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Object recognition test for studying cognitive impairments in animal models of Alzheimer's disease

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1.ABSTRACT

Animal models are essential resources in basic research and drug discovery in the field of Alzheimer's disease (AD). As the main clinical feature in AD is cognitive failure, the ultimate readout for any interventions or the ultimate goal in research should be measures of learning and memory. Although there is a wealth of genetic and biochemical studies on proposed AD pathogenic pathways, the aetiology of the illness remains unsolved. Therefore, assessment by cognitive assays should target relevant memory systems without assumptions about pathogenesis. The description of several tests that are available for assessing cognitive functioning in animal models can be found in literature. Among the behavioural test, the novel object recognition (NOR) task is a method to measure a specific form of recognition memory. It is based on the spontaneous behaviour of rodents and offers the advantage of not needing external motivation, reward or punishment. Therefore, the NOR test has been increasingly used as an experimental tool in assessing drug effects on memory and investigating the neural mechanisms underlying learning and memory. This review describes the basic procedure, modifications, practical considerations, and the requirements and caveats of this behavioural paradigm to be considered as appropriate for the study of AD. Altogether, NOR test could be considered as a very useful instrument that allows researchers to explore the cognitive status of rodents, and hence, for studying AD related pathological mechanisms or treatments.

2.INTRODUCTION

The goal of this review is to discuss the novel object recognition (NOR) test as a valid test to assess cognitive impairments in Alzheimer´s disease (AD) and to test new treatments. There are some reviews in literature precisely describing some behavioural tasks commonly used for testing cognition in mice, such as contextual fear conditioning, radial arm water maze, or Morris water maze, but do not include NOR testing. Other excellent previous reviews have focussed on the NOR test provided a detailed explanation about methodology, brain structures involved or even the effects of different drugs on this test. However, those reviews do not fulfil the requirements and caveats of this behavioural paradigm to be considered as appropriate for the study of AD.

In the present review, a brief introduction of the neuropathological alterations in AD is followed by the description of NOR test protocol and specific modifications of the test that are available in the literature. In a third section of the manuscript, the results found in the literature using NOR testing in different experimental models of AD, both transgenic and non-transgenic

are discussed. In summary, the NOR test is a simple method that does not need external motivation reward or punishment and it can be completed in short-run, so animals do not feel stressed. Its reliability makes this test suitable for evaluating new drugs in AD experimental models.

3. PATHOGENIC MECHANISMS IN ALZHEIMER´S DISEASE

AD is a neurodegenerative disorder characterized clinically by progressive cognitive decline. Currently, AD is the most common type of dementia worldwide: according to the Alzheimer´s Association (www.alz.org) one in nine people aged 65 and about one third of people aged 85 and older suffers from AD. Since age is the biggest risk factor, its prevalence is expected to greatly increase over the next few decades. The huge economic and health care burden of AD will increase if the progression of the disease does not slow in future years (1). The Alzheimer´s Association (www.alz.org) estimates that any new drug that could delay the onset of AD by just five years could decrease the number of AD patients by 43 %. The neuropathological hallmarks of AD are toxic isoforms of amyloid beta (A beta) peptide as well as phosphorylated Tau proteins. These two key features of AD (A beta and Tau) are strongly implicated in mitochondrial dysfunction, synaptotoxicity, inflammation and neuronal loss in the illness (2). Amyloid plaques consist of insoluble extracellular deposits of A beta peptide and appear in cortex mainly. Nowadays, soluble oligomers of A beta are considered key factors in the development of disease (3). Neurofibrillary tangles (NFT) that consist of aggregates of hyperphosphorylated Tau, begin to deposit in enthorhinal cortex and hippocampus (2).

Unfortunately, despite decades of research, the aetiology of AD is mostly unknown, and many fundamental questions remain unanswered. Research into AD therapy has been only successful in terms of developing symptomatic treatments. The approved compounds for the treatment of AD include the acetylcholinesterase (AChE) inhibitors donepezil, rivastigmine and galantamine as well as the NMDA (*N*-methyl-d-aspartate) receptor antagonist memantine. This symptomatic treatment is only moderately effective in stabilizing or improving cognitive and functional symptoms for some months and may slow further decline thereafter. Therefore, 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 (4). Development in AD research needs to address the crucial therapeutic endpoint, which is the amelioration and/or prevention of cognitive dysfunction. There is an insidious onset in clinical symptoms in AD, with an initial loss of short-term memory, followed by progressive impairment of multiple cognitive functions and behaviour. Episodic memory processes (memory concerning past events) and working memory (cognitive abilities that are started “on the go” and that are necessary for performing a task) are impaired early in the illness (5), and these forms of memory are evaluated clinically by explicit recognition memory tasks. Spatial memory, associated with navigation, is strongly involved in episodic memory, which also includes recognition memory mechanisms (6, 7).

Over the last decade, different behavioural paradigms have been developed for the evaluation of the cognitive functions in animal models, such as the Morris water maze, Barnes maze, passive avoidance, radial arm tests, fear conditioning or novel object recognition tests (8, 9). All these paradigms have strengths, caveats and specific requirements. Given the limited knowledge on disease aetiology, the assessment by cognitive assays offers the advantage of targeting memory without requiring assumptions about pathogenesis. Among the cognitive assays that test learning and memory processes in animals, this review will focus on the Novel Object Recognition (NOR) test. This cognitive task allows for the relatively fast assessment of several batches of mice in a short period. The aim of this review is to highlight and discuss practical considerations of this assay, as well as the protocols, guidelines and caveats of its use in various AD mouse models.

4. THE NOVEL OBJECT RECOGNITION TEST

NOR test is a useful paradigm for assessing cognitive status of rodents. NOR test is a task based on spontaneous behaviour, with the advantage that does not require any reward or punishment, and it can be performed quickly with a single learning phase (10). When exposed to a familiar object alongside a novel object, rodents spend more time exploring the novel than the familiar object. This apparent ‘unconditioned preference’ for a novel object indicates that a representation of the familiar object exists in memory and it forms the basis of the NOR task in the study of memory functions in rodents. It has been suggested that NOR eliminates possible bias in the interpretation of results deriving from behavioural modifications that could influence the memory performance and that are connected with drug-related changes of pain perception, stress susceptibility, thermoregulation, and anxiety (11-13). Due to these advantages, NOR test is widely used to test recognition memory in different experimental models of AD (14).

4.1. Methodology

Ennaceur and Delacour (10) firstly described the use of the novel exploration activity of rodents to test their memory. This test was derived from some visual task performed in primates for evaluating their cognitive abilities (15, 16). A detailed and comprehensive review on the methodology used, and methodological modifications

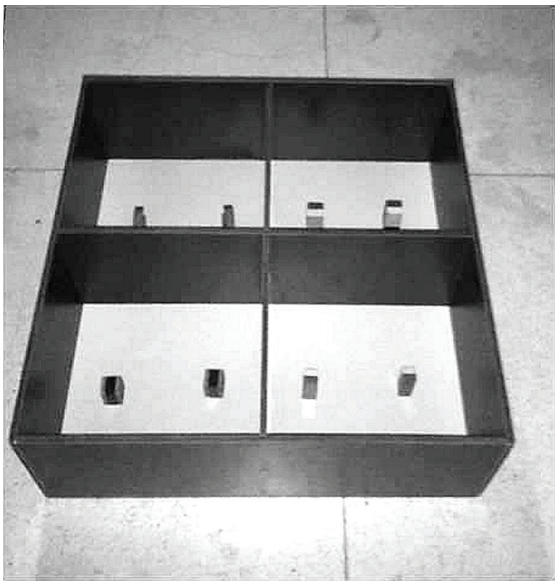

Figure 1. Picture showing a real setup for performing NOR testing. In this case, this setup corresponds to the familiarization phase (two similar objects). Lighting is an important issue. For a detailed explanation, please check point 2.1. Methodology

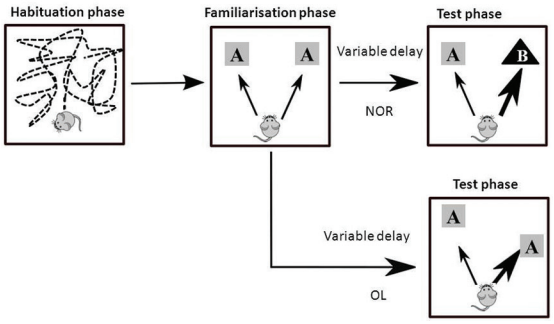

Figure 2. Experimental protocol used in novel object recognition (NOR) experiments. A variation of NOR is object location (OL). For a detailed explanation, please check point 2.1. Methodology

of this test can be found in (17). In the initial description of NOR apparatus, Ennaceur and Delacour (10) used an open field box (arena) made of wood. The shape of the arena usually is rectangular or quadrangular. Less common are circular arenas (18). Regarding dimensions, Ennaceur and Delacour (10) used an arena of 65×45×65 cm in size. Ideally, the arena should take into account the objective of work and be adapted to the features of animals, i.e., the study of Reger *et al.* (19), in which three sizes of arenas were used according to animals' ages. The floor of the apparatus can be covered with sawdust or paper beddings. This cover should be agitated between trials or regularly replaced. Some laboratories have started to move away from bedding material because of the potential for the bedding to trap scents and cause olfactory complications. Objects that have been used in the NOR test vary widely in shapes, sizes, textures, materials, colours, and appearance. Many objects have been used in this test: cans, bottles, tins, pots, pyramids, tower, cylinder, box, Playmobil or Lego toys, coffee mugs or pet toys. The objects can be made of metal, glass, porcelain, glazed ceramic, rubber, durable nontoxic plastic, or aluminium. These materials are non-porous can be easily cleaned and animals cannot easily gnaw them. Concerning the object height, it varies between 4.5. and 24 cm, as long as it is high enough to avoid animals climbing or resting on it during trials. The object should be heavy enough that animals cannot move it. Most of the NOR test occurred in sound-isolated room. Light conditions might vary among laboratories: although tests were made with constant illumination, its intensity can range from <10 lx to 30–40 lx. Lighting is an important issue, as high levels of light can cause anxiety in rodents and result in poor learning/reduced exploration of the objects, as shown in Figure 1, in which a picture of a real setup for performing NOR testing is displayed. Practical issues should be considered include the necessity to clean thoroughly chamber and objects after each testing in order to eliminate olfactory interferences and to balance the order of the objects between testing to avoid preference for one of the objects. Based on the limited information on object recognition (perception) in rats and mice, it is necessary to try to maximize the difference between the new and old objects in order to ensure their affordances to the sensorial modalities of rats and mice without inadvertent induced-preference for one of the two objects (20).

Nowadays, the most widely used protocol in AD research consists of three phases (Figure 2): the habituation phase, the familiarisation phase and the test phase. In the habituation phase, animals are placed in the arena, and are allowed to explore for a considerably long time. In the familiarisation phase, animals are placed in the same arena again but, containing two objects similar in shape, size, colour, texture, etc., equidistantly placed within the chamber. The procedure in the test phase is similar to the familiarisation phase, but a new unknown object replaces one of the old ones. In animals that are cognitively competent, the natural curiosity of rodents (21, 22) induces them to spend more time exploring the new object. Nonetheless, rodents do not always show this mentioned natural curiosity (23). Sometimes they do not discriminate between the two objects, or furthermore, a 'known' object preference can appear. Several hypotheses have been proposed to explain this behaviour (20). To keep the natural curiosity of animals, it is necessary to control the environmental conditions as much as possible. Some features such as the size of the arena or the colour of the objects can affect the results of the task. Objects should be as different as possible in shape and colour –but similar in size- and, it is recommendable to use objects tall enough, so that the animal does not climb onto them

(animals might stand over them with no exploration). Computer recording and analysis systems are useful for avoiding distractions in the rodents. If not possible, manual recordings of exploration times are also affordable. Manual recordings can be essential for the data interpretation (in case tracking software does not accurately define head position or the location of the objects). These measurements can be done post-testing using video-recorded test runs.

An important issue to consider is the evaluation of data in the NOR test. The points to consider are time spent by the animal in exploring individual objects during each phase and total time spent by the animal in exploring both objects during in each phase. Performance can then be evaluated through different indexes, as discrimination index, recognition index, or preference index depending on the aim of each study. Discrimination Index (DI), allows discrimination between the novel and familiar objects, i.e., it uses the difference in exploration time between exploration of familiar and novel object, but then dividing this value by the total amount of exploration of the novel and familiar objects. This result can vary between +1 and −1, where a positive score indicates more time spent with the novel object, a negative score indicates more time spent with the familiar object, and a zero score indicates a null preference (24,25). Recognition Index (RI) is the time spent investigating the novel object relative to the total object investigation, and it is the main index of retention (26, 27). Preference Index is a ratio of the amount of time spent exploring any one of the two objects over the total time spent exploring both objects, i.e., in the test phase, time in novel object/(total time exploring (novel+familiar))×100 (%). Therefore, a preference index above 50% indicates a novel object preference, below 50% familiar object preference, and 50% no preference (28, 29).

4.2. Modifications to the basic protocol

Several modifications have been described to improve the reliability and reproducibility of the assay (reviewed in (17)). Sample trial durations can last between 1 and 10 minutes, although some authors measure until reaching a threshold of total object exploration (30). The inter-trials delays last typically between 1 hour (31) and 24 hours (32). Longer delays are not recommended, as the task might be too demanding for even healthy animals, and this would decrease the sensitivity of the assay. Some authors also repeat several times the familiarisation phase to make animals to learn deeper (33). All these modifications in the NOR test protocol have led to the proposal for the standardisation of the NOR task, which seems to be necessary to allow for the comparison of results from different studies (34). In this sense, Leger *et al.* (34) recently proposed the use of a selection criterion based on a minimal time of exploration for both objects during each session (familiarization and test sessions).

Although the NOR test is the most used test for evaluating object recognition abilities in AD models, the object location (OL) test can be very useful too. This paradigm is analogous to the NOR, but in the test phase instead of changing the object, its location is changed (Figure 1). This performance evaluates the spatial memory rather than the recognition memory (35).

As ageing is the most important risk factor in AD (36), the NOR test has been used to evaluate cognition in aged rodents. Some authors pointed out a decrease of novel exploration associated with an increase of age (37). Cognitive impairment in the NORT test associated to ageing is a controversial point and some authors have reported cognitive competency in aged healthy rats (38). In aged animals, in case it is necessary to dissociate cognitive impairments associated with ageing from cognitive impairments associated to other causes, it might be necessary to build variations in the procedure to evaluate memory in aged rodents. In this sense, Platano *et al.* (33) proposed a modification of the NOR test, consisting of a 3 day lasting familiarisation phase with 5 object recognition trials. Following this procedure, memory consolidation improves and aged rodents show a competent performance. It has to be noted as well that evidence has been shown suggesting that object recognition in rodents is influenced by sex, gonadal sex hormones and gonadectomy (see review by (12).

Episodic memory refers to the ability to encode and recall events and experiences. The specific components of those memories consist of a particular object or person (memory for “what” happened), the context or environment in which the experience occurred (memory for “where” it happened), and the time at which the event occurred (“when” it happened). The ability to integrate these “what,” “where,” and “when” features of an event is considered fundamental to the subjective experience of human episodic memory (39). However, work over the past few decades on episodic-like memory across a number of animal species has suggested otherwise, and several mouse behavioural tasks designed at assessing episodic-like memory have been developed (40). These tests (41, 42) are designed to evaluate the three components of the episodic memory, i.e. the object recognition, spatial and temporal components respectively. One such task is the What-Where-Which Task (WWWhich), an adaptation of the NOR test. In this task, the animal must integrate the location of a particular object with specific contextual cues to form an episodic-like memory (43). Other adaptations have been employed to distinguish “what,” “where,” and “when” information, such as the What-Where-When task (WWWhen) that shows spatio-temporal context memory, the What-Where task that is used for object location-associative memory, or the What-Which task for object-context memory that involves association of object, location, and contextual information (41, 42).

The one-trial object recognition task was initially developed for rats (10), and later on adapted to mice with only minor modifications (44-46). However, cognitive aspects displayed in this task by both species do not need to be similar. In fact, other memory tasks such as the Morris Water Maze show consistent differences in the procedure protocol requested by the two species. Compared to rats, mice typically show lower levels of total exploration, both in terms of the number of contacts, as well as the time spent exploring the objects (12), probably due to a higher tendency to neophobia in mice (47). This fact conditions that NOR trials in mice last typically longer than in rats (12). There is also a study revealing differences between rat and mouse depending on the inter-trial delay (48). In this study, rats performed competently the task with 3 hours of delay whereas mice showed impairment with delays longer than 2 hours. In spite of this study, the NOR test is widely used to evaluate object recognition memory in rodents, both mice and rats, and lead itself well cross-species generalization (19, 26).

4.3. Brain regions involved

Several cerebral structures are crucial for the competent performance of the NOR test (reviewed by (12)). The results of the NOR paradigm are influenced by both hippocampal and cortical lesions (49, 50). The hippocampus receives inputs from the perirhinal cortex, which is itself the site of several information entrances as visual, olfactory, and somatosensory stimulus, all of them involved in object recognition (51). The perirhinal cortex is involved in the proper perception of the objects (52, 53) and when lesions in this brain region exist, the impairment in object recognition memory could be observed (24). The other main area that projects into the hippocampus (i.e. the enthorinal cortex) (54) also influences strongly the performance of the NOR test. The hippocampus, enthorinal and perirhinal cortex are highly integrated, but these structures show also some specific functionalities. The perirhinal cortex is involved in object recognition after short retention intervals and it is necessary to represent basic information about familiarity or novelty, whereas the hippocampus is responsible for long-term object recognition (19). Memory consolidation but not persistence seems to be hippocampus-dependent. Other structures like the nucleus basalis (37) and the nucleus accumbens (55) might also play a role in the appropriate performance of the NOR test. Interestingly, these structures have been shown to be damaged both in AD (56-59) and AD animal models (60, 61). To conclude this section, it is worth mentioning the neurotransmitter systems that have been described to be involved in NOR behaviour. As reviewed in (12), there seems to be a significant implication of the glutamatergic and cholinergic system in the NOR behaviour. The dopaminergic and serotonergic system seems to be also involved.

Brain circuitry involved in OL is different from NOR (62). Evidences showing an implication of the hippocampus seem to be much more solid (35, 62). The medial septum area and the nucleus basalis magnocellularis might also play a role in OL performance (63). The OL test can also detect an impairment induced by lesions in the nucleus accumbens (64).

5. NOVEL OBJECT IN ANIMAL MODELS OF AD

In 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*. The ideal AD animal model should present an increase in A beta 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). Aetiology of AD is multifactorial, with both genetic and environmental factors implicated in its pathogenesis. The disease is basically classified into 2 types, sporadic AD (SAD), the common form accounting for 90% to 95% of the cases for which no defined cause is known, and familial AD (FAD) that shows autosomal dominant inheritance. In SAD, advancing age appears to be the greatest risk factor for AD. Its incidence increases in people of 60 years and older. Other risk factors may include cardiovascular risk factors, insulin-resistance, chronic stress, depression or hypertension (65, 66).

Based on this premises, both genetic (transgenic) and non-transgenic models of AD are available. In most of the animal models, the first goal is to simulate the neuropathological findings of AD followed by the correlation of cognitive function.

5.1. Transgenic models of AD

Transgenic mice are the most commonly used animal models for studying AD. These models are an essential tool in studying *in vivo* pathophysiology. With the identification of the genetic factors involved in AD, the development of several transgenic mouse models has taken place. The common rationale employed in creating transgenic model of AD is the overexpression of the transgene carrying FAD mutations under different promoters. Genes, namely amyloid precursor protein (APP), presenilin-1 (PS1) or presenilin-2 (PS2) and Tau are used to construct transgenic mouse models of AD. Although about 90 multi- and single transgenic mice modelling AD have been created, only a few of them recapitulate most of the neurophatological characteristics of AD, and currently, there is no model that can reproduce all the aspects of AD (Table 1). Some given reasons are that only a few recent models of AD suffer from neurodegeneration (67), that the aberrant protein accumulation progression does not correspond to progression described in the brain of AD patients or that FAD account for only up to 5% of the total AD cases (68).

Table 1. Neuropathological characteristics of some common experimental models of Alzheimer's disease

Model	Description	Pathological increase in Aβ production	Aβ burdens	NFT	Synaptic deficit	Memory deficit in NOR	Reference
Transgenic models							
Tg 2576	APP swedish mutant (K670N/M671L)	Yes	Yes	No	Yes	Yes	(72-79)
J20	Double mutant: APP (KM670/671NL) and APP (V717I)	Yes	Yes	Yes	Yes	Yes	(80-85)
3×Tg	Triple mutation: APP (K670N/M671L), PS1(M146V), and Tau (P301L)	Yes	Yes	Yes	Yes	Yes	(86-92)
5×FAD	5 fold mutant: APP (K670N/M671L), APP (I716V), APP (V717I), PSEN1 (M146L), PSEN1 (L286V)	Yes	Yes	Yes	Yes	Yes	(93-94)
APPSwe/PS1dE9	Double mutant: APP (KM670/671NL) and PS1dE9	Yes	Yes	No	No*	Yes (ol)	(97-101)
Non-transgenic models							
Cholinergic lesions	Surgical or toxic depletion of cholinergic neurons	No	No	No	No	Yes	(109)
Aβ administration	Cerebral administration of Aβ toxic species.	Yes	No	No	Yes	Yes	(118,120)
Ageing	Senescent animals	Yes	Yes	No	Yes	Yes	(129-130)
Stress	Chronic exposure to variable hostile agents	Yes	No	No	Yes	Yes	(137-140)

*Controversial results. NFT: Neurofibrillary tangles, NOR: Novel object recognition, OL: Object location; APP: Amyloid precursor protein, Aβ: Beta -amyloid peptide

However, there is a very high degree of phenotypic similarity between FAD and sporadic late-onset AD, suggesting that mechanistic information obtained about FAD would also be directly relevant for SAD (69). In addition, it has to be considered also that the genetic background can affect their behavioural phenotype (70). Numerous works in literature describe the performance of the different transgenic models in NOR test. It is described here a brief overview of the results found in 5 of the most commonly used different AD transgenic mice using the NOR test.

One of the most widely-used models is the Tg2576 mouse line (Promoter: Hamster PrP Promoter, Symbol: Tg (APPSWE) 2576Kha, MGI ID: 2385631), which overexpresses human APP with two point mutations (K670N, M671L) that were originally identified in a Swedish family with FAD (71). These mutations are located by the beta secretase site and bias APP processing toward the amyloidogenic pathway, leading to higher overall levels of A beta (72) Tg2576 mice have numerous parenchymal A beta plaques by 11-13 months with some vascular amyloid, tau hyperphosphorylation, but without tangles. An important point to consider is the age at which cognitive deficits could be detected with the NOR test in this mouse model and the protocol used (inter-trial intervals). NOR test has been reported to be impaired in this transgenic model already at the age of 6 months, and still detectable at age of 12 months (73). Or even in younger mice if the protocol is demanding enough: cognitive deficits could be detected in the NOR test using a 4 and 24 h retention interval in 5-months-old Tg2576 mice, which could be reversed by acute inhibition of calcineurin with FK506 (74). Tg 2576 mice aged 9 months old showed deficits in the NOR test that were reverted by chronic propranolol treatment (75). Immunotherapy with oral vaccine using a recombinant adeno-associated viral vector carrying A beta cDNA, administered once at the age of 10 months, alleviated the progressive cognitive impairment at the age of 13 months (76). Interestingly, 4 months-old Tg2576 mice that were cognitively trained for 8 weeks and, after a lag of 8 months, long-lasting beneficial effects of the cognitive training on cognitive abilities could be detected using NOR test (24 h interval) (77). On the other hand, 14-month-old Tg2576 mice showed no deficit in NOR test using two item object arrays in 1, 3 and 24 h intervals. However, in the same set of experiments, these mutant mice failed in the OL memory, suggesting the effects of hippocampal damage in these rodents (78), likely to a sensitivity of Tg2576 mice to object-location analogue of visuospatial paired associate learning. This could be of particular interest as AD patients, in addition to the impairment in recognition memory, show a high sensitivity to paired-associated learning that involves object-location associations (79).

The J20 mouse model (promoter: Platelet-Derived (PDGF), Symbol: Tg (PDGFB-APPSwInd) 20Lms, MGI ID: 3057148) was developed by (80). The J20 model expresses human APP with the Swedish KM670/671NL, and London (V717I) mutation. This model is unique in that the first presented cognitive deficits are observed very early (at 2-3 months of age) in recognition memory (81). These deficits in recognition memory are

present when assessed at several other time points (82-84). However, they do not appear to progress with the age of the animal and there has even been one report of no recognition memory deficits in aged animals in advanced stages of the disease (85).

The 3×Tg-AD transgenic mouse (Promoter: Thy-1, Symbol: Tg (APPSwe,tauP301L) 1Lfa, MGI ID: 2672831) × (Promoter: Endogenous, Symbol: Psen1tm1Mpm, MGI ID: 1930937) carries AD transgenes for APPSwe, PS1M146V and TauP301L. It shows a neuropathology that include both plaque and tangle restricted to the hippocampus, amygdala, and cerebral cortex, extracellular A beta deposits by 6 months in frontal cortex (more extensive by 12 months) and Tau pathology manifested at 12 months (86). The NOR task is particularly efficient in 3xTg-AD mice in part because this model often shows signs of anxiety (87). The use of NOR testing is thus favoured as it involves less stressful tasks compared to water maze-based paradigm and other tests involving the generation of anxiety in the animals. Cognitive impairment in this strain in the NOR test have been observed at 9 (88) or 13 months (89). Different treatments have proven effective in reversing cognitive deficits in the NOR test in this mice model (90-92).

5XFAD mice (Promoter: Thy-1, Symbol: Tg (APPSwFlLon, PSEN1*M146L*L286V) 6799Vas, MGI ID: 3693208) with mutations in APP KM670/671NL (Swedish), APP I716V (Florida), APP V717I (London), PSEN1 M146L, PSEN1 L286V (67) show amyloid pathology that deposits at 1.5 months. A beta 42 also accumulates intraneuronally in an aggregated form within the soma and neurites starting at 1.5 months. By 4 months it is observed neuronal loss and deficits in spatial learning. 6-8 months old 5XFAD mice showed deficits in the NOR test (10 min, 30 min and 24 h after training) that were reversed by diosgenin treatment (93). Even more, 2-months-old 5XFAD mice, which could be referred as “prodromal stage AD”, showed cognitive impairments in the NOR test (5 min exploring, 1 h delay and 5 min retention test) that were reverted after 2 months of treatment with an 5-HT_4 agonist (94).

APPSwe/PS1dE9 mice (Promoter: PrP, Symbol: Tg (APPswe, PSEN1dE9) 85Dbo, MGI ID: 3524957) expressing mutant APP Swedish (APP KM670/671NL) and mutant presenilin 1 (PS1dE9) have high A beta 42 levels and induces amyloid deposition by 4-6 months of age (95, 96) and shows hippocampal functional deficits from 7 months (97). This genotype triggers a cognitive impairment in NOR in 7-month-old-females with an intertrial delay of 4 hours (98), but not with 1 hour (99). 7 months old APPSwe/PS1dE9 mice showed an improvement in spatial memory measured by OL task after administration of a brain-derived neurotrophic factor (BDNF) agonist on TrKB in only 1 hour delayed between first and second trial (not at 24 h delayed) (100) 6.5 months aged APPSwe/PS1dE9 mice had an impairment at 1 h and 4 h intertrial delays in the OL test that was reversed (at the 4 h interval) by a treatment of subcutaneous injections of a isoform of phosphodiesterase type 4 inhibitors (GEBR-7b) (101).

5.2. Non-transgenic models of AD

Trying to solve limitations in the use of transgenic AD models, some other strategies have been developed to model AD in animals (sporadic AD). Based on the cholinergic hypothesis, scopolamine induced amnesia and excitotoxic lesions of the basal forebrain have been used to assess cognitive deficits. Current symptomatic drugs for AD were successfully evaluated in these models, but their etiological relevance is low (102). Senescence-accelerated mice or mice exposed to well-known risk factors for developing AD, such as insulin-resistance or animals chronically stressed, are also commonly used for the study of sporadic AD (Table 1).

5.2.1. Models of AD based on the induction of a cholinergic deficit

Following the demonstration of cholinergic deficits in AD, the classical cholinergic hypothesis of memory was established, stating that the cause of the illness is a deficit in cholinergic neurotransmission (103). This theory was responsible for the appearance of the first successful AD therapies, the acetylcholinesterase inhibitors. Nowadays, depletion or blockade of this neurotransmitter system is still used to evaluate the efficacy of new AD treatments that addresses the cholinergic system.

The muscarinic antagonist scopolamine has been used to induce a functional cholinergic deficit in the brain, eventually causing a cognitive impairment in several behavioural tests, including NOR or OL test (to mention a recent example (104)). Acetylcholinesterase inhibitors currently used for the clinical treatment of AD, such as galantamine, have been shown to possess memory-enhancing effects in two conditions that reduced object discrimination: scopolamine-induced deficits and when a longer retention interval was used (105). Similarly, several candidates for the treatment of AD based on enhancing cholinergic neurotransmission have been shown to be effective, such as the M1 agonists (106), or cholinesterase inhibitors like memoquin (107). Other techniques have shown to be capable of diminishing cerebral acetylcholine levels, although there can be other concomitant damage mechanisms. Sodium azide, an inhibitor of the cytochrome oxidase IV, decreases cholinergic tone in the brain and causes cognitive impairment in the NOR test. In this model, the acetylcholinesterase inhibitor ladostigil reversed the cognitive dysfunction (108).

Selective lesions of specific cholinergic basal nuclei have been used to study the participation of

specific cholinergic pathways in memory. Lesions in cholinergic neurons of the medial septum that project to the hippocampal formation with the selective cholinergic immunotoxin 192-IgG-saporin induced no significant effect on NOR, but produced a significant impairment in OL, support a role for septo-hippocampal cholinergic projections in memory for the location of objects, but not for novel object recognition (109).

The surgical removal of the olfactory bulb of rodents causes AD related changes, including cognitive impairment, depressive-like behaviour and cholinergic alterations (110). Interestingly, olfactory bulbectomy increases A beta production too (111). Rivastigmine, another cholinesterase inhibitor used in the clinical treatment of AD, improves NOR test performance in bulbectomized animals, highlighting the importance of the cholinergic damage in this model (112).

5.2.2. Beta amyloid cerebral administrations as AD model

Intracerebral administrations of A beta have been used to test whether beta amyloid peptide is toxic for the brain. A beta 42 is considered the most pathological species of A beta. However, as natural A beta 42 exhibits low solubility, the much more soluble fragment A beta 25-35 is frequently used. This peptide fraction has shown to be the essential fragment that possesses toxic activity (113) and is preferred for intracerebral administrations in many experiments.

The intracerebroventricular administration of the peptide uses the ventricular system of the brain for spreading the A beta peptide across the brain. It has been successfully used to cause a cognitive impairment in the NOR test performance, and several therapies have been tested by this procedure. Many therapies reduce the A beta 25-35 damage and reverse the cognitive impairment in NOR test associated with A beta administration, such as the gamma secretase inhibitor BMS-299897 (114), the toxin Tx3-1 (115), the MAO-B inhibitor selegiline (116), the flavonoid derive silibinin (117), the PPAR-alpha inhibitor GW7647 or the palmitoylethanolamide (118).

The cerebral A beta accumulation plays a very important role in the development of the AD, but do not fulfil many pathological hallmarks of the illness, such vascular alteration (119). In this sense Choi *et al.* (120) combined the intracerebroventricular administration of A beta25-35 with an artery obstruction in the brain to induce a mild hypoxia and therefore a misuse of nutrients in the brain. The combination of both factors worsens the impairments of A beta 25-35 in the NOR test. It is to mention that in this experiment only the combination of both A beta 25-35 administration with the artery occlusion was capable of triggering a cognitive impairment in the Morris water maze paradigm.

An important limitation of the intracerebroventricular administration of A beta is the lack of temporal pattern in the progression of the pathology. Whereas the AD pathology seems to start in structures of the temporal lobe, the ventricular administration cannot reproduce this feature. Sipos *et al.* (121) tried to model the first steps of the AD pathology by administering A beta 42 selectively in the entorhinal cortex. This paradigm triggered a cognitive impairment in the NOR test that was not revealed in the Morris water maze paradigm.

5.2.3. Senescence models

Ageing is the most important risk factor for the development of AD. Almost every experimental model of the illness is combinable with ageing to form a more consistent approach of the illness. Therefore, delaying senescence associated impairments would constitute an alternative for preventing the development of AD pathology, suggesting that it can be also possible to evaluate possible AD therapies in aged animals (122). Several treatments have shown to improve the age associated cognitive impairment in NOR test and are, therefore, are likely to be active against AD, such as the phosphodiesterase 5 inhibitors, sildenafil and vardenafil (123) and the cholinesterase inhibitor metrifonate (124).

It has to be mentioned that murine animals might be far from the reality of the human senescence. Life expectancy in murine species is no longer than 30 months, so they might be not capable of developing some of the causal factors that appear along life in humans, who live much longer. Interestingly, senescence accelerated models have been developed to overtake this limitation. These strains, such as SAMP8, develop faster several markers of ageing (reviewed by (125)), including gliosis (126), learning deficits (127) and increased radical oxygen species production in the brain (65). Interestingly, SAMP8 shows an increase production of APP, A beta and p-Tau (128). This mouse strain has shown a cognitive impairment in the NOR test that can be reverted by pramlintide (129) or propranolol (130).

5.2.4. Stress related models

There is growing consensus that environmental stressors may increase the probability of risk for AD. Clinical data suggest that a stressful lifestyle can be a risk factor for AD (131) and stress-related psychiatric disorders (e.g. major depression) have been identified as a risk for developing AD (132). There is a dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis in AD and cognitive status was negatively associated with glucocorticoid levels (133, 134) . Csernansky *et al.* (135) also showed that initially higher serum glucocorticoids in the pre-dementia clinical stage of AD predict a more rapid cognitive decline. This view gained support from studies in transgenic mouse models of AD in which stress or glucocorticoids exacerbated AD like

Table 2. Examples of compounds found to ameliorate cognitive deficits in the novel object recognition (NOR) or object location test in Alzheimer's disease (AD) mouse models

Compound	Action	Behavioral task	Model tested	References
FK506	Ca^{+2}/calmodulin-dependent protein phosphatase calcineurin inhibitor	NOR	Tg2576 mice	(75)
Propranolol	β -blocker	NOR	Tg2576 mice	(75)
Oral vaccine Aβ	Inmunotheraphy	NOR	Tg2576 mice	(76)
Enriched environment/ physical activity	Cognitive stimulation	NOR	Tg2576 mice	(77)
Cannabidiol	CB receptor agonist	NOR	Tg2576 mice	(32)
MMBO	GSK-3 inhibitor	NOR	3×Tg-AD mice	(89)
DHA	Long-Chain Omega-3 Oil	NOR	3×Tg-AD mice	(90)
Diosgenin	Ligand of steroid-binding protein ($1,25D_3$-MARRS)	NOR	5XFAD mice	(93)
RS67333	5-HT_4 agonist	NOR	5XFAD mice	(94)
7,8-DHF	TrKB agonist	OL	APPswe/PS1dE9 mice	(100)
GEBR-7b	PDE4 inhibitor	OL	APPswe/PS1dE9 mice	(101)
Neuropeptide S	NPRS ligand	NOR/OL	Cholinergic lesion	(104)
Ladostigil	Acetylcholinesterase inhibitor	NOR	Cholinergic lesion	(108)
Memoquin	Anti-oxidant, others	NOR	Cholinergic lesion and Aβ cerebral administration	(107)
BMS-299897	Gamma secretase inhibitor	NOR	Bulbectomy-cholinergic lesion	(112)
Selegiline	MAO-B inhibitor	NOR	Aβ cerebral administration	(116)
Pramlintide	Amylin analog	NOR	SAMP8 mice	(129)
Propranolol	β -blocker	NOR	SAMP8 mice	(130)
SB271046	5-HT_6 receptor agonist	NOR	Stress related models	(137)
Venlafaxine	Selective serotonin/norepinephrine reuptake inhibitor	NOR	Stress related models	(138)
Mifepristone	GR antagonist	NOR	Stress related models	(143)
GLP-1	Incretin glucagon-like peptide	NOR	Model of sporadic AD based on induction of insulin-resistance	(147)
GIP	Incretin glucose-dependent insulinotropic polypeptide	NOR/OL	Model of sporadic AD based on induction of insulin-resistance	(148)

Aβ: beta-amyloid peptide; NOR: novel object recognition; OL: object location ; MMBO: 2-methyl-5-(3-{4-((S)-methylsulfinyl) phenyl}-1-benzofuran-5-yl)-1,3,4-oxadiazole; GSK-3: glycogen synthase kinase 3; DHA: docosahexaenoic acid; APP: amyloid precursor protein; AD: Alzheimer's disease; $1,25D_3$-MARRS: $1,25D_3$-membrane-associated rapid response steroid-binding protein; FAD: familial Alzheimer's disease; 5-HT_4: serotonin receptor 4; TrKB: tropomyosin related kinase B; PDE4: phosphodiesterase 4; CB: cannabinoid receptors; NPRS: neuropeptide S receptor; MAO-B: monoamine oxidase B; SAMP8: senescence mouse; 5-HT6: serotonin receptor 6; GR: glucorticoid receptor; GLP-1: glycogen-like peptide 1; GIP: glucose-dependent insulinotropic polypeptide

neuropathology (136). This strong relationship between those three factors -stress, depression and AD- suggests not only the existence of common physiopathological pathways in the course of these illnesses, but also the possibility of using animal models of chronic stress to reproduce and study these common physiopathological pathways.

Different stressors have been cognitively evaluated with the NOR test. Stressful events in the early stages of the life in rats (such as maternal separation) have been reported to cause adult cognitive impairment in the NOR test that could be reversed by the 5-HT_6 receptor agonist, SB271046 (137), and by the antidepressant drug venlafaxine (138). Chronic stressors during the adult life

have also been described as cognitively deleterious. Animals subjected to chronic mild stress procedure perform poorly in the NOR test, and venlafaxine has shown to reverse this cognitive impairment (139, 140). Other authors have found a similar effect of the chronic mild stress in males, but they fail to reproduce this finding in females (141, 142), showing that there are likely sexual differences that are poorly understood.

The hypothalamus-pituitary-adrenal axis is the main mediator in the chronic stress response in mammals, mainly by increasing the levels of circulating glucocorticoid levels in response to stressors. Interestingly, the stress triggered impairments in the NOR test can be prevented by a prior administration of a glucocorticoid receptor antagonist, the mifepristone. This effect has been seen in stress protocols, such as maternal separation (143) or chronic mild stress (139). Stress and glucocorticoids have also strong metabolic effects, such as increased glycaemia and increasing the risk of metabolic syndrome and diabetes, which have also been described as risk factors for developing AD (see point 5.2.5.). This way, the chronic oral administration of glucocorticoids can provoke a cognitive impairment in the NOR (75), apparently involving activation of the mineralocorticoid receptor and the JNK pathway (144).

5.2.5. Models of sporadic AD based on induction of insulin-resistance

There are several AD risk factors associated with metabolism, like obesity, hypercholesterolemia or diabetes mellitus. Metabolic hormones like insulin and leptin have not only been associated with metabolic disorders, but also with AD and cognitive impairment in animal models. Diabetes mellitus and hyperglycemia has been shown to be risk factors for the development of AD in later life (145). In fact, it is well known that a state of insulin-resistance induces cognitive impairment (75, 144, 146). Some authors have also detected a cognitive improvement with glucose pathways mediating agents. For instance, incretin glucagon-like peptide 1 plays a beneficial effect in the NOR test, but not in OL (147). The incretin glucose-dependent insulinotropic polypeptide might also may show an improvement in NOR and OL tests (148).

6. CONCLUSIONS

Evaluation of cognition in animal models has become a fundamental tool in multiple areas of translational neuroscience and is useful for studying both the mechanisms underlying neurological disorders and the efficacy of novel drugs in reversing cognitive deficits in disease models. This is particularly useful in the AD field, as the main clinical hallmark of the disease is memory loss. Toward this end, Table 2 lists some drugs that have provided positive results in the NOR test. However, it is important to keep in mind that NOR test or other cognitive tasks are incomplete analogues of human cognition. Indeed, this fact could contribute to the continuous clinical trial failures with drugs that showed promising efficacy in preclinical behavioural tasks. In any case, in the absence of practical alternatives, animal models will continue to be essential for testing new therapeutic strategies.

NOR test is a very useful instrument that allows researchers to explore the cognitive status of rodents. The NOR test is a simple method that does not need external motivation reward or punishment, but a little training or habituation is required, it can be completed in short time so animals do not feel stressed, and it can assess the recognition memory after only one trial, which gives it an advantage over other methods. Its application is not limited to a field of research and enables that various issues can be studied, not only memory and learning, but also preference for novelty, influence of different brain regions in the process of recognition, all of those relevant areas in the field of AD research. Its proven reliability, as well as its useful variations make this test suitable for evaluating new drugs and their effects in AD experimental models. We suggest here that combinations of different cognitive assays and experimental models will allow investigating different aspects of AD pathology and disease progression and further developing strategies in the treatment of AD.

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Abbreviations: NOR: Novel object recognition, AD: Alzheimer´s disease, A beta: amyloid, beta peptide

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