Video Article

The Successive Alleys Test of Anxiety in Mice and Rats

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Abstract

The plus-maze was derived from the early work of Montgomery. He observed that rats tended to avoid the open arms of a maze, preferring the enclosed ones. Handley, Mithani and File *et al.* performed the first studies on the plus-maze design we use today, and in 1987 Lister published a design for use with mice. Time spent on, and entries into, the open arms are an index of anxiety; the lower these indices, the more anxious the mouse is. Alternatively, a mouse that spends most of its time in the closed arms is classed as anxious. One of the problems of the plus-maze is that, while time spent on, and entries into, the open arms is a fairly unambiguous measure of anxiety, time in the central area is more difficult to interpret, although time spent here has been classified as “decision making”. In many tests central area time is a considerable part of the total test time. Shepherd *et al.* produced an ingenious design to eliminate the central area, which they called the “zero maze”. However, although used by several groups, it has never been as widely adopted as the plus-maze. In the present article I describe a modification of the plus-maze design that not only eliminates the central area but also incorporates elements from other anxiety tests, such as the light-dark box and emergence tests. It is a linear series of four alleys, each having increasing anxiogenic properties. It has given similar results to the plus-maze in general. Although it may not be more sensitive than the plus-maze (more data is needed before a firm conclusion can be reached on this point), it provides a useful confirmation of plus-maze results which would be useful when, for example, only a single example of a mutant mouse was available, as, for example, in ENU-based mutagenesis programs.

Video Link

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Introduction

The elevated plus-maze was derived from the early work of Montgomery.[27] He observed that rats tended to avoid the open arms of a maze, preferring the enclosed ones. Handley and Mithani, and File *et al.*[22,29] performed the first studies on the plus-maze design we use today, and in 1987 Lister published a design for use with mice[25]. It is now a standard assay for anxiolytic treatments[23], and several protocols have been published[36,24] http://www.jove.com/video/1088; http://currentprotocols.com/protocol/ph0538.

The elevated plus-maze consists of two opposite open and two opposite closed arms intersecting at a central area, in the form of a + in plan. Percentage time spent on, and percentage entries into, the open arms are an index of anxiety-like behaviour; the lower these indices, the more anxious the mouse is. Alternatively, a mouse that spends most of its time in the closed arms is classed as anxious.

Factor analysis has shown that while indices of open arm activity load heavily on a factor designated as anxiety-like behaviour, and closed arm entries largely reflect motor activity, the boundaries between factors are not definitive; also other factors such as exploration, risk assessment and decision making comprise the profile of behaviour on the plus-maze,[12,32]. Time in the central area is more problematic to interpret, although time spent here has been classified as "decision making"[32]. Although mice spend most of their time in the enclosed arms, and relatively little time on the open arms, in many studies much time is spent in the central area where the arms join. Although this may represent decision-making[32], it is ambiguous in terms of anxiety-like behaviour, although highly anxious mice might be expected to avoid the central area as well as the open arms. An early attempt to eliminate this ambiguous central area was to construct a circular maze with two closed quadrants alternating with two open ones[33]. In this "zero-maze" an animal is unambiguously in an open (anxiogenic) or a closed (nonanxiogenic) zone. However, although used by several groups, it has never been as widely adopted as the plus-maze. In our laboratory we found that whereas the plus-maze (and successive alleys) showed anxiolytic effects in hippocampal lesioned rats, these were not seen on a zero maze constructed exactly like that of Shepherd *et al.*[13]

The successive alleys apparatus was therefore devised with the primary aim of eliminating the central area of the plus-maze. Secondarily, the elements of other tests of anxiety-like behaviour were incorporated. By starting the animal in the enclosed alley 1, an emergence component was introduced, similar to that in Stone's "stovepipe" test[34]. The color of the alleys changes from black (alley 1) through grey (alley 2) to white (alleys 3 and 4). This originates from observations that mice prefer darker areas, as exemplified in the light-dark box test[11]. The walls of the successive alleys also slowly decline in height, unlike in the plus-maze where there is an abrupt transition between high walls and no walls. The width of the alleys progressively decreases to gradually increase the exposure of the mouse to the elevated position of the maze, in concert with the gradual decrease in wall height. All these factors were incorporated in an attempt to increase the sensitivity of the test and span a wider range of anxiety-

like behaviour than the plus-maze does. The successive alleys apparatus was an attempt to bracket a wider range of anxiety-like behaviour baselines, in the same way that the four baseline conditions of the social interaction test as originally performed by File[17] permit detection of subtle modifications of anxiety-like behaviour. File, in a study of the effects of nicotine, only saw differences in the two intermediate anxiety-like behaviour conditions, not the highest or lowest[20].

As well as the plus-maze, another widely used test of anxiety-like behaviour is the light-dark box[11]. Results from the light-dark box and the plus-maze do not always correlate with each other[35]. It is now considered that anxiety-like behaviour is not a unitary phenomenon[19]. The successive alleys test may therefore well give results different to those obtained using the light-dark box, and conceivably even from the plus-maze, since it is subtly different from the latter; however, results between the former and the latter would be expected to be broadly similar.

It has been shown that repeated testing on the plus-maze alters the type of anxiety observed.[8,21] It may be that exposing an animal to the plus-maze followed by the successive alleys eliminates the change in the type of anxiety-like behaviour measured if the plus-maze test is repeated. An example of this was seen when the plus-maze and successive alleys were used to test medial prefrontal cortex-lesioned and control mice in a temporally counterbalanced manner. The lesioned mice showed significantly lower anxiety-like behaviour in both types of apparatus, regardless of whether testing occurred with the successive alleys or the plus-maze first[15]. It is also possible that sequential use of the plus-maze and successive alleys might preserve the sensitivity to anxiolytic drugs seen when the plus-maze test is repeated.[18]

Rats can also be tested on a larger version of the successive alleys. The procedure is exactly the same as for mice.

As in the case of testing for hyponeophagia, the prior general experience of the animals, apart from elevated maze anxiety-like behaviour testing per se, may affect the result.[1,16]

Protocol

1. Method

1.1 Apparatus

The successive alleys apparatus consists of four successive linearly connected alleys of putatively increasing anxiogenic character and is made of painted wood (see **Figure 1** and, for comparison, **Figure 2** of a plus-maze). Details are shown in **Table 1**:

Alley	Length	Width	Wall height	Color
1	25.0	8.5	25.0	Black
2	25.0	8.5	5.0	Grey
3	25.0	3.5	0.8	White
4	25.0	1.2	0.2	White

Table 1. Construction details for the successive alleys apparatus. All dimensions are in cm.

The apparatus is elevated approximately 50 cm by clamping the back-extended floor of alley 1 to a laboratory bench, shelf or other support. The open end of alley 1 should be at least 10 cm away from the support to prevent mice trying to climb on to it. Provide adequate and ample padding under the alleys in case a mouse falls off.

The rationale for the different wall heights was: those of alley 1 need to be high to maximize its non-anxiogenic properties. Also high walls create a darker environment as long as the laboratory light source is not immediately overhead. The walls of alley 2 were judged from experience to appear less safe than alley 1 to mice, but to still provide some degree of protection. Alleys 3 and 4 had just enough height to maximize anxiety but still provide a grip if the mouse was in danger of falling off. In practice falls are rare, and no more frequent than on the plus-maze.

The apparatus should be handled carefully as the narrow Alley 4 is particularly susceptible to damage. If securely fastened, the apparatus should not vibrate as the mouse moves over it. To minimize both of these potential problems, the apparatus could be given a strengthening extra "spine" spanning its length under the floor. A successive alleys apparatus for rats can also be made. Details are shown in **Table 2**:

Alley	Length	Width	Wall height	Color
1	45.0	9.0	29.0	Black
2	45.0	9.0	2.5	Grey
3	45.0	6.7	0.5	White
4	45.0	3.5	0.3	White

Table 2. Construction details for the successive alleys apparatus for rats. All dimensions are in cm.

1.2 Procedure

* Bring the mice to the experimental room 5-20 min before testing to ensure that they are adapted to the room and are at a moderate level of arousal.

* As with all experiments where animals are tested once for spontaneous behavior on a novel apparatus, it is good practice for the alleys to be first given a mouse odor; place non-experimental mice on the apparatus for a few minutes, then clean the apparatus as during the main experiment. The objective is to ensure a slight but uniform background mouse odor, on the basis that it is impossible to totally remove animal odor (let alone prove that this has been achieved).

* Place a mouse at the closed end of alley 1 facing the wall. Start timers 1) for the overall length of the test + latency to enter arms, and 2) time spent in alley 1. When the mouse places all 4 feet on to the next alley it is considered to have entered it. Record total time spent in each alley (all four feet) and the number of entries (both forward and backward). Record the number of faecal boli and whether any urination has occurred.

* Test duration: 5 min. (This is the normal time; if you know or suspect that the animals you are testing will not be very anxious, shortening the test duration to 3 min may be advantageous. Note what time you *do* use).

* A combined event counter/timer is invaluable for this work as well as for timing the plus-maze. We have a battery-powered portable one with four buttons, each associated with an event counter and a timer (1/10 s), built in the Psychology department. Pressing button 1 registers an event (entry into alley 1) and starts a timer. Releasing the button stops the timer. Pressing button 2 registers an alley 2 entry and starts alley 2 timer, etc. However, one must subtract one entry from the total number of entries registered on alley 1 as the first entry was initiated by the experimenter, not the mouse.

* To summarize; for each alley record: the latency to enter it (apart from alley 1 of course), the number of entries into it and the total time spent there.

* If a mouse falls off, stop the clock and replace it on the alley from which it fell, facing alley 1.

* All urine and faeces are removed between animals and the apparatus cleaned with a damp, followed by a dry tissue. It is important to clean and disinfect the equipment between each mouse tested.

* In addition, always wear gloves when handling rodents.

Representative Results

Rats with complete, ventral or dorsal cytotoxic hippocampal lesions showed moderate or partial anxiolytic effects on the plus-maze, while ventral, but not dorsal, lesioned rats showed anxiolytic effects in the successive alleys[6,26]. However, in another laboratory, electrolytic dorsal hippocampal lesions did not have anxiolytic effects[35]; this is not improbable as the former study obtained only partial anxiolytic effects with dorsal cytotoxic lesions, and it is the ventral hippocampus that is thought to play the major role in anxiety-like behaviour[4,7].

Medial septal lesions also reduced anxiety-like behaviour in rats in the successive alleys[5]. In contrast, the zero maze did not detect any anxiolytic effects of complete cytotoxic hippocampal lesions[13]. C57BL/6JolaHsd mice spent a longer time on the most anxiogenic of the successive alleys (alley 4) than 129S2/SvHsd mice[9]. In contrast, a clear anxiety-like behaviour difference was not seen on the plus-maze, where 129 mice spent longer time in the central area, a result also seen in another 129 strain, 129/SvEvTac[2]. In unpublished work on mice with complete, dorsal or ventral lesions of the hippocampus, there was a general tendency to observe anxiolytic-like results of complete lesions. However, these were nowhere near as marked as in hyponeophagia tests, where the anxiolytic effect of complete or ventral lesions was strong and reliable. For example, the complete lesioned mice spent less time in the closed arms of the plus-maze, but not more in the open. The successive alleys test was done twice; only on the second trial were there significant anxiolytic effects of the complete lesions. Both the successive alleys and the plus-maze make demands on orientation processes, so the lesioned animals may be taking more time to "get their bearings" and hence the time available to demonstrate anxiolytic effects is reduced.

When mice with lesions of the medial prefrontal cortex were tested on the plus-maze and successive alleys apparatuses with the order of testing counterbalanced, there was no effect of which was tested first, but clear anxiolytic effects were present in both tests compared to controls[15] (see **Figures 3** and **4**). The lesioned mice made significantly more entries into each alley of the successive alleys compared to controls, and the open arms of the plus-maze, although interestingly entries into the closed arms of the latter were not higher than controls. In the open field the lesioned mice were significantly more active than controls, and marginally so in photocell activity cages.

The successive alleys test has also been shown to be sensitive to the effects of the anxiolytic drug chlordiazepoxide. In unpublished work 12 mg/kg i.p.chlodiazepoxide (CDZP) was shown to be active in male NIH mice, as used by Lister in the original description of the plus-maze for mice[25]. The CDZP-treated mice spent less time than controls in Alley 1 (158 ± 12.1 vs 212 ± 14.8 s, P=0.0108, and made more entries into Alleys 2-4 (see **Table 3**).

Alley	Control	CDZP	P
2	7.2 ± 1.2	18.4 ± 2.0	0.0001
3	1.0 (0-3.5)	5.0 (2.5-12.3)	0.0277
4	0.0 (0.0-1.0)	2.0 (0.0-5.0)	0.0604

Table 3. Entries into Alleys 2-4 of the successive alleys apparatus by mice treated with 12 mg/kg i.p. of CDZP or controls. Values are medians and (interquartile range) or means ± SEM according to whether data was non-parametric or parametric; Mann-Whitney U test or *t* test respectively. N=11/group.

Figure 1. The mouse successive alleys apparatus. It is made of painted wood and is clamped on to the bench.

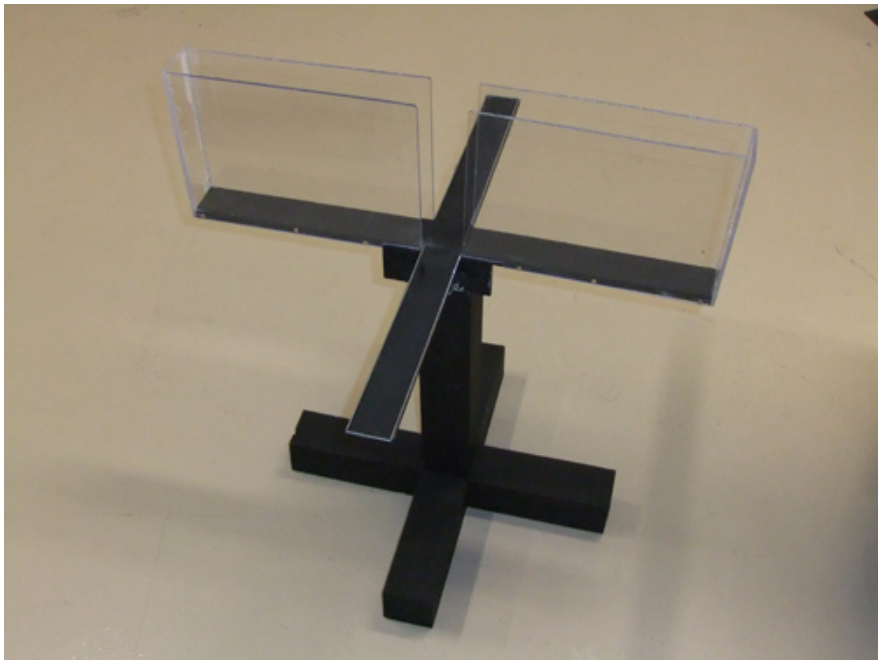

Figure 2. The mouse plus-maze. It is made of black painted wood with transparent Perspex walls on the enclosed arms.

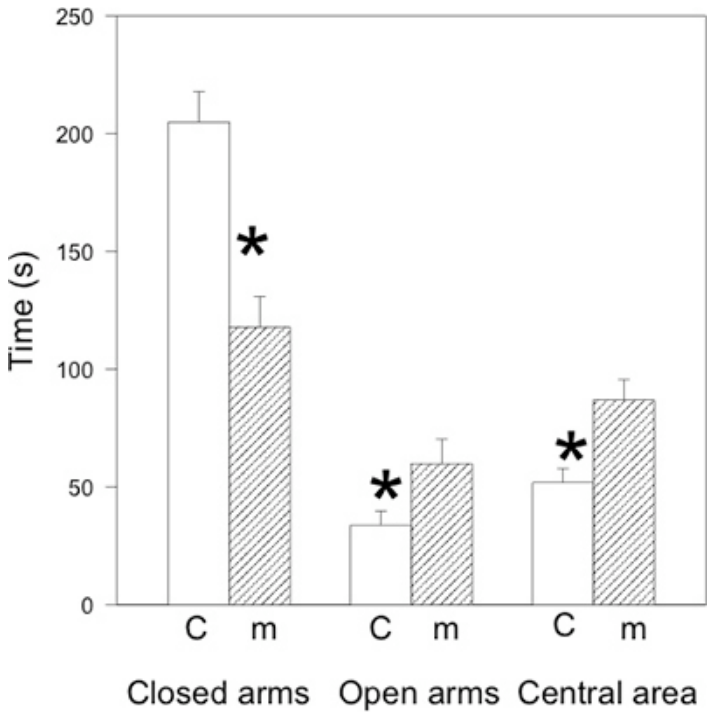

Figure 3. The anxiolytic effect of medial prefrontal cortex lesions (m) on the plus-maze compared to controls (C). * = P< 0.05 C vs m. Data originally published in reference 15.

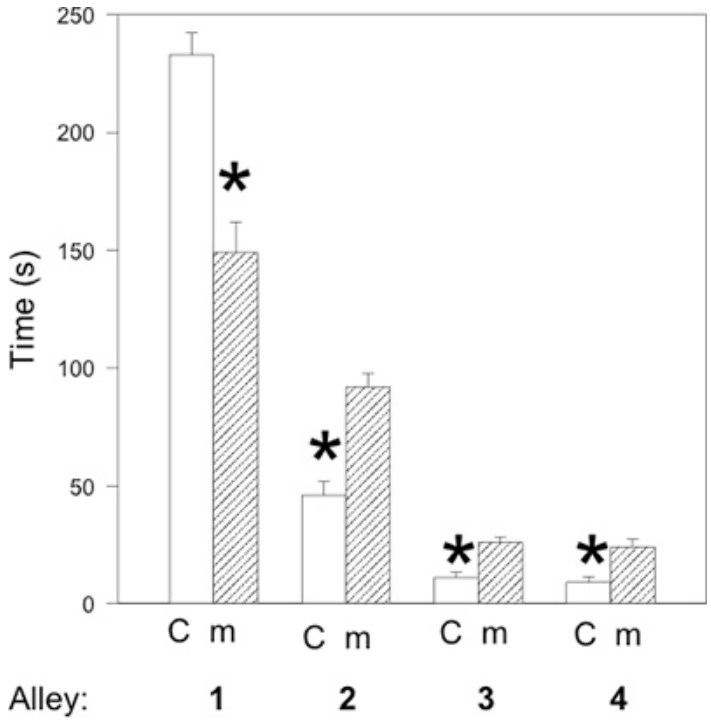

Figure 4. The anxiolytic effect of medial prefrontal cortex lesions (m) on the successive alleys compared to controls (C). * = P< 0.05 C vs m. Data originally published in reference 15.

Discussion

In conclusion, the successive alleys test produces generally similar results to the plus-maze when lesions of the hippocampal complex or medial prefrontal cortex are made, but further work is needed to assess the effects of lesions in other brain areas thought to be related to anxiety-like behaviour. Occasionally, results on the plus-maze differ markedly from those in the successive alleys; an example of this is our work on Kir6.2 KO mice[14]. When they were tested on both apparatuses, it allowed us to demonstrate that KO mice did not show a general unidirectional change in anxiety-like behaviour, since they spent more time than wild-type mice on the open plus-maze arms (showing less anxiety-like behaviour),

but more time in alley 1 of the successive alleys (showing greater anxiety-like behaviour). Thus, combined use of the plus-maze and successive alleys can eliminate false positive or false negative results, since the two tests, although similar, are not functionally identical.

As in the plus-maze, hyperactivity does not lead to a non-selective increase in the number of alleys entered. Studies have shown that the organization of behavior in mice varies independently of activity [28]. Medial septal lesioned rats, which were shown to be markedly hyperactive in a separate test in photobeam activity monitoring cages only showed significantly greater entries into alleys 2 and 3; entries into alleys 1 and 4 were not different to controls.[5] So the less anxious septal lesioned rats were shuttling back and forth between the two moderately anxiogenic areas of the successive alleys, showing what the apparatus was designed to detect, *i.e.* their position on a spectrum of anxiety. If they had been even less anxious, an increase in crossings between alleys 3 and 4 would have been seen.

Other evidence that hyperactivity does not lead to a false positive in the successive alleys test is that rats with dorsal hippocampal lesions are hyperactive but do not show anxiolytic effects in the successive alleys[7]. In a related test with linear apparatus, the black-white alley test, GluRA KO female mice show rather more anxiety-like behaviour than controls while males are similar to controls, yet both sexes are markedly hyperactive[3]. Kir6.2 KO mice were more active in their home cages than wild type mice but showed les anxiety in the successive alleys[14]. Notably, 8 KO mice jumped or fell off the plus-maze but none from the successive alleys.

One limitation of the successive alleys test as presently used is that, given the varying color of the floor, video-tracking systems would be difficult to use. However, the floor could probably be painted a uniform color as color is only one of the factors in the apparatus that generates anxiety.

The successive alleys test is unlikely, however, not to suffer from the variability problems associated with similar ethologically based tests such as the plus-maze[10,23], but testing a batch of animals on both tasks might help to clarify an inconclusive plus-maze test[30,31,37]; a multiple-test approach may have advantages when testing anxiety-like behaviour.

Disclosures

No conflicts of interest declared.

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