers to specific personal events characterized by “warmth and intimacy”, as described by William James in 1890 [211]. It is autobiographical, and events are recalled and placed in relation to the time of life of the individual in which they occurred [see [212]]. Conversely, non-declarative memory is a typical memory process aimed at recollecting how to perform an action by identifying or using a stimulus that had already been presented. Non-declarative memory is characterized by unconscious influences of past experiences, which are manifested by changes in the speed or the ways in which the same task is performed. It includes motor learning, perceptual representation system (to identify words and objects on the basis of their shape and structure), procedural memory (to acquire skills and habits through repeated exercises), and WM. In particular, the latter is considered a memory system entrusted with the task of manipulating the information temporarily in order to maintain it for basic cognitive activities such as comprehension, reasoning and problem solving. The term “working memory” was coined in 1960 in relation to the new theories comparing the mind with computer functioning [213]. Before, neuroscientific debate focused on STM as the ability to remember information for a short time (on the order of seconds) as opposite to the more durable LTM.

What is WM in rodents? How is it possible to differentiate WM from STM by using behavioral testing in animals? The first attempt was made in the late seventies [120, 121, 214] by using behavioral tasks such as the RAM in which WM was considered the capacity to remember which arm a rodent had visited in a session. Indeed, this form of memory is transient and it serves to carry out a specific task in a given time. In an interesting review, Dudchenko defines WM as “a short term memory for an object, stimulus, or location that is used within a testing session, but not typically between sessions and it is distinguishable from reference memory, which is a memory that would typically be acquired with repeated training, and would persist from days to months” [215]. Particular emphasis is also given to the concept of forgetting because WM, once used, is immediately forgotten. This confirms that WM is a type of STM typically used within a testing session, but not between testing sessions, because the information will be deleted after its use [215].

As mentioned before, WM temporarily retains information for manipulation in carrying out complex cognitive tasks [216]. Thus, it requires both spatial and procedural memory and relies upon the same brain structures described for MWM, particularly the hippocampus and medial prefrontal cortex. This cortical region is mainly involved in the temporary storage and processing of information lasting up to seconds, whereas the hippocampus is more critical when a longer storage is required [217].

3.2.3 RAWM in Alzheimer’s disease models—WM performance is sensitive not only to damage of the above-mentioned brain areas, but also to normal aging [218], and neurodegeneration such as AD neuropathology [219]. Indeed, testing WM deficits is essential in Tg mouse models of AD to establish the progression of the disease and the effectiveness of potential new drugs. Although WM might also be tested by a modified version of MWM [220], we have found RAWM to be the most reliable task for investigating WM in AD animal models. RAWM was first used in 1985 [197] by inserting an 8-arm radial maze into a water tank where rats were forced to swim and escape onto a bench in one of the

maze arms, with the position of the submerged platform changing for each trial. Since then, several protocols have been utilized and the RAWM task has also been modified to study reference spatial memory in a short term paradigm that lasts only 2 days, particularly suitable for AD models [33, 34, 204].

Several studies have been performed using RAWM to detect an impairment of WM in animal models of AD, but results are controversial especially with respect to the age at which RAWM detects a WM deficit [221]. In single Tg2576 transgenic mice the impairment of WM has been detected at 4.5 [222] or 8–9 months of age [223, 224]. Wilcock et al. [225, 226], conversely, found a RAWM impairment in APP mice at old age (from 24 months), but results are not available for younger animals that were not tested for RAWM. Results are also contradictory in APP/PS1 mice. In APPswe/PS1dE9 some studies demonstrated an impairment of RAWM performances starting at 6 months of age [227], whereas in other papers transgenic mouse performance was normal at 7 months of age and reduced at 13 months [228] or at 10–15 months [229]. The group of Morgan also showed that RAWM was impaired starting at about 15 months of age in both APP and APP/PS1(line 5.1) mice, whereas they learned and remembered the platform location at 6 and 11.5 months of age [230, 231]. Recently, Webster et al [232] have studied RAWM in four different age groups (7, 11, 15, and 24 months) in APP/PS1 knock-in mice characterized by the introduction of a Swedish FAD K670N/M671L point mutations, the humanization of the mouse β-amyloid sequence, and the introduction of a P264L mutation in the PS1 gene. These mice performed worse than WT controls in the RAWM task starting at 15 months of age, and the increase of errors paralleled the increase of age and the disease progression. In our laboratory, we have also demonstrated that the worsening of WM increased with age, in parallel with the accumulation of Aβ. However, WM was affected as early as 3 months of age in APP/PS1 (line 6.2) mice [83, 138, 140] as demonstrated by the increased number of errors at A4 and R compared with WT littermates. This impairment worsened in older animals (6–8 months). Conversely, reference memory tested by MWM started to be impaired at 6–8-months in Tg mice [83, 138, 140].

The choice of the experimental samples groups is fundamental. For example, if we aim to demonstrate the positive effect of a drug on AD onset and progression, we need to use 4 groups of animals: AD model without treatment; AD model with treatment; WT littermates without treatment; WT littermates with treatment. If littermate controls are not available, WT mice of the same sex, age and with the proper genetic background should be used. This simple experimental scheme provides important information: i) AD model without treatment should present synaptic or cognitive deficits at the appropriate age when such dysfunction is known to be present; ii) AD model with treatment, as the primary experimental group, may demonstrate a change in synaptic plasticity and memory measures due to the treatment; iii) WT without treatment should not present deficits, and synaptic and memory measures should be consistent with the known data on the strain; iv) WT with treatment usually do not present a change in synaptic plasticity and memory when the treatment selectively acts on a disease-related pathway; however, in some cases, an alteration of synaptic plasticity and memory is possible if the drug affects the physiological mechanisms at the basis of learning and memory. Moreover, if the treatment is expected to slow the onset of the disease, it should be started earlier than a drug designed to treat the overt disease.

In conclusion, despite the possible differences due to the protocol and the strain used, we believe that RAWM is a useful task to investigate early STM impairment in animal models of AD.

3.3 Morris Water Maze (MWM)

The MWM was first recognized by Richard G. Morris in 1981 [201] as a behavioral test for hippocampal-dependent spatial learning and long-term spatial memory in rodents. It is now one of the most used tools in behavioral neuroscience due to a number of advantages [233–236]: i) simple and fast execution, no pre-training period, and relatively low number of animals required; ii) possibility of differentiating between spatial learning and long-term spatial memory, and testing of non-spatial abilities such as visual and motor performance; iii) reduction of olfactory interferences, which are known to rely on the hippocampus; iv) absence of unpleasant procedures based on the delivery of an electric shock or food deprivation; v) low costs since the apparatus can be easily made. Along with many other research groups, we have successfully used MWM to test spatial memory impairment in models of AD [83, 138, 140] and aging [237], and to evaluate the effects of drugs with enhancing or deleterious effects on cognition.

3.3.1 Methodology—Several papers have described the MWM methodology (reviewed in [233, 236, 238, 239]) since the apparatus and/or the training procedures can exert significant influences on spatial performance. Here, we will describe the most commonly used apparatus and protocol that we have personally tested in recent decades with good results and a high degree of reproducibility in both normal, aged and genetically modified mice models of AD [32, 83, 138, 140–142, 237].

Briefly, the apparatus consists of: i) a plastic large circular pool (height: 75–80 cm; diameter 150–200 cm for rats and 90–120 cm for mice) filled with water (up to about 40 cm below the edge to prevent the animal jumps out), maintained at about 25°Cand made opaque to hide the submerged platform, e.g., by the addition of nontoxic white paint, powdered milk or synthetic opacifiers; 2) a submerged platform (1 cm under the surface of the water) measuring 10 × 10 cm, preferably made of Plexiglas, non visible to the swimming animal (hence the name “hidden test”); 3) stationary geometric cues made by different objects usually placed on the 4 cardinal points of the maze (we have found this to be more efficacious) or on the walls of the room but visible by animals during swimming; it is important that cues are not moved during the test; 4) a camera mounted on the ceiling and connected to a video tracking system and software for motion detection. If this system is based on the color difference between the maze and the animal, one should use a white animal on a black background or, in our case, a black mouse (e.g. of the common C57Bl/6 genetic background) in a white maze (see Fig. 3).

During the entire protocol the role of the experimenter should be carefully considered. It is preferable for the experiment to be conducted after a period of handling by only one experimenter. Moreover, the experimenter should remain stationary in a constant location (or leave the room) because he/she represents a distal cue for the swimming animals.

Finally, it is important to perform the experiment in at least three cohorts, possibly by different experimenters (we adopt a similar precaution also for FC and RAWM).

Rodents are tested individually. First, animals undergo a training period that can last from 2 to 10 days; this training reveals the ability of the animal to learn in relation to spatial cues. Animals are placed into the pool where they learn to locate the hidden platform beneath the surface of the water. In our protocol, mice are trained for 3 days in 2 daily sessions each consisting of three 1 min trials. Conventionally, the maze is divided in 4 quadrants by a cross whose vertices represent the 4 cardinal points: North (N), South (S), East (E) and West (W). For each trial the animal is placed in the water starting from a different randomly chosen quadrant (that does not contain the platform), whereas the platform is always positioned in the same place (SW). If the animal fails to find the platform within the given time (60 sec), the trial is considered over and the animal is placed on the platform for 15 seconds before starting with the second trial. Sessions are held 4 hrs apart, so that the mice have to rely on LTM of the platform location. Typically, the time taken to reach the hidden submerged platform (escape latency) is recorded; this time measure progressively decreases, which produces a characteristic learning curve. However, since the ability to reach the platform can be based on a praxis strategy (animals learn sequence of movements), a taxon strategy (animals recognize proximal cues), or a spatial navigation strategy (animals map the spatial environment and recognize distal cues) [240], the video tracking system should also allow the measurement of non-mnemonic behaviors or strategies (length of the swimming path, path directionality, or cumulative distance to platform [241]).

After this acquisition training, on the 4^{th} day (or in any case, 24 h after training) a probe trial is usually performed. The platform is removed from the pool and mice are allowed to freely swim for a certain period of time (we perform four 1-min trials, for each animal starting from a different cardinal point). For the probe analyses, the maze is virtually divided into 4 quadrants, the target quadrant (TQ; i.e. the one previously containing the platform), and 3 non-target quadrants (AL = adjacent left, AR = adjacent right, and OQ = opposite quadrant). The percent time spent in each quadrant seeking the platform is analyzed (usually, a normal mouse will spend about 35–40% or more of the time in the TQ). Moreover, it is possible to examine the number of times it crosses the platform area and the travelled trajectory.

As with the RAWM, a visible test is used to assess motivational, visual and motor abilities. We usually perform this test with the same protocol as the hidden acquisition training during the 5^{th} and 6^{th} day. An impairment of the hidden acquisition test with normal performance on the visible test indicates that the mouse has a spatial memory impairment that is not related to motivational, visual and motor skills.

3.3.2 Factors that may influence MWM performance—MWM was originally designed to study spatial memory in rats [201, 233]. However, mice have recently become the most used animals for studying the pathophysiology and therapeutic targeting of diseases due to the availability of transgenic models [242]. In this regard, it should be noted that mice perform more poorly than rats in MWM [243], partially due to the fact that their performance can be affected by thigmotaxis (the tendency to swim near the wall of the maze) and passivity (mice tend to float more compared to rats) [236, 244]. In these cases,

the experimenter could intervene by prodding the mouse with an object. Alternatively, the mouse can be left in the water until the end of the trial and then tested again on the same day or the day after. If it continues to fail, the mouse should be removed from the data analysis. Maintaining a low water temperature increases the aversive environment and thus lessens the potential impact of thigmotaxis and passivity. In any case, it should be noted if an entire experimental group consistently exhibits atypical behavioral tendencies that can influence MWM results, especially when testing transgenic mice [242]. Among WT mice, C57Bl/6 animals are considered the better performer of MWM experiments [208, 245, 246], whereas albino mice, due to their visual impairment, tend to show impairments in MWM [247].

In general, lighting is another factor to be considered when performing tasks that depend upon the ability to process visuo–spatial information. Light should be soft and diffuse, and there should be no light reflections in the water. It is important to keep in mind that rodents are nocturnal animals that usually rely on olfactory capabilities rather than visual acuity [123, 248, 249], with the former not being involved in MWM.

Steroid hormones also affect hippocampal function and influence animal responses to stress [250–252]. Indeed, it is important to minimize the amount of stress with a correct period of pre-training handling, soft lighting, and an appropriate manipulation during the test (i.e. lift mice out of the water on the hand rather than by the tail). If very high-anxiety behavior is noticed, anxiety-specific tests [253, 254] can be conducted, especially in transgenic mouse models, to exclude anxiety-related effects on performance in the memory tests.

Behavior can be also influenced by viral, bacterial and nematode infections [255–258]; thus, a veterinary health control must be up to date.

Another important factor is age. MWM performance declines with increasing age of the animals [237, 259, 260]. Part of this decline may be due to age-related changes in swimming abilities, locomotion and exploration. On the other hand, it is very difficult to perform MWM in very young mice (<1 month) due to their tendency to jump out of the pool.

3.3.3 Brain regions involved in MWM—It is well known that the integrity of the hippocampus is necessary for spatial memory; however other brain structures might influence spatial memory by influencing spatial navigation, motor performances or movement organization [reviewed in [239]]. First, integrity of input and output hippocampal connections (i.e. fimbria-fornix, entorhinal and perirhinal cortices) is required [261–267]. Other critical brain structures include: thalamic structures [92, 268–274], mammillary body [275–280], amygdala [281–284], locus coeruleus [285–288], cerebellum [289–293], prefrontal cortex [294–300], anterior cingulate cortex [301–303], and the striatum [304–307]. The cholinergic basal forebrain, and in particular the nucleus basalis of Meynert, provide the major cholinergic projections to the cerebral cortex and hippocampus and has been linked to memory (reviewed in [308]). Lesions or degeneration of these structures are considered central during aging and AD (reviewed in [309, 310]); indeed, lesions of the forebrain cholinergic system do induce MWM deficits [311]. These should be taken into account when designing a protocol, especially with drug manipulations or the use of genetically modified mice that can present impairments of several brain structures.

3.3.4 MWM in Alzheimer's disease models—MWM has been often utilized to investigate the presence of rodent cognitive impairment that can be correlated with the cognitive decline in AD patients. However, given that LTM deficits start late in the disease process in AD, MWM deficits can similarly manifest later in AD animal models (see below).

It is possible to use non-transgenic, single transgenic or multi-transgenic models of the disease and each model has its strengths and weakness. Among the most commonly used transgenic mouse models, many manifest an increase of Aβ with plaque formation that parallels the onset of LTM deficits (reviewed in [312]). In our laboratories, we have often used the double transgenic APP(swe)/PS1(M146L) line 6.2 [83]. These animals have the advantage of presenting synaptic and memory deficits at 3–6 months of age together with amyloid increase and deposition. However, it is important to note that while RAWM impairment is already present at 3–5 months of age, MWM starts to be deficient at 6–8 months of age [83, 138, 140]. Thus, it is important to choose the appropriate age in relation with the behavioral test to be performed. Unfortunately, due to commercial issues, it is very difficult to acquire this strain, especially in Europe, so many research groups are using more readily available models such as 3xtg-AD [70]. These models show all of the anatomopathological hallmarks of the disease (senile plaques, neurofibrillary tangles, neuronal loss), together with synaptic and cognitive deficits. In these mice, we detected a MWM impairment, especially in the hidden test, at 4 months of age (manuscript in preparation).

4. Conclusions

Animal behavior evaluation has become a fundamental tool in multiple areas of translational neuroscience and is useful for studying i) the physiological mechanisms underlying neurological disorders, ii) the functional modifications induced by genetic manipulation or chemical treatment, and iii) the efficacy of novel drugs in reversing phenotypes in disease models. This is particularly useful in the AD field, as a clinical hallmark of the disease is memory loss. Furthermore, the "*primum movens*" of AD is still unknown. Thus, using cognition-based behavior as a readout can help avoid assumptions about the disease pathogenesis that might be disproved by future studies. Towards this end, Tables 3 and 4 list some AD approved drugs and experimental compounds, that have provided positive results in various behavioral assays performed in our laboratories and might therefore be used as positive controls.

Although cognitive assays of mouse models can provide important information on the validity and efficacy of targets and compounds, it is important to keep in mind that these tasks are incomplete analogs of human cognition. A key limitation is that many cognitive functions are unique to humans or cannot be adequately measured in experimental models (e.g. WM related to language or math). Indeed, these differences might explain, at least in part, the recent high-profile clinical trial failures with drugs that showed promising efficacy in preclinical behavioral tasks. However, in the absence of practical alternatives, mouse models will continue to be essential for accessing AD-type pathology *in vivo* and testing therapeutic strategies. Cognitive assays will provide the most utility in combination with

other experimental arms that support a strong mechanistic correspondence between behavior and molecular target.

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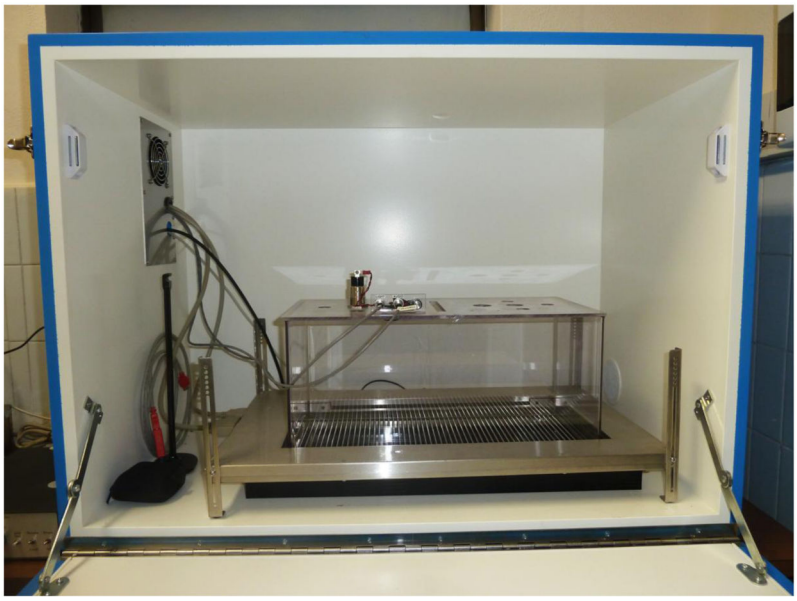

Figure 1.
Picture of the fear memory apparatus used in our laboratories.

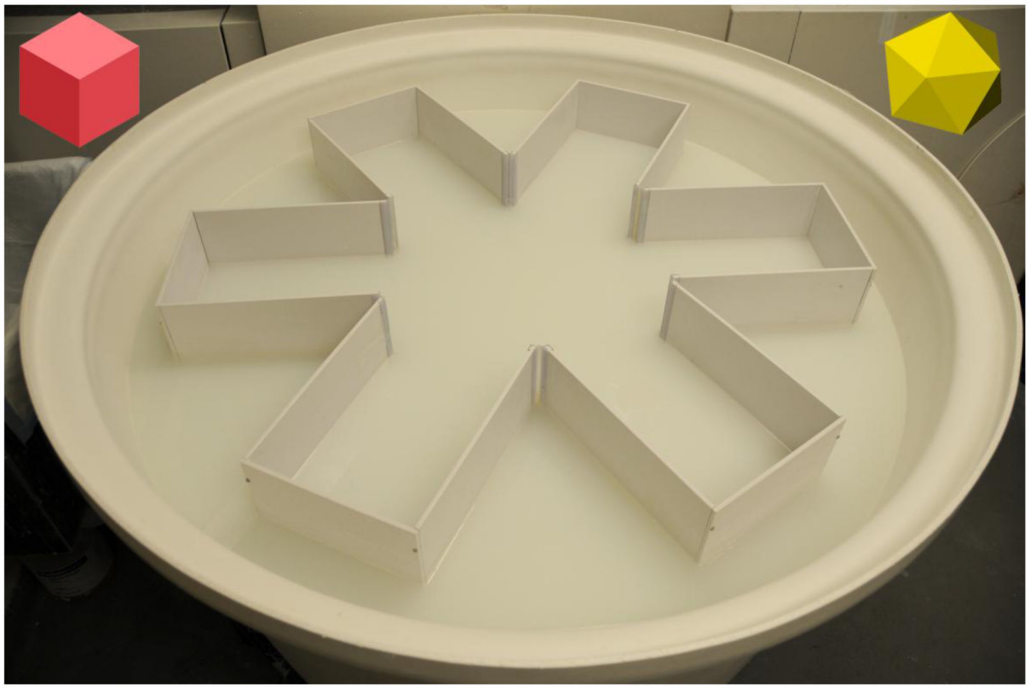

Figure 2.
Picture of the RAWM apparatus used in our laboratories.

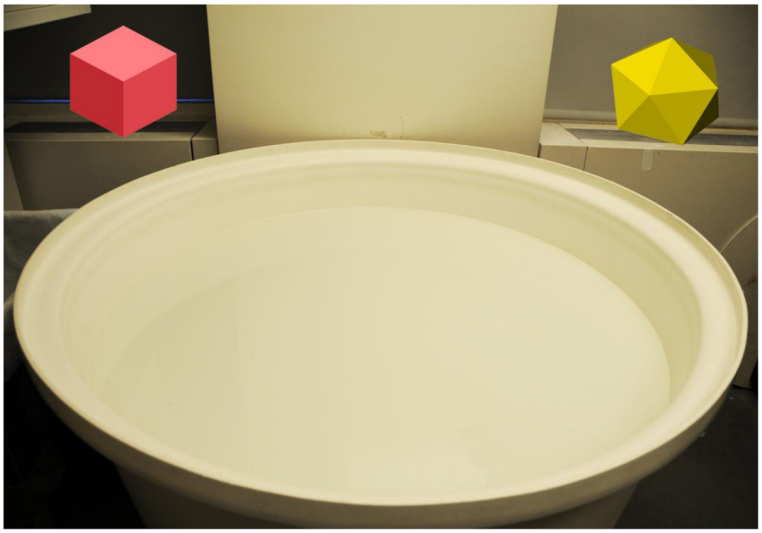

Figure 3.
Picture of the MWM apparatus used in our laboratories.

Table 1

Transgenic mouse models of Alzheimer's disease.

Model	Description	Outcome	Plaques	NFTs	Neuron loss	Synaptic deficits	Memory deficits	Notes	Example	References
Single transgenic										
APP (Swedish)	Mutations at β-secretase cleavage site (aa 670/1)	Enhanced cleavage by β-secretase; overall more Aβ (all forms)	YES: 9–12mo	NO	NO	YES	YES	Mostly dense cored plaques, some tau hyperphosphorylation. Synaptic and memory defects generally precede amyloid deposits.	Tg2576 (Swedish)	Hsiao et al. 1996 Science [56]
APP (Indiana, London)	Mutations at gamma-secretase cleavage site (aa 717)	Enhanced cleavage by gamma-secretase; increased Aβ 42:40 ratio	YES: 9–12mo	NO	NO	YES	YES	Higher levels of diffuse amyloid deposits.	PDAPP (Indiana)	Games et al. 1995 Nature [55]
APP (Arctic, Dutch, Flemish, Italian)	Mutations within Aβ sequence (aa 692/3)	Enhanced Aβ oligomerization, formation of protofibrils	YES: 9–12mo	NO	NO	YES	YES	Pronounced cerebral amyloid angiopathy.	TgAPParc, APPDutch	Ronnback et al. 2011 Neurobiol Aging; Herzig 2004 et al. Nat Neurosci [57,58]
APP (Japanese)	Mutation within Aβ sequence (aa E693delta - deletion of glutamate 22)	Enhanced Aβ oligomerization with no fibrillization or plaques	NO	NO	YES (>24mo)	YES	YES	Intracellular accumulation of Aβ oligomers from 8mo; includes tau hyperphosphorylatoin, gliosis.	TgAPP (E693)	Tomiyama et al. 2010 J Neurosci [59]
multiple APP mutations	Combination of single APP FAD mutations	Combination of effects on APP processing/Aβ	YES	NO	NO	YES	YES	Increased amyloid pathology over single APP mutation models.	TgCRND8, J20	Chishti et al. 2001 J Biol Chem; Davis et al. 2004 J Biol Chem [60,61]
Amyloid-β (Aβ)	Human Aβ40 or 42 fusion protein with BRI	Overexpression and secretion of only Aβ, no extra APP cleavage products	YES: 3mo	NO	NO	YES	YES	Reactive gliosis and amyloid pathology only with Aβ42 model; similar overexpression level as Tg2576	BRI-Aβ42	McGowan et al. 2005 Neuron [62]

Model	Description	Outcome	Plaques	NFTs	Neuron loss	Synaptic deficits	Memory deficits	Notes	Example	References
Presenilin-1	FAD point mutations or exon 9 deletion in human PS1	Altered APP cleavage; enhanced Aβ 42:40 ratio	NO	NO	NO	YES	NO	Accelerated neurodegeneration in older mice >13mo; PS2 model also available with rare mutation, not commonly used	PS1 (M146V/L), PS1(dE9)	Duff et al. 1996 Nature [51]
Tau	Point mutations in human MAPT (FTD mutations)	Increased tau phosphorylation/aggregation	NO	YES (>6mo)	YES	YES	YES	Significant motor defects, brainstem and spinal cord pathology in some strains. Inducible promoter models may be more appropriate for cognitive behavioral studies.	JNPL3, MAPT (P301L), MAPT(VLW)	Ramsden et al. 2005 J Neurosci; Lim et al. 2001 Mol Cell Neurosci; Terwel et al. 2005 J Biochem [63–65]
Multi-transgenic										
APP/PS1	Double transgenic (APP FAD mutant overexpression, PS FAD mutant expression or knock-in)	Accelerated phenotype and pathology but minimal neurodegeneration	YES: 3–6mo	NO	NO	YES	YES	Significant hippocampal neuron loss in some subtypes (APP(swe +lon)/PS1)	APP(swe)/PS1(M146L), APP(swe)/PS1(A246E)	Holcomb et al. 1998 Nat Med; Borchelt et al. 1997 Neuron [66,67]
APP/Tau	Double transgenic (APP FAD mutant overexpression, tau FTD mutant overexpression)	Accelerated phenotype and pathology but minimal neurodegeneration	YES: 9mo	YES	YES	YES	YES	Increased amyloid deposition compared to Tg2576/reports of high death rate	APP(swe)/tau (P301L), APP (swe)/tau (VLW)	Lewis et al. 2001 Science; Ribe et al. 2005 Neurobio Dis [68, 69]
APP/PS1/Tau	Triple transgenic; FAD APP and FTD tau transgenes in PS1 FAD knockin	Accelerated phenotype and pathology including NFTs	YES: 3–6mo	YES	YES	YES	YES	Early intraneuronal deposits; plaques precede tangles	3xTgAPP [APP(swe)/PS1(M146V)/MAPT(P301L)]	Oddo et al. 2003 Neuron [70]

Table 2

Non-transgenic mouse models of Alzheimer's disease.

Model	Description	Outcome	Plaques	NFTs	Neuron loss	Synaptic deficits	Memory deficits	Notes	Example	References
Non-transgenic										
Acute Aβ injection	Direct infusion of Aβ into the brain (e.g. dorsal hippocampi, lateral ventricles)	Acute local Aβ elevation	NO	NO	NO	YES	YES	Aβ source/ preparation/ conformation is crucial	Cell-derived Aβ, Aβ dimers from human brain, 200 nM synthetic Aβ oligomers	Walsh et al. 2002 Nature; Shankar et al. 2008 Nat Med; Puzzo et al. 2012 Neurobiol Aging; Watterson et al. 2013 PLOS One; Fiorito et al. 2013 Eur J Med Chem [21, 30, 32–34]
Acute tau injection	Direct infusion of tau into the brain (e.g. dorsal hippocampi)	Acute local tau elevation	NO	NO	NO	YES	YES	Different tau isoforms can be used, as well as naturally-derived tau from brain extracts	Tau oligomers from human brain, recombinant tau oligomers	Moe et al. 2008, 2009, 2010, 2010; Lasagna-Reeves et al. 2012 Sci Reports [71, 101–104]
Aged animal models	Aged mice, rats, dogs, non-human primates	Natural age-related deficits	YES (dogs, non-human primates)	NO	NO	YES	YES	Exhibit cognitive deficits, brain hypermetabolism, cholinergic defects, altered calcium homeostasis, oxidative stress, neophobia.	Aged mice (>18–20 months old)	Gallagher et al. 1993 Behav Brain Res; Gower et al. 1993 Behav Brain Res [72, 73]
Senescence-accelerated prone mice (SAMP8)	Spontaneously mutated inbred mouse strain	Shortened lifespan and accelerated aging phenotype; elevated levels of endogenous APP and Aβ	NO	NO	NO	YES	YES	Some tau hyperphosphorylation along with decreased spine density and synaptic proteins; increased gliosis and oxidative stress.	SAMP8	Yagi et al. 1988 Brain Res; Okuma et al. 1998 Jpn J

Model	Description	Outcome	Plaques	NFTs	Neuron loss	Synaptic deficits	Memory deficits	Notes	Example	References
										Pharmacol J Pharmacol.

Table 3

Approved Alzheimer's disease drugs found to ameliorate cognitive deficits in Alzheimer's disease mouse models

Compound	Action	Behavioral Task(s)	Model(s) Tested	Reference
Memantine	NMDAR antagonist	FC, MWM	3xTg-AD	Martinez-Coria et al., 2010 [313]
		MWM	APP/PS1	Minkeviciene et al., 2004 [314]
		MWM	APP (APP23)	Van Dam et al., 2005; Van Dam and De deyn, 2006 [315,316]
		MWM	APP (Tg2576)	Chen et al., 2010 [317]
		FC	Ts65Dn (Down syndrome mouse)	Costa et al., 2008 [318]
Donepezil	AChE inhibitor	FC	intracerebroventricular Aβ injection	Tsunekawa et al., 2008 [319]
		FC	APP (Tg2576)	Dong et al., 2005 [185]
		MWM	APP (APP23)	Van Dam et al., 2005; Van Dam et al., 2008 [315,320]
Donepezil, Memantine		MWM	APP/PS1	Nagakura et al., 2013 [321]
Galantamine	AChE inhibitor, nAChR modulator	FC	intracerebroventricular Aβ injection	Wang et al., 2007 [322]
		MWM	intracerebroventricular Aβ injection	Takeda et al., 2009 [323]
		MWM	APP (APP23)	Van Dam et al., 2005; Van Dam and De deyn, 2006 [315,316]

Table 4

Example experimental compounds found to ameliorate cognitive deficits in Alzheimer's disease mouse models

Compound	Action	Behavioral Task(s)	Model(s) Tested	Reference
Ciproxifan	H3 antagonist	MWM	APP (Tg2576)	Bardgett et al., 2011 [324]
AF267B	M1 muscarinic agonist	MWM	3xTg-AD	Caccamo et al., 2006 [325]
Nicotine	nicotinic receptor agonist	RAWM MWM	chronic Aβ infusion APP/PS1	Srivareerat et al., 2011 [326] Inestrosa et al., 2013 [327]
A-582941	α7-nAChR agonist	FC, MWM	3xTg-AD	Medeiros et al., 2013 [328]
CHF5074	γ-secretase modulator	FC	APP (Tg2576)	Imbimbo et al., 2010 [329]
TAK-070	β-secretase inhibitor	MWM	APP (Tg2576)	Fukumoto et al, 2010 [330]
GRL-8234	β-secretase inhibitor	MWM	APP (Tg2576)	Chang et al., 2010 [331]
PBT2	Aβ aggregation inhibitor	MWM	APP/PS1	Adlard et al., 2008 [332]
Bexarotene	retinoid receptor X agonist	FC, MWM	APP/PS1	Cramer et al., 2012 [333]
Rolipram	PDE4 inhibitor	FC, RAWM, MWM	APP/PS1	Gong et al., 2004 [140]
Sildenafil	PDE5 inhibitor	FC, RAWM, MWM	APP/PS1	Puzzo et al., 2009 [138]
Compound 7a	PDE5 inhibitor	FC, RAWM	APP/PS1; Aβ42 intrahippocampal infusion	Fiorito et al., 2013 [33]
E64	cysteine protease inhibitor	FC, RAWM	APP/PS1	Trinchese et al., 2008 [143]
BDA-410	calpain inhibitor	FC, RAWM	APP/PS1	Trinchese et al., 2008 [143]
Trichostatin A	histone deacetylase inhibitor	FC	APP/PS1	Francis et al., 2009 [144]
MW108, MW181	p38αMAPK inhibitor	FC, RAWM	Aβ42 intrahippocampal infusion	Watterson et al., 2013 [34]