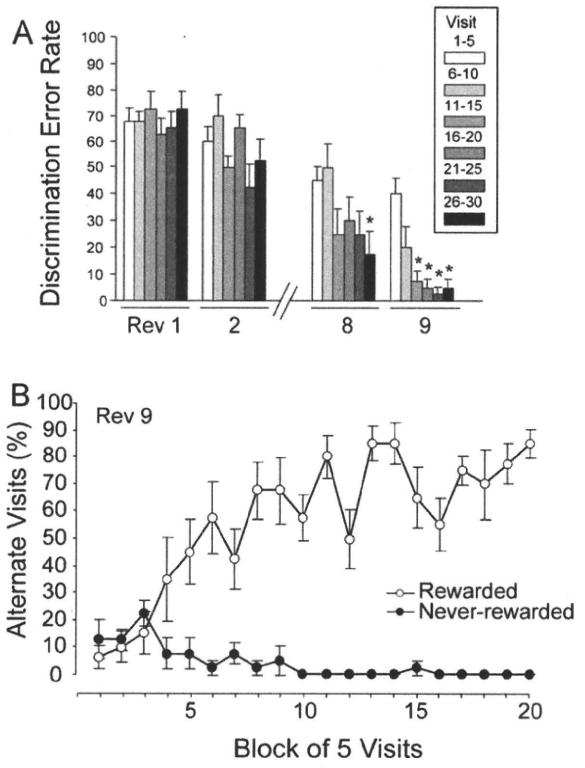

Fig. 7. Rapid adaptation to the reversed contingency in B6-UT mice. (A) Discrimination error rates (B6-UT) of the first 30 visits in the first sessions of Revs 1, 2, 8, and 9. Each bar represents the mean ± S.E.M ($n = 8$) of discrimination error rates compiled for every five-visit block. A significant linear trend of error reduction was observed in the first 30 visits in Revs 8 and 9 ($p < 0.05$, repeated measures ANOVA). The asterisks above the bars indicate a statistically significant difference in discrimination error rate (ANOVA and Dunnett's test, $^*p < 0.05$) compared with that in the first 5 visits in Rev 8 or 9. (B) Intra-session rapid progression of shuttling behavior between rewarded corners (B6-UT, mean ± S.E.M, $n = 8$) in the first session of Rev 9.

improvement over a series of reversal tasks, and has been observed in several animal species [67–70]. In this context, we speculate that the rapid adaptation to reversal learning shown after a series of reversals observed in this study reflects a long term learning effect which was attributed to an adaptation to "reversal rule" itself, or a reversal "learning-set".

4.2. Comparison among laboratories, ages and strains

Comparisons of the results from three independent laboratories showed that essentially the same results in the behavioral sequencing task and its serial reversals can be replicated in all the three strains (C57BL/6, DBA/2 and ICR) and in all of our laboratories (UT, JMU and UZH) (see Supplemental Fig.S2 and Figs. 4 and 6). However, despite the overall concordance in trends of the data, there were some differences among the laboratories particularly in the minimal discrimination error rates. Although the minimal error scores were relatively constant at each laboratory, best at UT (approx. 5–10%), somewhat higher at JMU, and highest at UZH (approx. 20–30%), the possible reasons for such differences as observed in the three laboratories are considered to be threefold. First, the highest minimal error scores in UZH might be attributable to the imposed shortening of the deprivation time, and with this, the motivation of the mice. The error visits of mice in UZH can be thought to reflect the exploratory patrolling of corners, which occurs spontaneously also under non-rewarded conditions. Thus, a sufficient deprivation time appears to be a critical factor in the protocol used. Second, the relatively small differences between UT and JMU may be attributable to the presence or absence of external LED light cues that indicated the task period (22:00–1:00), the light of which was not originally present in the IntelliCage system. This extra cue was installed at UT, but not at JMU or UZH, and it is considered to give mice a chance to reduce the minimal error scores at UT, but not at JMU or UZH. Third, a difference in the schedule of acclimatization, particularly the period after the time restriction was started (5, 4 and 3 days in UT, JMU and UZH, respectively) could be another factor that might produce the observed differences among the three laboratories. Although there were some uncontrolled conditions among the laboratories as mentioned above, it should be pointed out here that this study was not initially intended to carry out inter-laboratory comparisons. If such comparisons were planned, much more stringent experimental controls, such as raising all the mice in the same place, synchronized shipping and other requirements described in detail elsewhere [24,71], should have been made.

We compared the age and strain differences in learning performance using the UZH data. We could not detect any significant age-related difference in learning performances between the 3- and 12-month cohorts of C57BL/6 and between those of DBA/2. While some studies showed that mice in "middle-age" (8–18 months) had age-related cognitive or learning declines in the tasks of spatial learning, working memory, and contextual fear conditioning [72–77], other studies showed that middle-aged mice did not have a learning deficit, or even had a better learning performance than those of young mice [23,75,78]. Since most of the studies that showed the age-related cognitive and learning declines used mice over 20 months of age, further research using middle-aged mice to clarify its aging effect in details is necessary. Additionally, the possible effect of environmental enrichment by housing and testing mice in IntelliCage might contribute to preventing their age-related decline of learning performance. In the previous studies using mice, environmental enrichment (i.e., socially housed in a large cage with toys and/or running wheels) was found to have the capacity to cancel age-related learning impairments [73,74]. However, modifications of the task protocol may increase the sensitivity to detect more subtle signs of cognitive inflexibility in the middle-aged mice. This point should be given careful attentions in future studies. Second, strain difference analysis was also carried out on the data obtained at UZH (young B6-UZH vs. young D2-UZH, and aged B6-UZH vs. aged D2-UZH). As a result, significant main effects of strain for discrimination error rate were detected in Rev 1, 2, 3, and 4 between the young cohorts, and in Rev 1,2, and 3 between the aged cohorts, consistently showing higher discrimination error rates in C57BL/6 than in DBA/2 mice ($p < 0.05$, repeated measures ANOVA). These results were in line with a previous study by Krackow *et al.* [24] where a simple place learning task paradigm in IntelliCage was used, and better performance in reversal learning was shown in DBA/2 than in C57BL/6 mice. Although the strain difference in learning ability seems to be consistent, it may be difficult to simply speculate that the DBA/2 mice learned the task better than the C57BL/6 mice in this study. This is because a persistent significant difference in the average number of visits per session was also observed between those strains throughout the experiment (data not shown). The tested C57BL/6 mice showed consistently about 40% higher number of visits on average than the DBA/2 mice, and thus, the higher error rate shown in C57BL/6 than in DBA/2 might be attributed to differences in basic behavioral characteristics such as hyper locomotion or impulsivity. However, it is notable that all the cohorts of mice tested in this study showed similar and stable learning and relearning curves of discriminative performance in this behavioral flexibility test.

4.3. Applications of IntelliCage system

The use of IntelliCage has demonstrated distinct advantages over conventional manual or semi-automated testing methods for

individual mice. This fully automated system can minimize the negative effects of human handling, and thus mice can focus on events or tasks within their cage. IntelliCage also provides social context and environmentally enriched housing for mice.

On the other hand, some limitations for analyzing mice behavior are present in this task due to the more naturalistic approach that makes it difficult to exclude the possible effects of variability from social and individual behavioral contexts on the experimental results. In this regard, the variability in the social context and environmentally-enriched housing might induce some behavioral variability [71]. However, a recent report by Krackow *et al.* [24] showed that such behavioral variability did not seem to diminish the reproducibility of the main results in the specifically designed inter-laboratory comparisons using IntelliCage. The inter-laboratory comparison across three laboratories in this study also strongly indicates an excellent reproducibility of the main test results. By contrast, it is difficult to clearly evaluate whether and to what extent the behavioral context in the task, on individual animal basis, might affect the overall main result of the present study. For example, it is up to each mouse to decide when to start, stop, and resume their responses during the period of the task, which is difficult to be controlled strictly. However, we would speculate that the prolonged monitoring of individual animals over several weeks minimized the possibility of misreading the daily variability of task performance on individual animal basis, thus, allowed us to detect subtle differences in genetic or epigenetic individual characteristics [19–27].

The protocol we developed for the present study added significant assets to the IntelliCage system, in the following two aspects. First, the readout of learning effect in the behavioral sequencing task can be readily achieved because the observed learning curves of discrimination error rate were clear and consistent among cohorts. Second, the serial reversal learning paradigm for assessing behavioral flexibility in IntelliCage was established.

5. Conclusion

In conclusion, we established a test protocol for mice to assess behavioral flexibility as an advanced cognitive function in a group-housed environment and validated its reproducibility by inter-laboratory comparisons. It provides unique and detailed analyses of place discrimination in the behavioral sequencing task and behavioral flexibility in the subsequent serial reversals. Thus, the current protocol for IntelliCage not only lessens the shortcomings of the existing methods used to assess cognitive behavior of mice, namely, efficiency, reproducibility and standardization, but also offers a uniquely successful paradigm for unveiling the otherwise overlooked higher-order cognitive functions in this animal species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbr.2011.02.037.

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Legends to Supplemental Figures

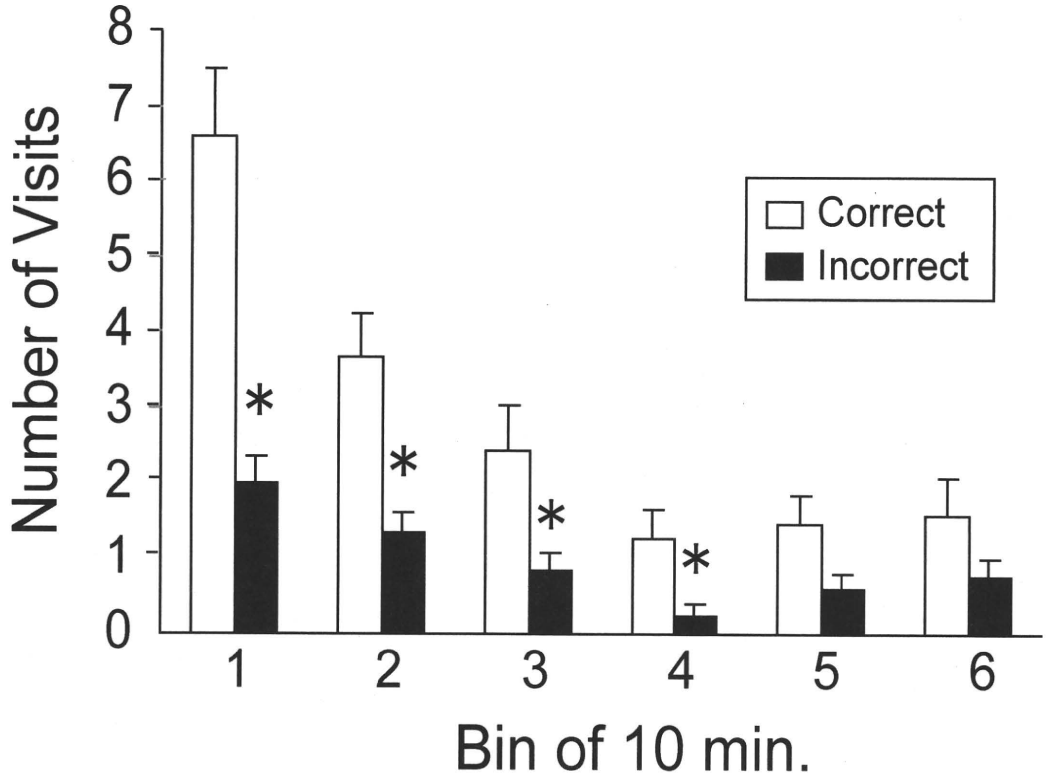
Supplemental Figure S1. Probe trial for the validation of behavioral sequencing task as a place learning paradigm. For this additional test, another cohort of C57BL/6 mice (n=14) was used at UT. The mice were acclimatized to the IntelliCage apparatus and trained for the behavioral sequencing task with the same schedule used for other cohorts studied at UT (B6-UT and D2-UT) until session 7. On the following day, the cage and all the corner chambers were fully replaced with a different set of IntelliCage apparatus. Then, the mice were subjected to a probe trial. In the probe trial, the LED light cue was turned on at 22:00 to signal the start of the water-availability period in the past sessions, but they could not receive water even when visiting the previously learned rewarded corners. The probe trial was carried out based on the following hypothesis: if the mice are utilizing non-spatial cues such as odors in the cage to discriminate the corners, then changing a set of IntelliCage apparatuses will diminish their preference to visiting the correct corners. In addition, in the probe trial the mice were not given a reward even when they visited the correct corner. The reason was to observe whether the mice would show their preference to visit the correct corner by utilizing the memory that they acquired before the probe trial, but not the memory newly acquired from the probe trial.

As a result, the mice showed a significantly strong preference to the correct corner in the probe trial without a reward. This preference indicates that the mice certainly had "learned" the corner discrimination based more on spatial strategy instead of the local cues before the probe trial. Each bar represents the mean ± S.E.M of number of visits to correct (learned as rewarded) and incorrect (learned as never-rewarded) corners in every ten minutes after the beginning of the probe trial. Asterisks above bars indicate significantly smaller number of visits to incorrect corners compared to that of correct corners in each bin of 10 minutes (t-test, $*p<0.05$).

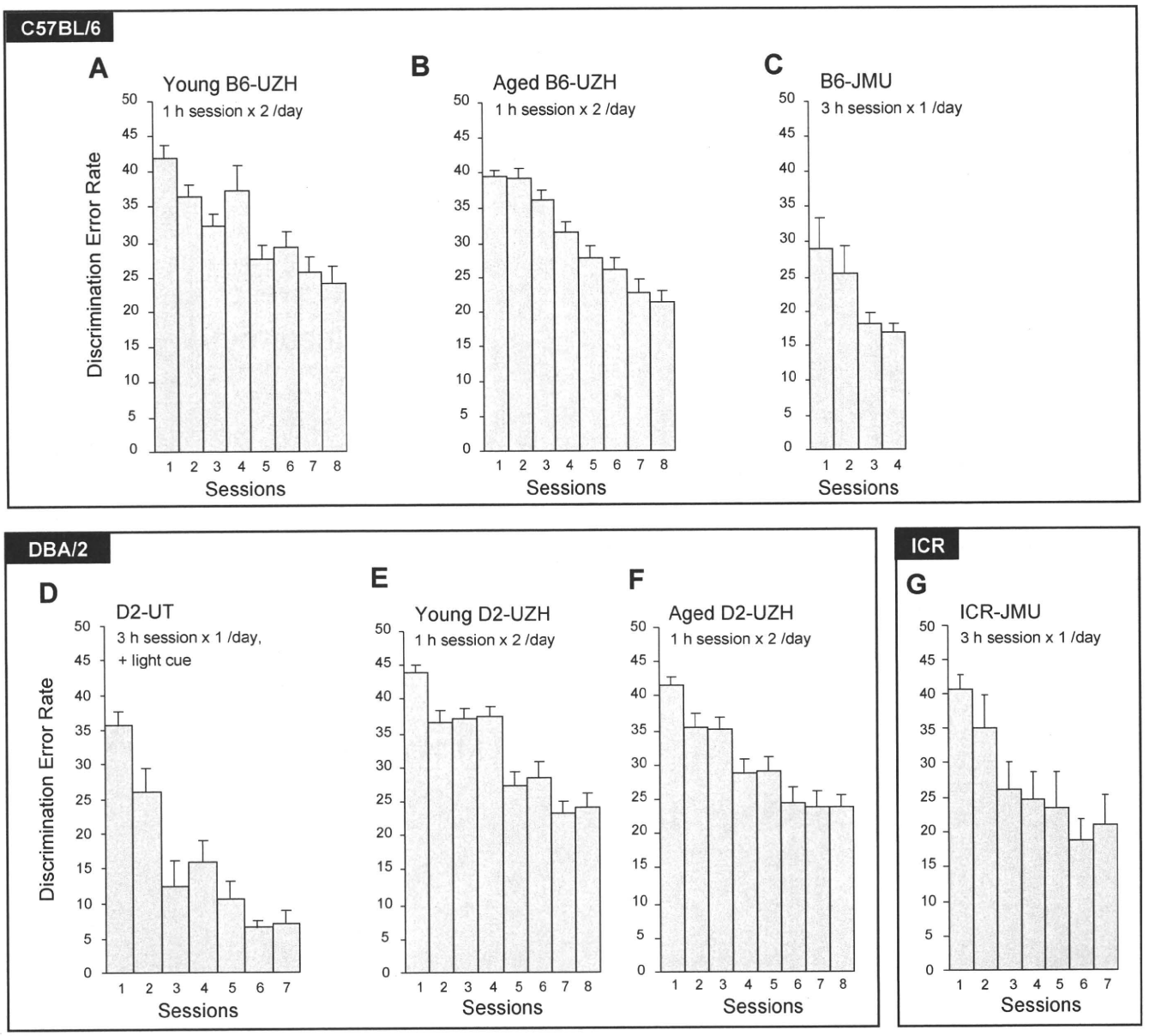
Supplemental Figure S2. Reduction of visits to never-rewarded corners (errors) in the acquisition phase in (A) young B6-UZH, (B) aged B6-UZH, (C) B6-JMU, (D) D2-UT, (E) young D2-UZH, (F) aged D2-UZH and (G) ICR-JMU. Discrimination error rate (mean ± S.E.M, n=11, 12, 15, 8, 12, 11, and 11, respectively from panel (A) to (G)) was defined as the number of visits to the two never-rewarded corners within the first 100 visits in each session, which makes the chance value of discrimination error rates 50%. Statistical analysis showed significant linear trends of error reduction ($p<0.05$, one-way repeated measures ANOVA with session as a factor) in all the cohorts.

Supplemental Figure S3. Shuttling behavior measures in sessions 1, 7, 8 (the first session of Rev 1), and 15 (the first session of Rev 2) of B6-UT mice. Means ± S.E.M (n=8) of ratio of alternate visits were plotted. Ratio of alternative choices = (number of alternate visits between the rewarded or never-rewarded corners)/block of five visits x 100.

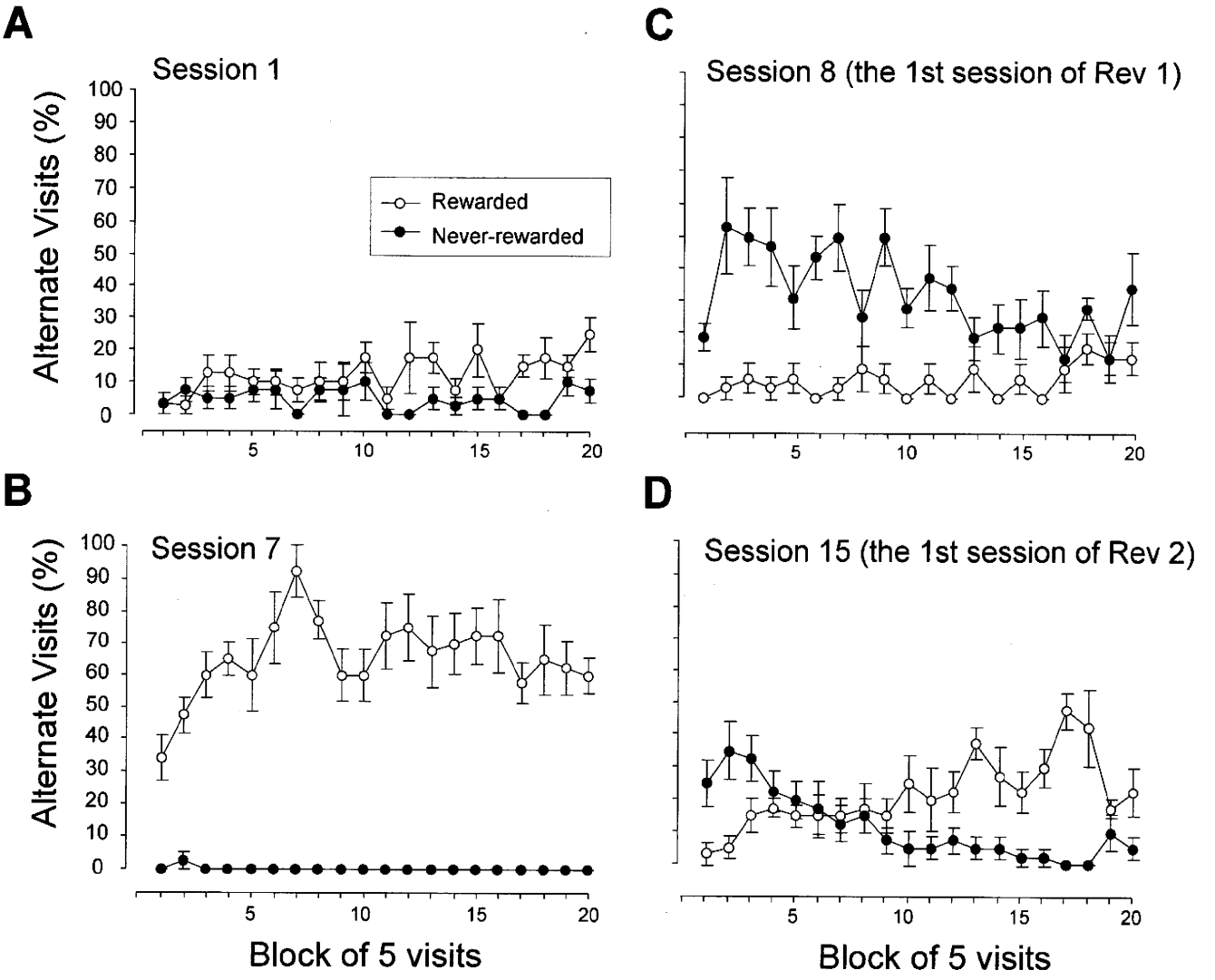
Supplemental Figure S4. Profiling of corner visit of an individual mouse of B6-UT in sessions 1 and 7 (the first and last session in the acquisition phase, respectively), and the first sessions of Revs 1, 2, and 9. Blue (navy and sky) and red (red and pink) boxes indicate a visit to rewarded and never-rewarded corners, respectively. The patterns of moving from the previously visited corner to the indicated corner are further coded in the box. Navy blue and red boxes with a diagonal line indicate that the mouse visited the rewarded/never-rewarded corner from the diagonally opposite rewarded/never-rewarded corner. Navy blue and red boxes without a mark indicate that the mouse visited the corner from the adjacent corners. Sky blue and pink boxes without a mark indicate that the mouse continuously revisited the same corner.



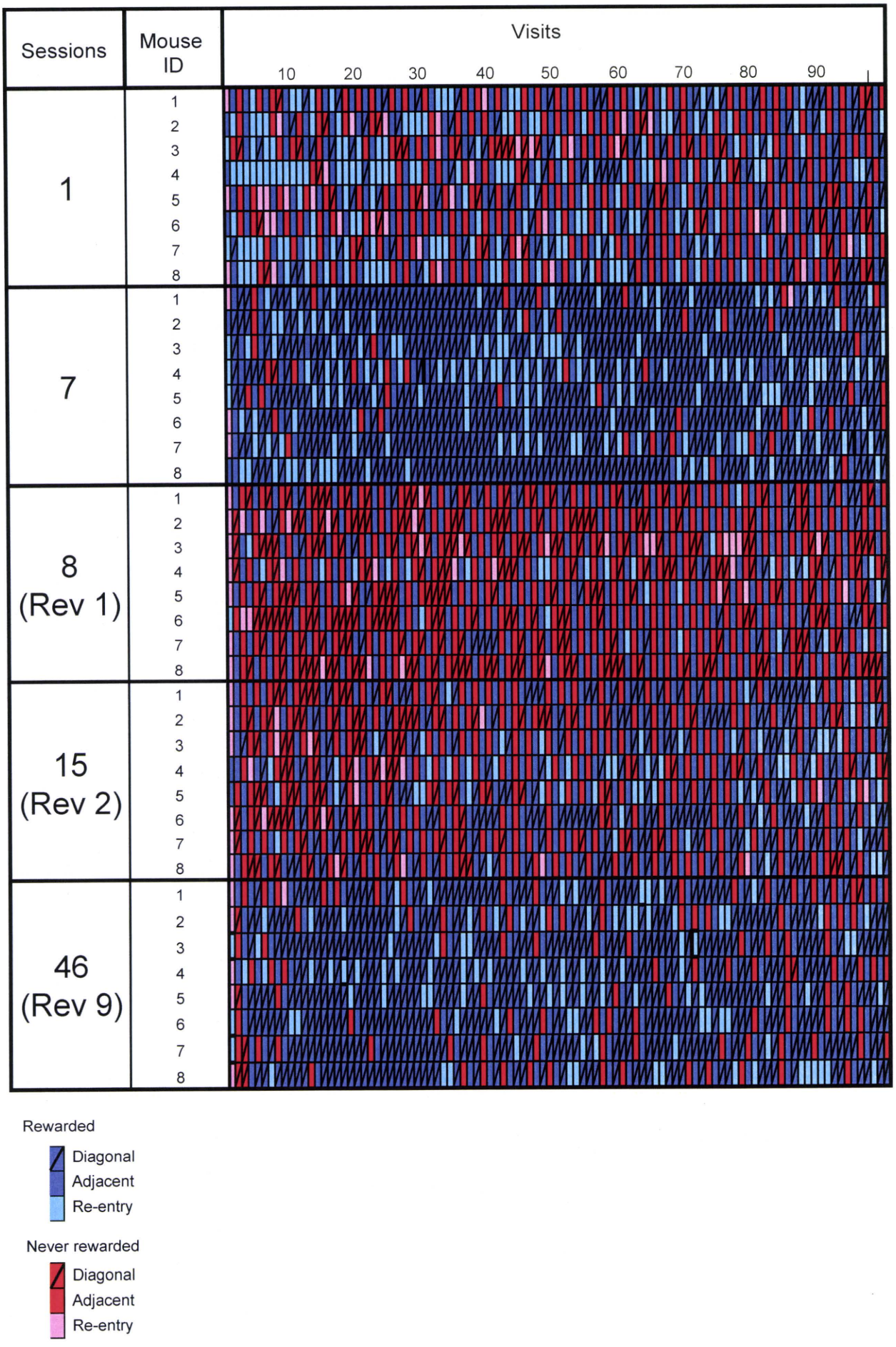
Supplemental Figure S1



Supplemental Figure S2



Supplemental Figure S3



Supplemental Figure S4