Chapter 6

Behavioural analyses of SAP102 mutant mice

In humans, loss of SAP102 function has significant behavioural consequences. This chapter presents the results of a battery of tests to determine the effect of SAP102 loss on behaviour in the mouse.

6.1 Loss of SAP102 causes a deficit in spatial learning

Male patients with truncating SAP102 mutations have learning difficulties and SAP102 is highly expressed in the hippocampus, where functioning NMDA receptors are required for normal spatial learning. To see whether SAP102 is required for spatial learning the mice were tested in a water maze, a widely used task in which performance is both hippocampal- and NMDAR-dependent (Frankland and Bontempi, ; Morris et al., 1986; Morris et al., 1982). The apparatus consists of a large circular pool of opaque liquid with a small platform hidden just below its surface. To escape from the water, the mouse must use distal spatial cues to navigate its way to the platform. To facilitate direct comparisons with the spatial learning capabilities of the PSD-95 mutant mice, the SAP102 mice were tested using the same water maze apparatus and experimental protocol (Migaud et al., 1998). These experiments were performed and analysed by Jamie Ainge (Division of Neuroscience, University of Edinburgh) and Lianne Stanford (Wellcome Trust Sanger Institute).

Figure 6.1a shows a timeline of the water maze experiment. SAP102 hemizygous mice and wild-type littermate controls were initially tested with a raised, visible platform for three days with four trials per day, to habituate them to the apparatus and tested for differences in visual or motor abilities which could confound the spatial learning aspect of the task. Performance of the mice in the visible platform training is shown in the left panels of the plots in figure 6.1b, c and d.

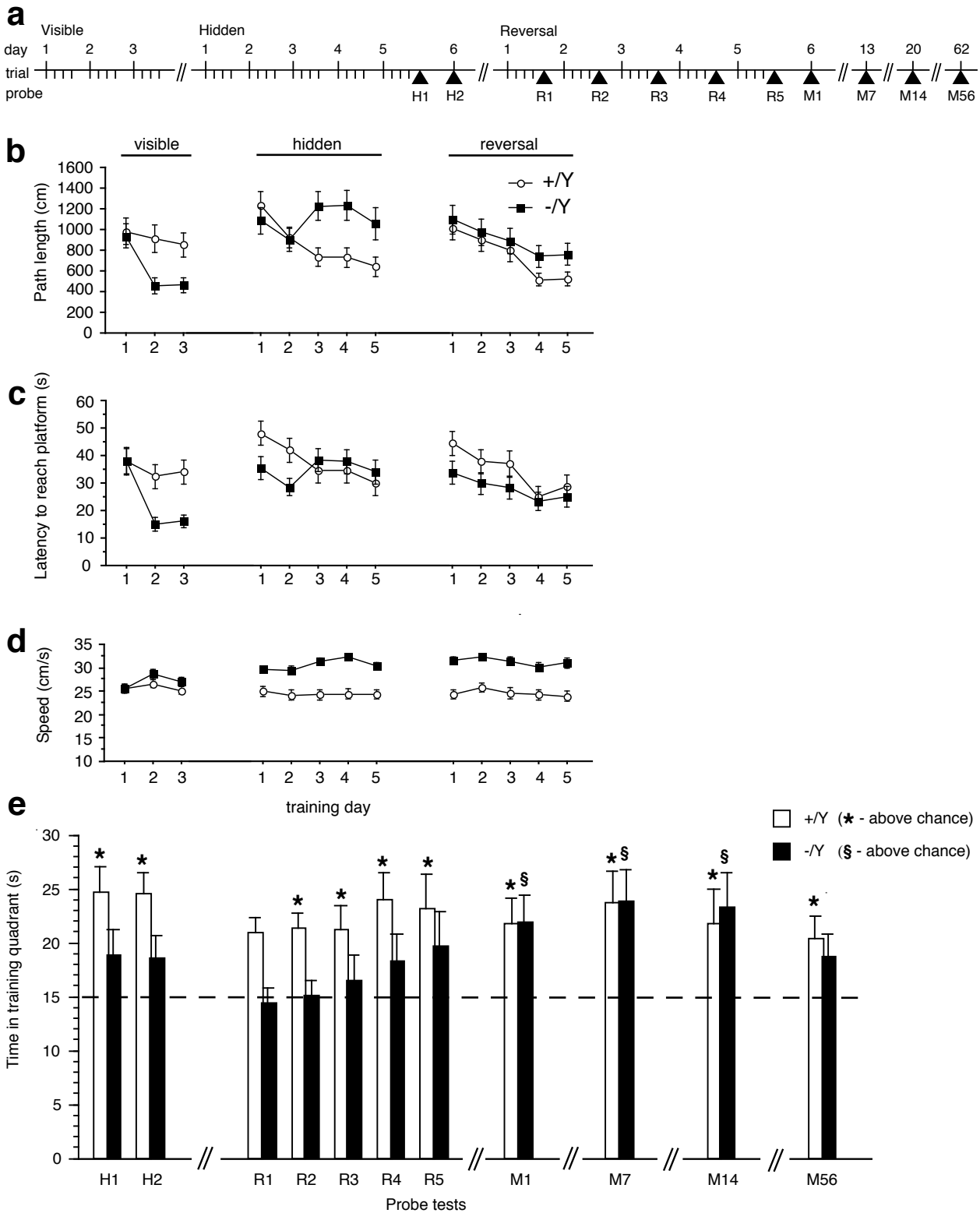

Figure 6.1 SAP102 mice display a spatial learning deficit in the water maze. (a) Mice were given three days training with a visible platform, followed by five days training with a hidden platform, followed by five more days of training with the hidden platform in the opposite location. Probe tests, in which mice were placed in the pool for 60 s without the platform present, were performed immediately (H1) and 24 hrs (H2) after the final hidden platform training trial, after every day of reversal training (R1-5) and 1 day (M1), one week (M7), two weeks (M14) and eight weeks (M56) after the final reversal training trial. **(b)** Mean path length to reach the platform during each day of training. **(c)** Mean latency to reach the platform during each day of training. **(d)** Mean swimming speed during each day of training. **(e)** Time spent in training quadrant during each transfer test.

The overall path length taken by the mice decreased across the three days of visible training [$F(2,48) = 6.64$, $p = 0.003$] indicating that they learnt the task, but there was no significant difference between wild-type and SAP102 -/Y mice [$F(1,24) = 2.83$, $p = 0.11$]. The plot (figure 6.1b) shows that mutant mice improved more rapidly over the three days than wild-type controls and the interaction between genotype and day approaches significance [$F(2,48) = 3.00$, $p = 0.06$].

Consistent with the path length data, the latency to reach the platform (figure 6.1c) decreased across all mice during the visible platform training [$F(2,48) = 8.50$, $p = 0.0007$]. Again there was no overall effect of genotype [$F(1,24) = 2.91$, $p = 0.10$] but the plot shows that mutant mice improved more than the wild-types over the three days, producing a significant genotype x day interaction [$F(2,48) = 3.41$, $p = 0.041$]. The average swimming speed of the mice in the visible platform training stayed constant over the three days [$F(2,48) = 2.58$, $p = 0.086$] and there was no difference between mutant and wild-type individuals [$F(1,24) = 1.55$, $p = 0.23$]. In summary, overall the mice improved their performance in reaching the visible platform with three days of training. There was no evidence of any SAP102-related motor or vision deficit - indeed, the mutant mice swam at the same speed as wild-types but rapidly learnt to take a more direct line to the platform, reducing their path length and latency over that of the controls.

The spatial learning ability of the mice was tested over five days of training with a platform hidden beneath the liquid surface. Performance during these trials is shown in the middle parts of the plots in figure 6.1b, c and d. Figure 6.1b shows that wild-type, but not mutant, mice reduced their path lengths with training and this was confirmed by the presence of a day x genotype interaction [$F(4,100) = 3.06$, $p = 0.02$] and separate statistical analysis of the two groups [$F(4,46) = 3.06$, $p = 0.03$ for wild-types]. A similar pattern was observed in latencies to reach the platform (figure 6.1c) with wild-type but not mutant mice improving with training [$F(4,46) = 3.36$, $p = 0.017$ for wild-types]. However the strikingly longer path lengths taken by mutants in the last

three days of training interestingly did not produce longer latencies on those days. This is explained by the fact that the mutants swam faster than wild-types throughout the training period [$F(1,24) = 19.73$, $p = 0.0002$] as shown in figure 6.1d. In summary, it appears that, unlike wild-types, SAP102 -/Y mice are unable to learn to swim more directly towards the hidden platform. However they swim faster than wild-type mice so that their latency to reach the platform is unimpaired.

Mice were subjected to probe tests 10 min and 24 hrs after the final hidden platform training trial. In a probe test, each mouse is placed in the pool without a platform for one minute. The proportion of time spent in the quadrant of the pool where the platform used to be (the 'target' quadrant) is a good measure of its spatial ability (Gerlai, 2001). The results of these tests are shown in figure 6.1e (H1 and H2). No differences were observed between mutant and wild-type mice in amount of time spent in each quadrant [$Fs(3,78) < 1.4$, $ps > 0.25$)], however, analysis of each genotype separately showed that wild-types spent more time in the training quadrant in both tests than chance would predict [$ts(13) > 2.87$, $ps < 0.013$), while mutants did not ($ts(13) < 1.36$, $ps > 0.20$). This suggests the mutant mice may have a mild deficit in spatial learning.

To examine the spatial abilities of the knockout mice in more detail they were then trained to find a hidden platform in the reverse position, that is, on the opposite side of the pool to its previous location. For this task, the process of acquisition of spatial knowledge by the mice was tracked by performing a probe test at the end of each of the five days of reversal training. Performance during this training period is shown in the right-most panels of figures 6.1b, c and d. Overall the path lengths [$F(4,100) = 5.60$, $p = 0.0004$] and latencies [$F(4,100) = 4.62$, $p = 0.002$] of the mice improved during this training period. There was no effect of genotype on either measure [$F(1,24) = 0.67$, $p = 0.42$ for path length; $F(1,24) = 0.65$, $p = 0.43$ for latency] but a general tendency towards the mutants having longer path lengths but lower latencies was present (figures 6.1b and

c). Again the mutants swam faster than wild-types throughout [$F(1,24) = 12.10$, $p = 0.002$], as shown in figure 6.1d.

Mice were subject to a probe test at the end of each day of reversal training (R1 – R5). as well as 1 day (M1), one week (M7), two weeks (M14) and 8 weeks (M56) after the last day of training. The time spent in the training quadrant by wild-type and mutant mice for each of these tests is shown in figure 6.1e. A full mixed ANOVA showed that there was no significant difference between the two groups of mice during any of these tests [$Fs(3,75) < 2.165$, $ps > .099$]. To examine this further, t-tests were performed for each probe test, comparing time spent in the training quadrant to chance level (15 s). Wild-type mice performed better than chance from the second day of reversal training (R2) and maintained this differential for the remainder of the tests. In contrast the mutant mice did not perform above chance level until 24 hours after the last training trial (M1). They maintained their above-chance performance in the 1-week (M7) and 2-week (M14) probe tests but after 8 weeks (M56) were again no better than chance. These results indicate that SAP102 loss causes an initial mild deficit in spatial learning which can be overcome by training. Once spatial information has been encoded it can be retained for a period but decays more rapidly than in wild-type mice.

6.2 SAP102 knockout mice display an activity deficit in T-maze and olfactory habituation dishabituation tasks

The spatial learning abilities of SAP102 null mice were further tested using spontaneous alternations in an unrewarded T-maze task. This experiment was performed in collaboration with Lianne Stanford and the results analysed by her. Each mouse was placed in the bottom arm of a T-shaped maze and allowed to make repeated choices between entering the left and right arms upon reaching the T-junction. Exploratory tendencies mean the mice tend to choose the less

familiar arm on each trial, resulting in a series of spatially-related choice alternations which are dependent on the hippocampus (Gerlai, 1998).

Figure 6.2 shows the results of the T-maze test. There was no difference in the number of choice alternations performed by hemizygous and wild-type mice [$t(19) = 0.42$, $p = 0.67$, figure 6.2a], however, SAP102 mice took significantly longer to complete the 11 trials [$t(19) = 2.6$, $p = 0.02$, figure 6.2b].

In a third, non-spatial, learning task, SAP102 mice were given three presentations of the same olfactory stimulus followed by three presentations of different olfactory stimulus. Since mice use urine as a means of identification and territorial marking and are very sensitive to slight variations in its odour, urine samples from two different strains of inbred mice were used as the olfactory stimuli. Learning the identity of each stimulus would result in a gradual reduction in time spent sniffing as each odour became familiar over repeated presentations (Brown et al., 1987; Wrenn et al., 2003). This experiment was performed in collaboration with Lianne Stanford and the results analysed by her.

The results of the olfactory habituation-dishabituation task are shown in figure 6.3. A log transformation of the data was performed prior to statistical analyses to satisfy the requirement of a normal distribution. There was a significant genotype effect on sniffing time across the whole experiment [$F(1, 37) = 8.24$, $p = 0.007$]. Simple effects analyses showed that wild-type mice spent different amounts of time sniffing each stimulus [$F(6,132) = 17.49$, $p < 0.0001$]. Post-hoc analyses showed that the time they spent sniffing the blank was different from that of the first presentation of odour A and the three presentations of that odour were different from one another.

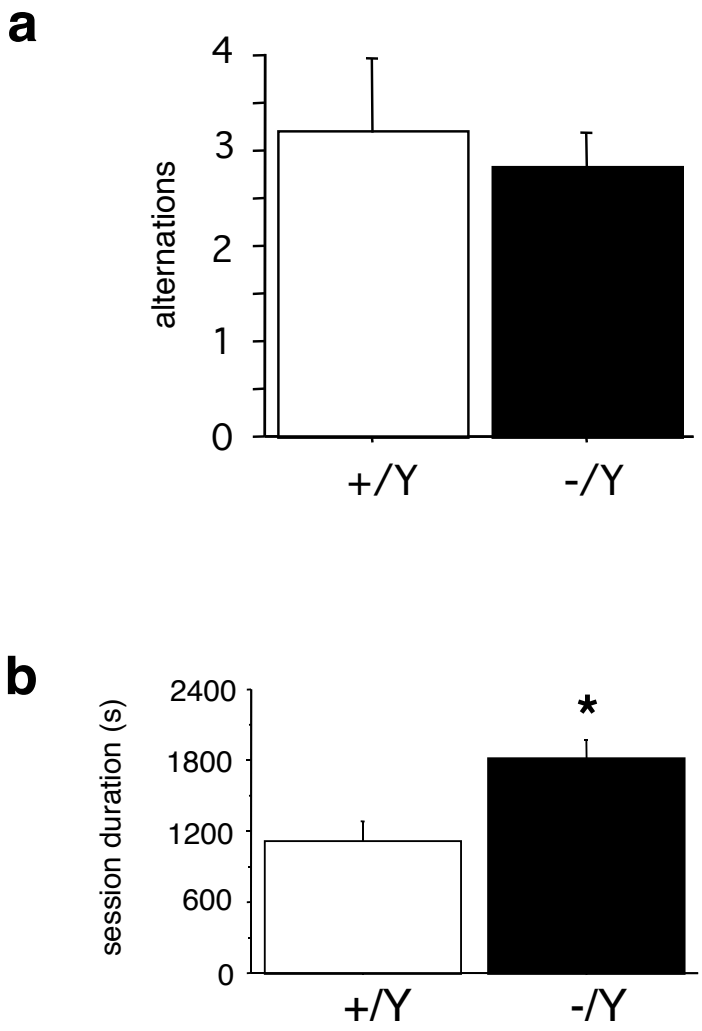

Figure 6.2 SAP102 mutant mice take longer to complete a T-maze task. Mice performed one forced choice trial followed by 10 trials of free choice between the unrewarded left and right arms of the maze. **(a)** No difference in spontaneous alternations in direction choice at the T-junction between wild-type and hemizygous SAP102 mice [$t(18) = 0.65$, $p = 0.67$]. **(b)** Hemizygous mice take longer to complete the 11 trials [$t(18) = 2.6$, $p = 0.02$].

The third presentation of odour A was different to the first presentation of odour B and the three presentations of the latter were different to one another. These analyses confirm that the wild-type mice distinguished between the two odours and habituated to each one, as shown in figure 6.3.

In contrast, the simple effects analysis showed that there was no difference in time spent sniffing each odour stimulus by the mutant mice [$F(6,90) = 1.05$, $p = 0.39$] and this was confirmed by post-hoc analyses. Since they spent no more time sniffing any of the odour stimuli than the vehicle their learning ability in this task could not be assessed.

In both the T-maze and habituation-dishabituation tasks SAP102 mice displayed a reduction in activity levels. The remainder of this chapter describes attempts to elucidate the cause of this phenotype.

6.3 Motor ability in SAP102 mutant mice

The performance of SAP102 mutant mice in the visual platform task in the water maze suggested their swimming ability was unimpaired (see figure 6.1a). However, since SAP102 is expressed in the cerebellum, an important centre for motor control, and motor impairment is an obvious potential cause of an activity deficit, the mice were subjected to a grip strength test for muscular strength and a rotorod task for motor coordination. Mice with targeted mutations in genes involved in motor control show poor performance on these tasks (Aiba et al., 1994; Chen et al., 1995).

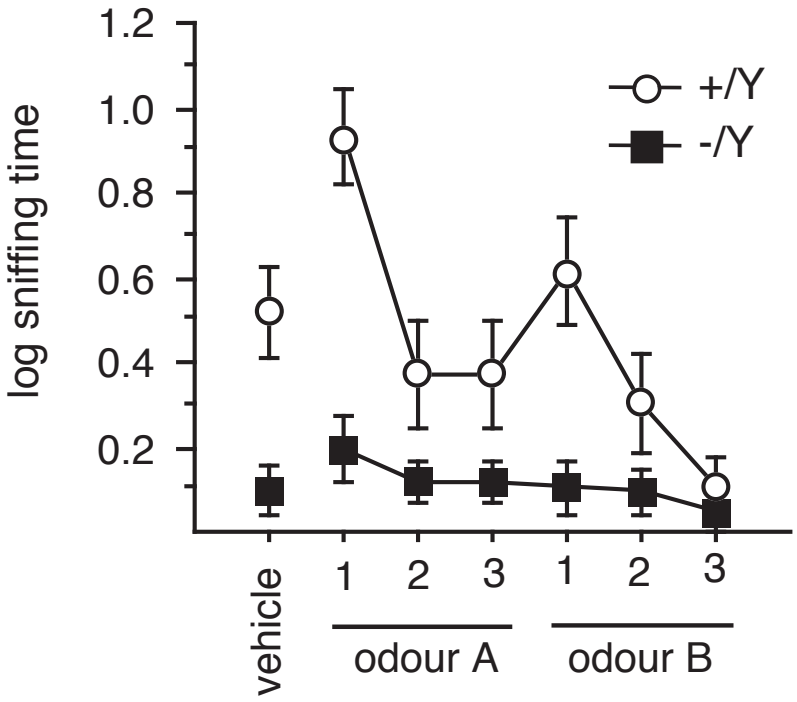

Figure 6.3 SAP102 mutant mice display impaired performance in an olfactory habituation-dishabituation task. Male hemizygous mice and wild-type littermate controls were subject to repeated exposures to two different odours. Wild-type mice initially spent more time sniffing the first odour than they did the vehicle but rapidly habituated and spent less time sniffing on its second and third presentations. Sniffing time increased upon presentation of the second odour but again rapidly decreased upon the second and third presentations. In contrast, SAP102 -/Y mice spent no more time sniffing any of the odour stimuli than they did the vehicle. See section 6.2 for statistical analyses.

The grip strength test was performed and analysed in collaboration with Lianne Stanford. Mice were held by the base of the tail and placed with their front paws gripping a horizontal, elevated bar. They were then drawn horizontally and smoothly backwards until they released their grip, while the maximum force exerted on the bar was measured by means of an attached force transducer. Mice with inherited neuromuscular deficits show impairment on this task (Levedakou et al., 2004). However, figure 6.4a shows there was no difference in the force exerted by SAP102 mutants and wild-type mice in this test, suggesting loss of SAP102 does not result in a deficit in muscular strength.

The motor coordination test was performed in collaboration with Lianne Stanford and the results analysed by her. In this test mice were given a series of trials on a non-accelerating rotorod. Each mouse was subjected to three trials at each of 4, 16 and 32 rpm, in that order, every day for three days. The results are shown in figure 6.4b. Both wild-type and mutant mice rapidly mastered the 4 rpm trials and by the third day could stay on the rotating rod for nearly the maximum 60 s. There was a main effect of day at this speed [$F(2,38) = 13.01$, $p < 0.0001$], suggesting performance improved with training, but no effect of genotype [$F(1,39) = 0.42$, $p = 0.52$] and no interaction between day and genotype [$F(2,78) = 1.08$, $p = 0.34$], indicating the mutation had no effect on performance at this speed. At 16 rpm both groups of mice performed considerably less well but improved with training, producing an effect of day [$F(2,35) = 22.19$, $p < 0.0001$]. Again there was no effect of genotype [$F(1,36) = 3.93$, $p = 0.06$] or day x genotype interaction [$F(2,72) = 2.37$, $p = 0.10$]. At 32 rpm both genotypes performed poorly, consistently falling off the rod after approximately 10 s, but there was still a significant day effect [$F(2,38) = 6.94$, $p = 0.002$]. There was no main genotype effect [$F(1,39) = 0.28$, $p = 0.60$] but an interaction between genotype and day was present [$F(2,78) = 3.26$, $p = 0.04$]. Analysis of the two genotypes

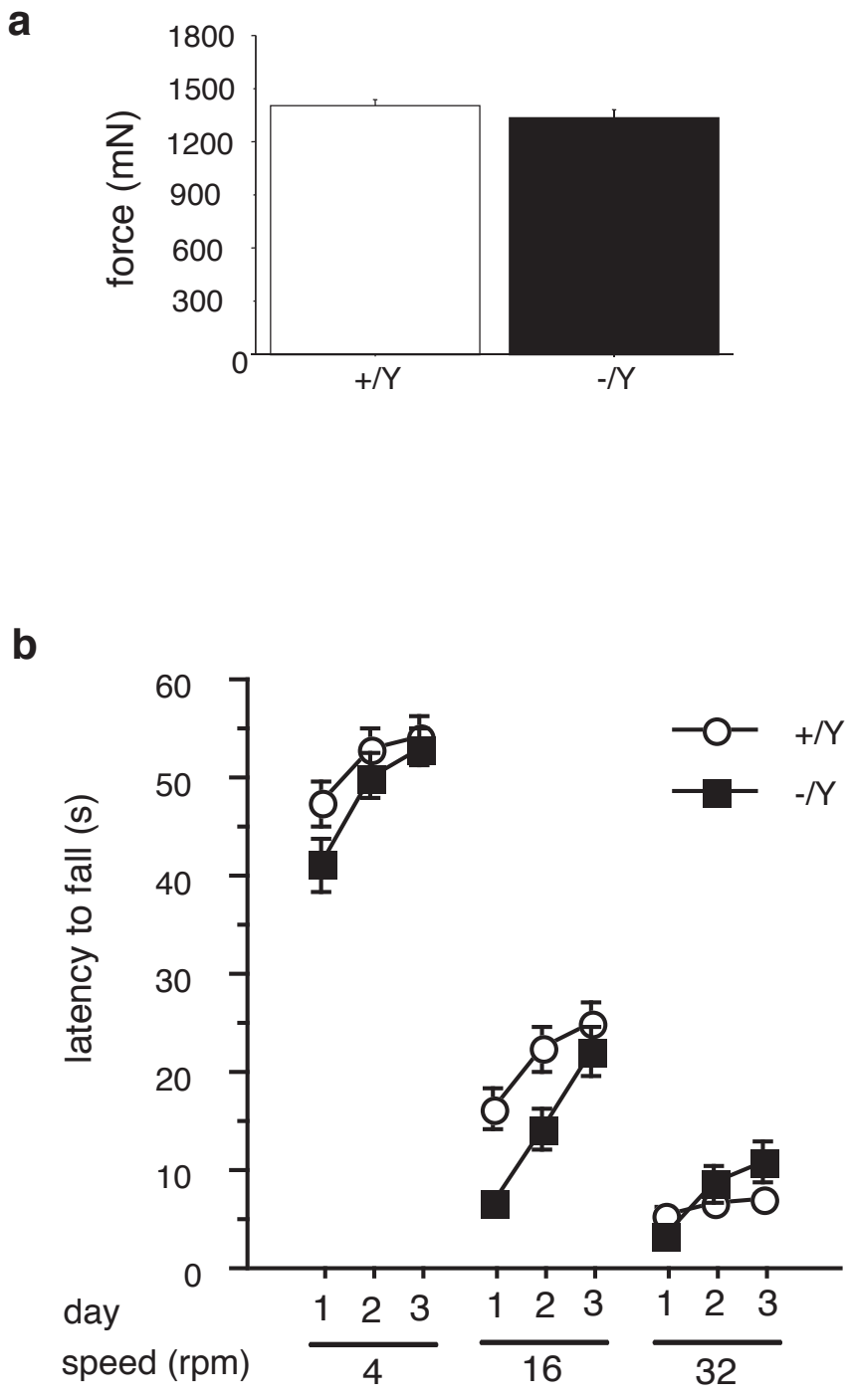

Figure 6.4 Motor abilities in SAP102 mutant mice. (a) A grip strength test shows loss of SAP102 has no effect on muscle strength ($t = 1.55$, $p = 0.12$, $n = 20$). **(b)** Rotorod test for motor coordination. Mice were given one trial at each of 4, 16 and 32 rpm each day for three days. Latency to fall from the rotating cylinder was recorded. See section 6.3 for statistical analyses.

separately showed a significant day effect in hemizygous mice [$F(2,38) = 5.9$, $p = 0.006$] but not in wild-type controls [$F(2,40) = 0.95$, $p = 0.40$]. Figure 6.4b shows that this effect is the result of mutant mice improving over time while wild-type performance remains static.

6.4 No change in anxiety behaviour following loss of SAP102

Another potential cause of reduced activity levels in SAP102 null mice is elevated anxiety levels. To examine this possibility the behaviour of the mice during a 15 min exposure to an open field was analysed, the results of which are shown in figure 6.5. This experiment was performed in collaboration with Lianne Stanford and the results analysed by her, Hayley Cooke and Magaret Green. For the analysis, the field was divided into three rows of three equal-sized rectangles. The centre rectangle was denoted the inner zone and the remainder the outer zone (figure 6.5a). Mutant mice were no different to wild-types in their latency to enter the inner zone (figure 6.5b), total time spent in the inner zone (figure 6.5c), number of stretch-attend postures (figure 6.5d) or time spent grooming (figure 6.5e), all important anxiety related behaviours (Gerlai et al., 2002; Gordon and Hen, 2004; Parks et al., 1998). They did, however, display differences consistent with a more general deficit in activity, including more time spent immobile (figure 6.5f), fewer crossings of the lines defining the nine rectangles (figure 6.5g) and fewer incidents of rearing, both supported (against a wall, figure 6.5h) and unsupported (figure 6.5i).

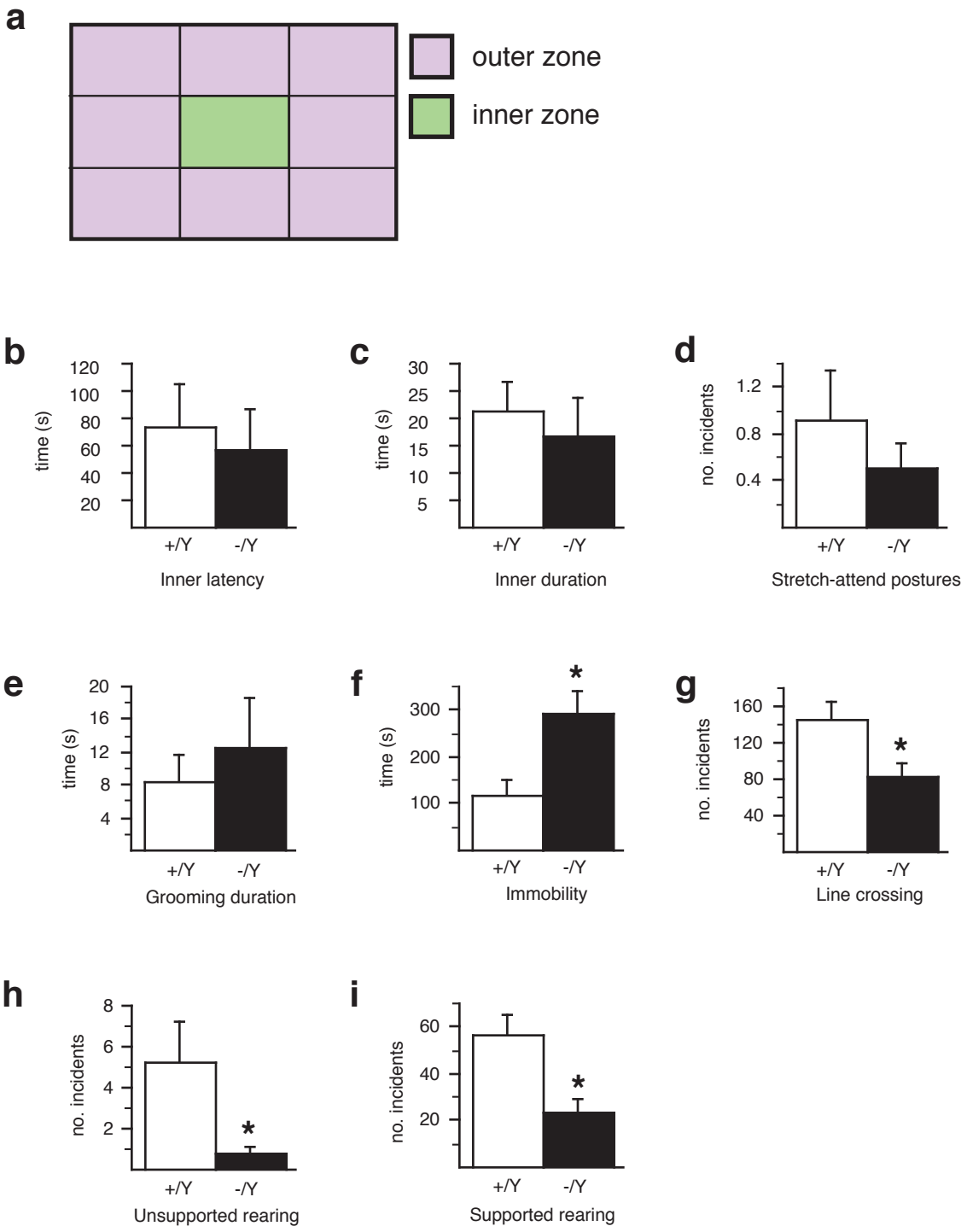

Figure 6.5 SAP102 mutant mice display decreased locomotion but no elevated anxiety in an open field. **(a)** Diagram of the open field layout. Limits of the inner and outer areas of the field are shown. **(b) - (e)** No difference in anxiety-related behaviours in SAP102 hemizygous males compared to wild-type controls, including **(b)** Latency to enter the inner zone [$t(38) = 0.35$, $p = 0.73$], **(c)** total time spent in inner zone [$t(38) = 0.5$, $p = 0.62$], **(d)** number of stretch-attend postures [$t(38) = 0.81$, $p = 0.43$] or **(e)** grooming behaviour [$t(38) = 0.63$, $p = 0.53$]. **(f) - (i)** SAP102 mutants show significantly decreased locomotion in several behavioural measures, including **(f)** increased time spent immobile [$t(38) = 3.05$, $p = 0.004$], **(g)** reduced crossings of internal dividing lines [$t(38) = 2.52$, $p = 0.02$], **(h)** less frequent unsupported rearing [$t(38) = 2.1$, $p = 0.04$] and **(i)** less frequent supported rearing [$t(38) = 3.12$, $p = 0.003$]. Asterixes above -/Y columns indicate $p < 0.05$ compared to wild-type.

The open field data were suggestive of an activity deficit without a change in anxiety levels, but many of the behavioural measures used in its analyses are equally indicative of both these phenotypes (Weiss et al., 2000), making the results somewhat ambiguous. To obtain a clearer distinction between anxiety and activity the mice were further examined in an elevated plus maze test, in which the animals spent 5 min exploring a plus-shaped maze supported 45 cm above the floor, with two arms shielded by high, vertical, opaque sides and the other two arms open (figure 6.6a). This experiment was performed in collaboration with Lianne Stanford and the results analysed by her.

There was no difference between wild-type and SAP102 hemizygous mice in their preference for the closed arms of the maze (figure 6.6b), the crucial measure of anxiety behaviour in this test (Rodgers and Johnson, 1995). In addition, both genotypes performed equal numbers of stretch-attend postures (figure 6.6c) and head-dips over the sides of the open arms (figure 6.6d). The only measure on which mutant and wild-type mice differed in the task was the total distance travelled, the mutants covering less distance than their wild-type counterparts (figure 6.6e), confirming that SAP102 loss causes an activity deficit which is not the result of a change in anxiety levels.

It appears that the observed differences in locomotor activity in the SAP102 mutants is not a consequence of changes in musculoskeletal ability or anxiety levels and may instead be a result of motivational changes caused by the mutation.

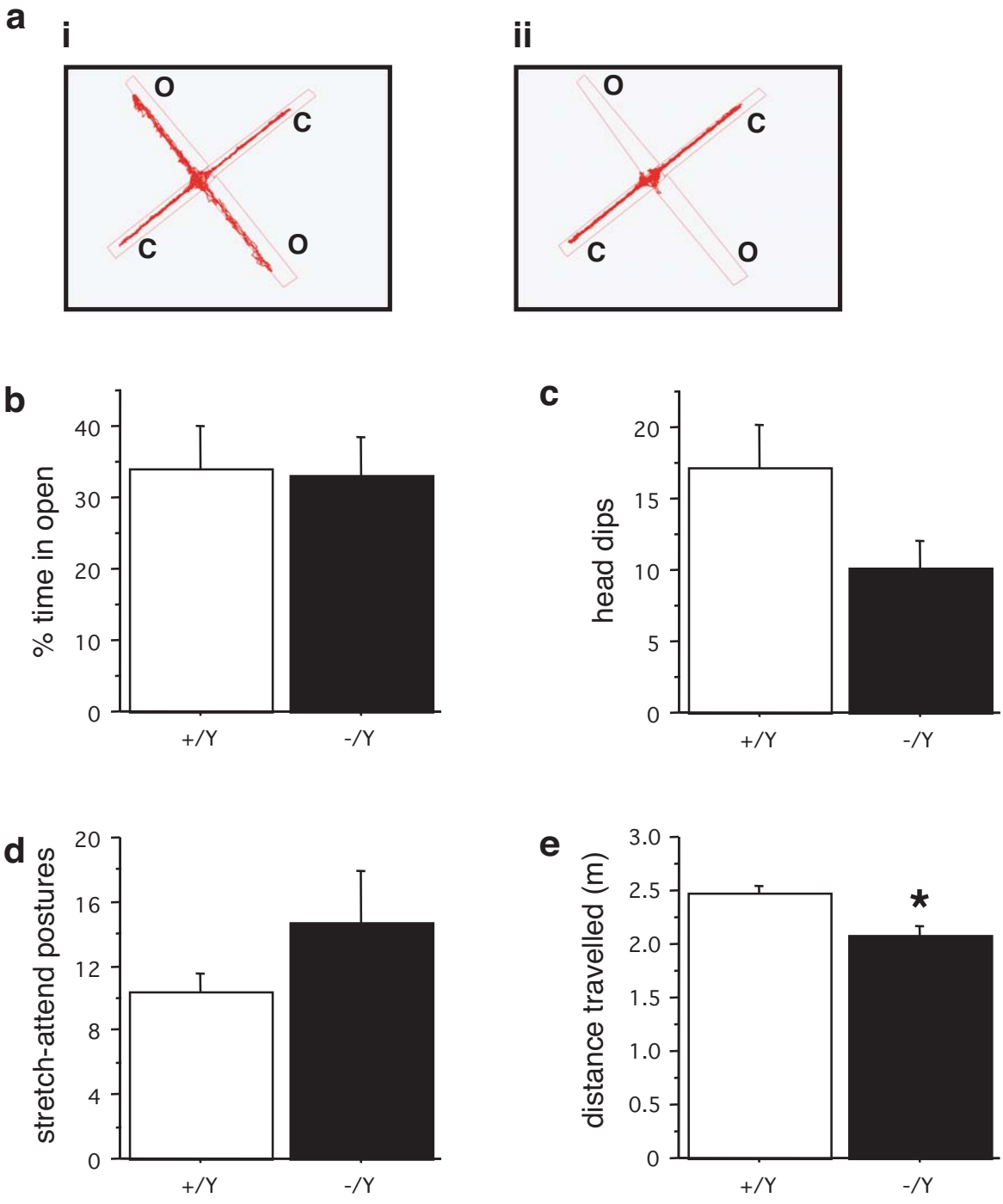

Figure 6.6 SAP102 mutant mice display no alterations in anxiety levels in an elevated plus maze. (a) The maze is an elevated, cross-shaped configuration in which two arms are covered by high, opaque side walls (c) and two are open (o). The red traces show the movement of two wild-type mice during 5 min in the maze, one spending approximately equal time each arm **(i)** and one staying exclusively in the closed arms **(ii)**. **(b)** The proportion of time spent in the open arms, a measure of anxiety, is no different between wild-type and hemizygous mice [$t(21) = 0.14$, $p = 0.90$]. **(c)** No difference between wild-type and hemizygous mice in the number of head dips over the sides of the open arms [$t(21) = 1.67$, $p = 0.11$], **(d)** nor in the number of stretch-attend postures [$t(21) = 1.40$, $p = 0.18$]. **(e)** Hemizygous mice travelled less total distance in the maze than wild-type controls [$t(21) = 2.71$, $p = 0.01$].

6.5 Discussion

The results of the water maze task show that loss of SAP102 causes a deficit in spatial learning that can be overcome with training. This contrasts with PSD-95 mutant mice, whose performance is severely impaired and cannot be improved by overtraining (Migaud et al., 1998). This is first evidence of an *in vivo* distinction between the functional roles of NMDAR-associated MAGUK proteins. The success of additional training in improving the learning performance of SAP102 mutant mice is also an encouraging result for treatment strategies in the human disorder, suggesting that patients may be able to minimise their cognitive impairment with additional practice or tuition. Knowledge of this property of SAP102 could be usefully broadened by examining whether it applies to memory in other tasks, for example retention of motor learning on the rotorod.

In contrast to the approximately 30 % alternation level observed here in mice of both genotypes, previously published experiments on inbred mouse strains have found wild-type 129S6 mice perform at chance level (50 % alternations), while various substrains of C57 mice perform at or above chance (Spowart-Manning and van der Staay, 2004). A study of targeted mice carrying an I213T substitution in presenilin 1 in a hybrid B6 x 129X1/SvJ background found wild-type controls displayed 68 % while mutant mice displayed 58 % alternations, both above chance level (Spowart-Manning and van der Staay, 2004). The former study reported a mean duration of approximately 60 s per trial across most inbred strains tested, while our wild-type mice on average took 110 s and the SAP102 mutants 164 s. It is possible that the low alternation levels seen in the 129P2 x MF1 background are creating a ‘floor’ effect, masking a deficiency in this task caused by loss of SAP102.

It is interesting to note that SAP102 knockout mice had a faster average swim speed than wild-types on every day of the hidden platform and reversal training, while their distance travelled was

often greater and their latency to reach the platform similar to wild-type. This suggests the mutant mice may be using a different search strategy which results in a less direct path to the platform but not in latency because the mutants are swimming more quickly.

SAP102 mutant mice display a deficit in activity which manifested itself in longer latency to complete the T-maze task, severe lack of response to olfactory stimuli and reduced movement in both an open field and an elevated plus maze. Grip strength and rotorod tests show this deficit is not the result of a lack of muscle strength or motor coordination. SAP102 mice also display what could be interpreted as enhanced motor performance when swim speed is measured in the water maze task. While the mutants' impairment in the habituation-dishabituation task in isolation could be attributed to loss of olfactory ability resulting from lack of SAP102 in the olfactory bulb, this would not explain the results of the other three tests which do not depend on olfaction. Results from the open field and elevated plus maze show that neither is the activity deficit the result of elevated anxiety levels in SAP102 mutants.

A further, as yet untested, possibility is that loss of SAP102 modifies activity by altering motivation levels. In relation to this it is interesting to note that the lowered activity was observed in passive, exploratory-type tasks, but not in tasks requiring active movement to avoid or escape from a negative stimulus – water in the water maze, hanging by the tail in the grip strength test, falling off the rotorod. In the visual platform training in the water maze and 32 rpm trials of the rotorod, mutants even displayed enhanced performance compared to wild-type controls. It may be that SAP102 mutants have a greater-than-normal range of motivation levels: lower than wild-type in passive situations, manifested as a reluctance to move, but increasing to levels higher than wild-type mice in situations requiring action to avoid or escape from unpleasant circumstances.

Whatever its cause, the activity phenotype clearly poses problems when assessing the mice on certain behavioural tasks, as seen in the results of the olfactory habituation test where no conclusions on the learning ability of the mice could be drawn. Further behavioural tests need to be carefully chosen and/or modified to allow analysis of the ability of interest independently of any altered activity level. For example, contextual and cued fear conditioning could be used to examine hippocampal-dependent and hippocampal-independent associative learning respectively, with freeze time prior to conditioning subtracted from freeze time after conditioning to adjust for any tendency of the mutant mice to remain immobile.