Refining learning and memory assessment in laboratory rodents. An ethological perspective

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Summary. - In neuroscientific research, the importance of a multi-level approach in studying behaviour, ranging from the molecular to the behavioural level, has been increasingly recognised. In fact, behaviour represents the ultimate output of the brain, and behavioural phenotyping may provide functional information that may not be detectable using molecular, cellular, or histological evaluations. To correctly exploit the study of behaviour in the field of neuroscience, the principles and strategies of ethology must be carefully considered, so as to design appropriate experiments and accurately interpret data. In particular, ethological-type scoring, which entails both the close observation of the studied behaviour and the taking into account of its possible evolutionary history and adaptive significance, can contribute to standardising experimental protocols, so as to improve the quality of data and the welfare of the experimental animal.

Key words: behaviour, water maze, radial maze, semi-naturalistic, rat, mouse.

Riassunto (Valutazione delle capacità di apprendimento e memoria in roditori di laboratorio: una prospettiva etologica). - Il comportamento rappresenta il risultato finale dell'attività cerebrale e la descrizione del fenotipo comportamentale fornisce informazioni rilevanti che integrano quelle ottenute mediante valutazioni molecolari, celluari o istologiche. Poiché le misurazioni comportamentali basate sui principi dell'etologia implicano osservazioni accurate e una considerazione della storia evolutiva e del significato adattativo del comportamento in esame, permettono di disegnare protocolli sperimentali in grado di migliorare sia la qualità dei dati raccolti, che il benessere psicofisico animale. L'applicazione di questo approccio nello studio delle capacità di apprendimento e memoria consente di migliorare le strategie metodologiche nella misurazione delle capacità cognitive dei roditori.

Parole chiave: comportamento animale, labirinto ad acqua, labirinto radiale, condizioni seminaturalistiche, ratto, topo.

An ethological perspective in neurobehavioural studies

As pointed out by Charles Darwin in The expression of emotions in man and animals, each species has evolved its own behavioural repertoire which has been shaped by its unique evolutionary history [1]. As a consequence, different species can react differently to the same stimulus or manifest different behavioural displays in response to the same internal drive [2, 3]. Thus studying the behavioural patterns of a given species from an ethological perspective requires that the eco-ethological constraints that shaped these patterns be taken into account, in that these constraints reflect the context in which the behavioural patterns should be interpreted [4, 5]. This point is crucial in studying behaviour from an ethological perspective and must thus be considered in both designing experiments and interpreting data.

In evaluating specific behavioural competencies, the stimuli provided should be, from an eco-ethological standpoint, relevant to the natural habitat of the given species [4, 6]. For instance, rats can learn to associate food with a nausea-inducing substance in a single trial, whereas many trials are needed to teach them to avoid food associated with an electric foot-shock: the relevance of this propensity appears to be evident when considering its possible adaptive value (i.e., it allows rats to avoid food that previously made them sick) [7].

The widespread use of animals in the study of human pathologies [8-11] has given rise to issues concerning the comparability of different species in terms of selected behavioural responses and the appropriate evaluation of the clinical relevance of behavioural alterations found in experimental models [11, 12]. Again, the ethological perspective is crucial. For instance, in studying the behaviour of mouse models of human neuropathologies, the analysis of

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human-like symptoms should be primarily based on the functional similarity of the observed behaviour rather than on the equivalency of the behaviour. The crucial point is not whether the mouse shows a given behavioural impairment, but rather, how a behavioural impairment manifests itself in a mouse [12, 13]. Ethological studies have provided detailed descriptions of the behavioural repertoire of mice, allowing the specific behaviours to be accurately analysed, so that deficits in specific behavioural abilities can be identified [5, 9, 10, 14, 15].

The study of behaviour from an ethological perspective entails considering each behaviour as part of a complex repertoire. Thus to thoroughly describe the behavioural phenotype of rodents, testing must be multi-tiered, with each tier being increasingly complex in terms of the responses scored [16]. The lower tiers are used to assess simple behavioural and functional responses, such as reflexes, postures, motor coordination, and sensory abilities, whereas the higher tiers are used to test more complex behaviour, including learning and memory, attention, and anxiety. With this approach, it can be determined whether or not impairment in complex responses is actually due to deficits in simple behavioural or physiological functions, such as motor coordination or altered pain sensitivity [16-18]. Furthermore, a selected behavioural competency cannot be accurately assessed without investigating different endpoints, since distinct behavioural competencies may influence each other in generating a selected response. For example, the performance of a mouse in a learning test, although clearly related to its learning abilities, can also be affected by other factors, such as emotional states [19, 20] and, in a plus maze test, performance can be related to the levels of both activity and anxiety [21, 22].

Another important aspect of behavioural studies are the ethological needs of the animals, which can in part be met through the ethological refinement of experiments, as pointed out by Russel and Burch in their book *The principle of humane experimental techniques* [23], a milestone in the improvement of the wellbeing of laboratory animals. A direct consequence of enhanced psychophysical welfare is a more reliable behavioural response and a consequent increase in the quality of data [24, 25].

The assessment of learning and memory in laboratory rodents

Since rodents must learn and remember their environment's spatial characteristics in order to survive in the wild, many learning tests consist of spatial learning tasks, such as those provided by the water maze and the radial maze. The water maze, which was first described in the early 1980s [26, 27] and is currently the most commonly used behavioural test in neuroscience [28], consists of a circular pool filled with water, with an escape platform hidden just below water level. After having been placed in the water, the animal must first learn, during repeated trials, to find the platform in order to escape from the water. Performance is further explored in a probe trial, without the platform, and through reversal training, with the platform placed in a different position. Learning and memory abilities are mainly measured by scoring latency and the length of the path that the animal swims to reach the platform and, in the probe trial, by scoring the time that the animal spends swimming close to the original location of the platform [27].

The eight-arm radial maze, which was originally developed for rats by Olton and Samuelson [29] and which is also quite popular, is a "dry" maze with eight arms radiating from a central compartment. In the simplest version, a small portion of food, which is not visible from the central compartment, is placed at the end of each arm. At the beginning of the test, the animal is placed in the central area and allowed to enter any arm and eat the food. Since the food is not replaced, the animal must remember those arms that have already been visited. This test has been extensively used for rats and mice [30, 31], and different versions have been developed to more accurately assess learning and memory. For example, to discriminate between working and reference memory, food is placed in only four of the eight arms: multiple entries in baited arms are considered as errors in working memory, whereas entries in non-baited arms are considered as errors in reference memory [32]. Many other experiments have been successful in assessing spatial learning, including the T-maze [33] and the Barnes maze [34], and nonspatial learning, such as fear conditioning [35], avoidance experiments [36], and the social transmission of food preferences [37].

Mice vs rats. Species differences matter

Rats, in particular *Rattus norvegicus*, have been used in research since the mid-1800s [38], and a vast amount of knowledge on the physiology and behaviour of this species has been obtained, making it, historically, the most commonly used species in the study of behaviour. However, because of recent advances in genetics and the greater amenability of mice to genetic manipulation, neuroscientific research is often conducted using mice, so that many of the behavioural tests originally developed for rats have begun to be used in studies on mice. However, the difference of species-specific competencies of rats and mice have not always been adequately taken into consideration [39-41]. For instance, there exist important differences between the two species in terms of their natural habitats: rats live in subterranean burrows near water, such as on river banks, which is reflected in the greater competency of rats in experiments involving swimming. Although mice can learn water-based tasks, their learning performance is worse when compared to rats [39], although the differences decrease when parameters such as the size of the pool [20, 41] and the height of its walls [42] are considered. By contrast, the two species have shown similar levels of spatial learning ability when subjected to a dry-land maze, such as a radial-arm maze [40]. These data corroborate the hypothesis that, though similar, the learning abilities of the two species greatly depend on the eco-ethological relevance of the stimuli and settings employed [4-5]. Other eco-ethological factors may also contribute to differences in performance in a water maze; for example, it has been hypothesised that different predatory avoidance responses may affect the strategy to cross open-spaces and thus to reach the platform [43]. Moreover, nonethological factors may also play an important role in learning performance. For example, in water-based tests, the animal is exposed to water for a few minutes. If the water is too cold (less than 24-26 °C) mice, unlike rats, can become severely hypothermic. Thus when testing mice in these experiments it is crucial to monitor temperature in order to obtain reliable data [44].

Although these considerations should not preclude the use of tests developed for rats in mouse studies, or vice versa, tests should be modified according to the species-specific competencies yet taking into account the potential effect of these modifications on behavioural performance.

Rodent performance. Are the selected behavioural phenomena being assessed?

As mentioned, a selected response can be generated by various competencies even in the best designed behavioural tests [5]. For instance, the data obtained on mice subjected to a water maze can be greatly affected by thigmotaxis (i.e., keeping in contact with, or very close to, the walls of the pool). In fact, a factor analysis of swimming navigation conducted on a large number of animals has clearly demonstrated that poor learning in mice is often the result of marked thigmotaxis [19], which is generally considered to be an emotional response (i.e, more anxious animals are afraid of crossing open spaces) [45]. In the first trials, when released into the pool all of the mice swam along the walls, whereas in subsequent trials many of them developed a goal-directed search strategy, although some of the mice continued to swim along the walls. Distinguishing thigmotaxis from other behavioural

components, which can be done with specific software packages (e.g., Wintrack, http://www.dpwolfer.ch/pers/ Wintrack/Wintrack.htm [46]), is important because failing to do so could result in a limited interpretation of the data. Specifically, the poor performance shown by mice with thigmotaxis in the probe trial should not be solely considered as the result of a spatial memory deficit; it instead suggests that emotional responses should be thoroughly analysed [19, 47]. Since the water maze is an aversively motivated learning test, the acquisition of the task is still a stressful event, even when aversive factors are minimised [48, 49].

To understand whether or not the data collected with a given experiment actually reflects the behaviour of interest, the careful observation of behaviour during the test, as opposed to the mere collection of data, can also be quite helpful [5]. For instance, careful observation of mice tested for spatial learning using a radial maze not equipped with doors at the entrances to the arms revealed that many of the mice simply entered the arm adjacent to the one just visited, moving in a clockwise or counter-clockwise direction. Although this is a form of learning, it is clearly not a spatial learning strategy. To solve this problem, the apparatus was modified so as to confine the mouse to the central part of the maze for a few seconds after returning from each arm [33, 50]. In this way, the mice perform the task without developing strategies that deeply bias the spatial learning performance, although this procedure continues to be used only rarely [33, 50, 51]. The above considerations should also be applied when using other spatial learning tests, such as the Barnes maze [34].

The semi-naturalistic approach

Although a semi-naturalistic approach to studying learning and memory has been extensively used in birds [52-55] and less frequently in rodents, the need to design more ethologically-oriented experiments for evaluating the cognitive abilities of rodents has been discussed [15, 56, 57]. In particular, emphasis has been placed on the potential use of a semi-naturalistic approach in studies on genetically modified mice with neural dysfunction [13, 47]. In fact, many researchers are now aware of just how artificial the laboratory environment is: rodents can show "abnormal" behavioural responses which interfere with the resolution of the given learning task.

Understanding the natural learning behaviour of rodents (in naturalistic or semi-naturalistic settings such as large outdoor pens) may be important for: a) understanding cognitive processes sensitive to natural selection (and consequently to genetic manipulation); b) recognising the ecological relevance of selected behavioural responses; and c) developing new testing strategies and validating commonly used laboratory tests [58]. Although the spontaneous behaviour in outdoor 234

enclosures has been observed for populations of rodents [56, 59], only in recent years has it been possible to obtain data on individual animals, following the development of automated systems that use microchips for small mammals [60]. The use of radio-telemetric devices has made it possible to automatically monitor individual visits to feeding stations and to develop a computerised system of a food controlled schedule in an outdoor pen partially resembling a classic radial maze task [58]. The data gathered in this experimental environment, which is more similar to the natural habitat in terms of physical and social features, have indicated not only that spatial and temporal adaptation to the availability of food is influenced by circadian and territorial factors but also that mice in the pen visit a greater number of different feeding boxes during the food controlled schedule, which is one of the behavioural responses on which the laboratory radial-maze task is based.

Recently, genetically modified mice, namely mutant mice lacking neurotrophin receptor TrkB in the forebrain, were subjected to the radial-maze task adapted to a naturalistic setting. Although these mice were impaired in both the semi-naturalistic and the laboratory version of the test [61], the data obtained with the semi-naturalistic task provided additional information. In particular, the mutant mice learned to accomplish the radial-maze task just as well as the control mice, yet they failed to adapt to a subsequent new regimen of *ad libitum* food availability, indicating that they suffered from a lack of behavioural flexibility, as opposed to a cognitive deficit [62].

Overall, these data suggest that the use of a seminaturalistic environment can reveal behavioural responses that are not detected by common laboratory tests. Moreover, the use of a semi-naturalistic environment appears to be beneficial not only to the study of cognitive abilities but to neuroscientific research on the whole. For instance, adult neurogenesis in rat hippocampus appears to be more likely if animals live in a complex environment (the visible burrow system developed by Blanchard and Blanchard [63]), as opposed to being housed in standard laboratory conditions [64].

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REFERENCES

1. Darwin C. *The expression of emotions in man and animals.* Oxford: Oxford University Press; 1872.

- Katz PS, Harris-Warrick RM. The evolution of neuronal circuits underlying species-specific behavior. *Curr Opin Neurobiol* 1999;9:628-33.
- 3. Crews D. Species diversity and the evolution of behavioral controlling mechanisms. *Ann N Y Acad Sci* 1997;807:1-21.
- Kamil AC, Mauldin JE. A comparative-ecological approach to the study of learning. In: Bolles RC, Beecher MD (Ed.). *Evolution and learning*. Hillsdale, NJ: Lawrence Erlbaum Associates, Publishers; 1988. p.
- Martin P, Bateson P. Measuring behaviour. An introductory guide. Cambridge, UK: Cambridge University Press; 1993.
- Capone F, Puopolo M, Branchi I, Alleva E. A new easy accessible and low-cost method for screening olfactory sensitivity in mice: behavioural and nociceptive response in male and female CD-1 mice upon exposure to millipede aversive odour. *Brain Res Bull* 2002;58:193-202.
- 7. Garcia J, Koelling RA. Relation of cue to consequence in avoidance learning. *Psychonomic Sci* 1966;4:123-4.
- 8. Bucan M, Abel T. The mouse: genetics meets behaviour. *Nat Rev Genet* 2002;3:114-23.
- Crawley JN. What's wrong with my mouse? Behavioral phenotyping of transgenic and knockout mice. New York: Wiley-Liss; 2000.
- Branchi I, Ricceri L. Transgenic and knock-out mouse pups: growing need for behavioral analysis. *Genes Brain Behav* 2002;1:135-41.
- Watase K, Zoghbi HY. Modelling brain diseases in mice: the challenges of design and analysis. *Nat Rev Genet* 2003;4:296-307.
- Cenci MA, Whishaw IQ, Schallert T. Animal models of neurological deficits: how relevant is the rat? *Nat Rev Neurosci* 2002;3:574-9.
- 13. Gerlai R, Clayton NS. Analysing hippocampal function in transgenic mice: an ethological perspective. *Trends Neurosci* 1999;22:47-51.
- 14. Crusio WE, Gerlai RT (Ed.). *Handbook of molecular-genetic techniques for brain and behavior research*. Amsterdam: Elsevier; 1999.
- 15. Alleva E, Fasolo A, Lipp HP, Nadel L, Ricceri L (Ed.). *Behavioural brain research in naturalistic and semi-naturalistic settings*. Dodrecht: Kluwer; 1995.
- 16. Crawley JN. Behavioral phenotyping of rodents. *Comp Med* 2003;53:140-6.
- 17. Rogers DC *et al.* Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. *Mamm Genome* 1997;8:711-3.
- Bignami G. Economical test methods for developmental neurobehavioral toxicity. *Environ Health Perspect* 1996;104(Suppl 2):285-98.
- Wolfer DP, Stagljar-Bozicevic M, Errington ML, Lipp HP. Spatial memory and learning in transgenic mice: fact or artifact? *News Physiol Sci* 1998;13:118-23.
- van der Staay FJ. Effects of the size of the morris water tank on spatial discrimination learning in the CFW1 mouse. *Physiol Behav* 2000;68:599-602.

- Rodgers RJ, Johnson NJ. Factor analysis of spatiotemporal and ethological measures in the murine elevated plus-maze test of anxiety. *Pharmacol Biochem Behav* 1995;52:297-303.
- File SE. Factors controlling measures of anxiety and responses to novelty in the mouse. *Behav Brain Res* 2001;125:151-7.
- Russell WMS, Burch RL. The principle of humane experimental techniques. London: Methuen; 1959.
- Alleva E, Vitale A. We urgently need more data to improve the lives of laboratory animals. *Nature* 2000;405:116.
- Vitale A, Alleva E. Ethological and welfare considerations in the study of aggression in rodents and nonhuman primates. In: Haug M, Whalen RE (Ed.). *Animal models of human emotion and cognition*. Washington, DC: American Psychological Association; 1999. p. 283-95.
- Morris R. Spatial localization does not require the presence of local cues. *Learn Motiv* 1981;12:239-60.
- Morris R. Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 1984;11:47-60.
- D'Hooge R, De Deyn PP. Applications of the Morris water maze in the study of learning and memory. *Brain Res Brain Res Rev* 2001;36:60-90.
- Olton DS, Samuelson RJ. Remembrance of places past: spatial memory in rats. J Exp Psychol 1976;2:97-116.
- Walsh TJ, Chrobak JJ. The use of the radial arm maze in neurotoxicology. *Physiol Behav* 1987;40:799-803.
- 31. Olton DS. The radial arm maze as a tool in behavioral pharmacology. *Physiol Behav* 1987;40:793-7.
- Olton DS, Becker JT, Handelmann GE. Hippocampus, space, and memory. *Behav Brain Sci* 1979;2:313-65.
- 33. Crusio WE. Methodological considerations for testing learning in mice. In: Crusio WE, Gerlai RT (Ed.). Handbook of molecular-genetic techniques for brain and behavior research. Amsterdam: Elsevier; 1999. p. 638-51.
- Barnes CA. Memory deficits associated with senescence: a neurophysiological and behavioral study in the rat. J Comp Physiol Psychol 1979;93:74-104.
- Anagnostaras SG, Gale GD, Fanselow MS. Hippocampus and contextual fear conditioning: recent controversies and advances. *Hippocampus* 2001;11:8-17.
- Pierce JM. Animal learning and cognition. Erlbaun (UK): Psychology Press; 1997.
- Galef BG Jr, Wigmore SW. Transfer of information concerning distant foods: A laboratory investigation of the "Informationcentre" hypothesis. *Anim Behav* 1983;31:748-58.
- Sharp PE, La Regina MC. *The laboratory rat.* Boca Raton, FL: CRC Press; 1998.
- Whishaw IQ. A comparison of rats and mice in a swimming pool place task and matching to place task: some surprising differences. *Physiol Behav* 1995;58:687-93.
- Whishaw IQ, Tomie JA. Of mice and mazes: similarities between mice and rats on dry land but not water mazes. *Physiol Behav* 1996;60:1191-7.

- Frick KM, Stillner ET, Berger-Sweeney J. Mice are not little rats: species differences in a one-day water maze task. *Neuroreport* 2000;11:3461-5.
- Carman HM, Mactutus CF. Ontogeny of spatial navigation in rats: a role for response requirements? *Behav Neurosci* 2001;115:870-9.
- 43. Schenk F. Comparison of spatial learning in woodmice (*Apodemus sylvaticus*) and hooded rats (*Rattus norvegicus*). J Comp Psychol 1987;101:150-8.
- 44. Iivonen H, Nurminen L, Harri M, Tanila H, Puolivali J. Hypothermia in mice tested in Morris water maze. *Behav Brain Res* 2003;141:207-13.
- 45. Belzung C. Measuring rodent exploratory behavior. In: Crusio WE, Gerlai RT (Eds.). *Handbook of molecular-genetic techniques for brain and behavior research*. Amsterdam: Elsevier; 1999. p. 739-49.
- Wolfer DP, Lipp HP. A new computer program for detailed offline analysis of swimming navigation in the Morris water maze. *J Neurosci Methods* 1992;41:65-74.
- 47. Lipp HP, Wolfer DP. Genetically modified mice and cognition. *Curr Opin Neurobiol* 1998;8:272-80.
- Francis DD, Zaharia MD, Shanks N, Anisman H. Stress-induced disturbances in Morris water-maze performance: interstrain variability. *Physiol Behav* 1995;58:57-65.
- McMahan RW, Sobel TJ, Baxter MG. Selective immunolesions of hippocampal cholinergic input fail to impair spatial working memory. *Hippocampus* 1997;7:130-6.
- Dubreuil D, Tixier C, Dutrieux G, Edeline JM. Does the radial arm maze necessarily test spatial memory? *Neurobiol Learn Mem* 2003;79:109-17.
- 51. Olton DS, Collison C, Werz MA. Spatial memory and radial arm maze performance of rats. *Learn Motiv* 1977;8:289-314.
- 52. Clayton NS, Krebs JR. Lateralization in memory and the avian hippocampus in food-storing birds. In: Alleva E, Fasolo A, Lipp HP, Nadel L, Ricceri L (Ed.). *Behavioural brain research in naturalistic and semi-naturalistic settings*. Dodrecht: Kluwer; 1995. p. 139-58.
- Clayton NS, Bussey TJ, Dickinson A. Can animals recall the past and plan for the future? *Nat Rev Neurosci* 2003;4:685-91.
- 54. Bingman VP, Strasser R, Jones TJ, Gagliardo A, Ioale P. Homing pigeons, hippocampus and spatial cognition. In: Alleva E, Fasolo A, Lipp HP, Nadel L, Ricceri L (Ed.). *Behavioural brain research in naturalistic and semi-naturalistic settings*. Dodrecht: Kluwer; 1995. p. 207-24.
- 55. Shettleworth SJ. Comparative studies of memory in food storing birds. In: Alleva E, Fasolo A, Lipp HP, Nadel L, Ricceri L (Ed.). Behavioural brain research in naturalistic and semi-naturalistic settings. Dodrecht: Kluwer; 1995. p. 159-92.
- 56. Berdoy M. Making decision in the wild: constraints, conflicts and communication in foraging rats. In: Galef BGJ, Mainardi D, Valsecchi P (Ed.). *Behavioral aspects of feeding. Basic and applied research in mammals.* Chur, CH: Harwood; 1992. p. 289-313.
- 57. Galef BGJ. Reciprocal heuristics: a discussion of the relationship of the study of learned behavior in laboratory and field. *Learn Motiv* 1984;15:479–93.
- Dell'Omo G, Ricceri L, Wolfer DP, Poletaeva II, Lipp H. Temporal and spatial adaptation to food restriction in mice under naturalistic conditions. *Behav Brain Res* 2000;115:1-8.

- 59. Valsecchi P, Singleton GR, Price WJ. Can social behaviour influence food preference of wild mice, *Mus domesticus*, in confined field populations? *Aust J Zool* 1996;44:493-501.
- Dell'Omo G, Shore RF, Lipp HP. An automated system, based on microchips, for monitoring individual activity in wild small mammals. *J Exp Zool* 1998;280:97-9.
- 61. Minichiello L *et al.* Essential role for TrkB receptors in hippocampus-mediated learning. *Neuron* 1999;24:401-14.
- 62. Vyssotski AL *et al.* Long-term monitoring of hippocampusdependent behavior in naturalistic settings: mutant mice lacking neurotrophin receptor TrkB in the forebrain show spatial learning but impaired behavioral flexibility. *Hippocampus* 2002;12:27-38.
- Blanchard RJ, Blanchard DC. Antipredator defensive behaviors in a visible burrow system. J Comp Psychol 1989;103:70-82.
- 64. Gould E. Adult neurogenesis in the mammalian brain. Paris, France: FENS meeting; 2002.