

Characterization of Shank1 Knockout Mice

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Abstract

Shank, a scaffold protein in the post-synaptic density (PSD), links many other scaffold proteins, but has only been studied *in vitro*. Scaffold proteins in the PSD connect several types of ion channels, and are therefore believed to play an important role in synaptic plasticity, the molecular correlate of learning and memory. We investigated Shank1 behaviorally by producing mice deficient in Shank1 (Shank1^{-/-} mice) and comparing them to their wildtype littermates. We used a variety of behavioral tests, including the open field, rotarod, wire hang, and the eight-arm radial maze. Relative to their wildtype littermates, Shank1^{-/-} mice performed poorly in tests of motor coordination. In contrast, knockout mice were superior performers in the eight-arm radial maze, committing fewer errors in reference and working memory than their wildtype littermates. After a prolonged break, Shank1^{-/-} mice briefly performed as poorly as wildtypes in the radial maze, but rapidly returned to their previous minimal level of error. Shank1 deletion presumably makes PSD protein structures of knockout neurons more flexible, enabling knockout mice to learn the eight-arm radial maze quickly, but also to forget it quickly. This is the first instance in which deleting a learning and memory-related gene leads to an improvement in learning.

Introduction

The post-synaptic density (PSD) is a specialized region of the neuron rich in uncharacterized proteins, many of which are likely to be involved in the mechanisms of learning and memory. A postsynaptic neuron receives a chemical input from neighboring neurons across a small specialized junction known as the synapse; the PSD is the protein-rich region of the postsynaptic neuron immediately adjacent to the synapse (Naisbitt et al., 1999). Several proteins in the PSD have been shown to interact with each other, and a comprehensive schematic of PSD protein interactions is slowly emerging (Sheng and Kim, 2000; Kim and Sheng, 2004). In general, glutamate receptors, one of the major chemical receptors in the postsynaptic membrane, are linked to each other and to their binding partners by means of scaffold proteins such as GRIP, PSD95, and Shank (Kim and Sheng, 2004), although the functional consequences of these linkages are not well-understood (Sheng and Kim, 2000). There is, however, new information suggesting that these scaffold proteins, particularly Shank, are regulated by neural activity (Kim and Sheng, 2004); this implies that Shank may be involved in synaptic plasticity and, by extension, learning and memory.

Shank, named for its SH3 domain and ankyrin repeats, also contains a PDZ domain, a SAM domain, and a proline-rich region (Lim et al., 1999); all of these protein motifs are known to interact with specific components of other proteins in the PSD. The presence of these domains indicates that Shank is able to bind to and interact with many different classes of proteins. Three Shank genes, and therefore at least three Shank proteins, have been identified: Shank1 is expressed exclusively in the brain, Shank2 is expressed in the brain and somewhat in the kidneys, while Shank3 is expressed mostly in the heart (Lim et al., 1999). Since Shank1 is the only isoform expressed exclusively in brain tissue, it is the logical choice for deletion in a mouse model. This paper describes behavior testing in three cohorts of Shank1 knockout mice, and the implications of the results of this testing for the effects of Shank proteins *in vivo*.

Methods

Experimental Design

Three cohorts of mice were tested with various rodent behavior apparatuses in order to characterize the phenotype of a Shank1^{-/-} (Shank1 null) mutant. Mice used in this study were males in a genetic background of C57BL/6 crossed with 129SvJae (gift of R. Jaenisch) to minimize confounding effects of neighboring loci. Genotypes were identified by PCR (polymerase chain reaction). Mice were between three months and five months old when testing commenced, and were housed with littermates. Except where noted, mice were allowed free access to food and water. All behavior testing was performed in a blinded fashion. Generation and maintenance of the colony, as well as all behavior procedures, were performed in compliance with guidelines of the MIT Committee on Animal Care.

Initial Phenotypic Characterization

Open field test

The open field test is used to ascertain the baseline locomotor activity of a mouse. Mice are placed in the center of a well-lit box, and various movement parameters are measured by computer. Mice were randomly assigned to groups of two or three and then placed in the open field testing apparatus (AccuScan, Columbus, Ohio). Testing began immediately after mice were placed into the testing apparatus and continued for 30 minutes. Digipro software (AccuScan, Columbus, Ohio) recorded all data, including horizontal activity, vertical activity, and time spent in the center, as described (Crawley, 2000). Mice were acclimated to the testing room for 15 minutes prior to the beginning of the test, and had not experienced a cage change for at least 24 hours.

Rotarod test

Mice were placed on a rotating rod (UGO Basile, Comerio, Italy). Testing started when the rod began to rotate, accelerating from 4 to 40 revolutions per minute, and ended automatically when the mouse fell off the rod. Any mice still on the rod after 300 seconds were removed. Each mouse was tested three times a day for two consecutive days.

Wire hang test

Each mouse was placed on a wire cage top, which was slowly inverted and suspended a foot above an empty cage. The time it took each mouse to fall from the cage top was recorded. Any mouse still gripping the cage top after 60 seconds was removed.

Behavior Testing

Radial maze test

The eight-arm radial maze, a test of spatial memory was performed as previously described (Miyakawa et al., 2001), with modifications. As is depicted in Figure 1, the same two arms of the maze are always baited with a treat pellet for a given mouse. During acquisition training, each mouse was randomly assigned to one of two groups: maze arms 2 and 5 were always baited for group 1, while maze arms 1 and 6 were

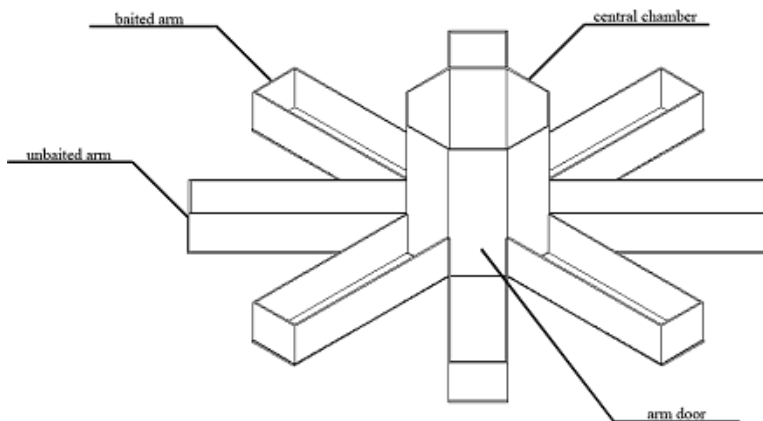


Figure 1. The eight-arm radial maze. Mice were placed in the central chamber, then allowed to explore the arms at will. Two arms were consistently baited for each mouse, and the remaining six arms remained unbaited. Maze doors were controlled by a computer, which also recorded the order of arms a given mouse entered.

always baited for group 2. A mouse was placed in the center of the maze and allowed to choose between the eight arms using prominent visual cues placed around the room. If the mouse entered an incorrect arm, a "reference memory" error was recorded, and if a mouse entered an arm which he had already visited, a "working memory" error was recorded. A given trial was terminated after both pellets were consumed, or after 20 minutes had elapsed. Mice were food-restricted beginning two weeks before training; each animal's weight was maintained at 80-85% of its unrestricted body weight. Two cohorts of mice were studied, and data from these cohorts was pooled (cohort 1: +/+, n=7; -/-, n=7; cohort 2: +/+, n=8; -/-, n=7). Animals were trained with one trial per day for fourteen consecutive days, then two trials per day for thirteen consecutive days (trials 15-40) and four trials per day for eleven consecutive days (trials 41-84).

Radial maze retention test

After twenty-eight days without exposure to the maze, mice were retested with the same bait configuration for four trials per day on two consecutive days. Retention training was performed with four trials per day on two consecutive days (trials 85-92). Image RM software (O'Hara and Company, Tokyo, Japan) controlled maze doors and recorded data.

Intensive radial maze test

The third cohort of mice (+/+, n=9; -/-, n=9) was trained on five consecutive days followed by two off days. They received one trial per day for four days (trials 1-4), then two trials per day for two days (trials 5-20) and four trials per day for ten days (trials 51-60).

Results

Three cohorts of mice were tested for general motor skills using the open field test, rotarod test, and wire hang test in order to establish motor differences between Shank1 knockouts and wildtypes. The mice were also tested in the eight-arm radial maze to assess the effect of Shank1 deficiency on the ability to learn. As shown below, Shank1 knockouts learned the eight-arm radial maze (Figure 1) faster than wildtype littermates, and their final performance in the maze was superior to that of wildtypes.

Improved radial maze performance in Shank1^{-/-} mice

In cohorts 1 and 2, knockouts consistently made fewer reference memory errors in the maze than their wildtype littermates (Figure 2). In the final four maze trials, knockout mice made an average of 0.95 reference memory errors, whereas wildtypes made an average of 2.20 ($t=2.55$, $df=17$, $p=0.02$; two-tailed t-test assuming unequal variances). Averaged over all trials, knockouts made significantly fewer reference memory errors than wildtypes ($F(1,27)=10.98$, $p=0.003$; one-way ANOVA).

In contrast to reference memory errors, a working memory error was recorded when a mouse re-entered a baited arm after it already consumed the pellet in the arm during the current trial. Shank1^{-/-} mice made significantly fewer working memory errors over all trials than Shank1^{+/+} mice ($t=3.83$, $df=20$, $p=0.001$; paired two-sample t-test for means).

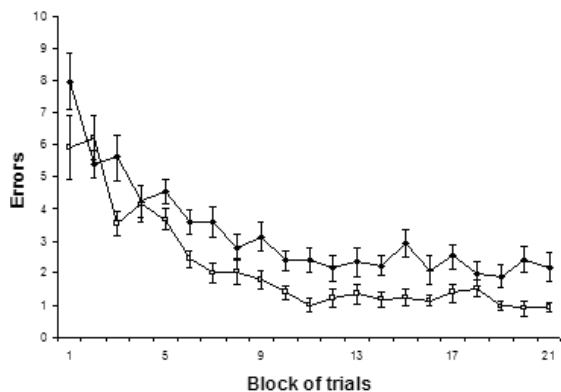


Figure 2. Superior performance of Shank1 knockouts (□) versus wildtypes (◆) in the eight-arm radial maze. The same two arms were baited for a given mouse during all trials; errors represent the mouse's entry into an unbaited arm. Data is blocked for simplification into groups of four trials, and is pooled from two cohorts. Data points depict the group means for each block of four trials plus or minus SEM.

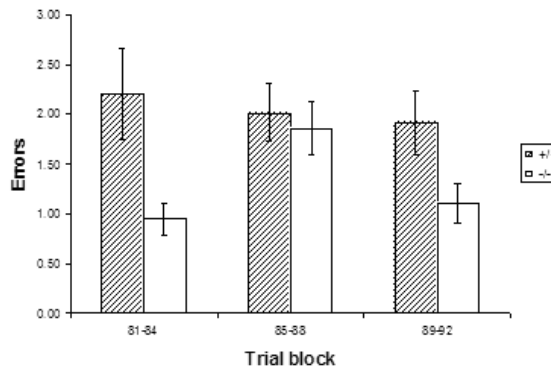


Figure 4. Performance in the eight-arm radial maze before and after a twenty-eight day vacation. Mice were allowed a twenty-eight day break from the radial maze, then returned for two days of four trials per day. Results are blocked by day of testing and are pooled between two cohorts. Trials 81-84 occurred before the vacation; trials 85-92 occurred after the vacation. Bars represent the group means plus or minus SEM.

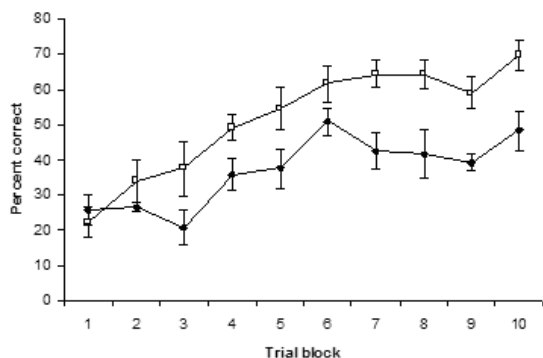


Figure 3. Percent of wildtype (◆) and knockout (□) mice whose first arm choice was baited. Two maze arms were baited consistently for each mouse; the mouse could choose to enter any of the eight arms first. Data is in blocks of eight; data points represent the group means plus or minus SEM.

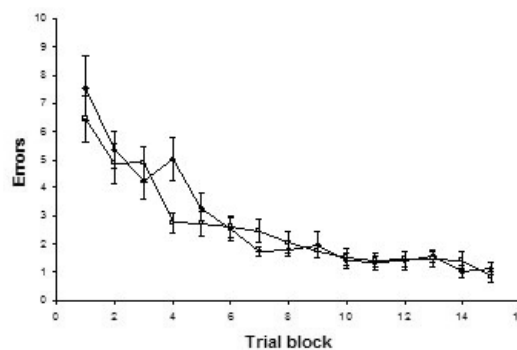


Figure 5. Performance of wildtype (◆) and knockout (□) mice after intensive training. Cohort 3 was exposed to an intensive training protocol which involved more condensed testing interspersed with two-day breaks. Results are shown in blocks of four trials; data points represent the group means plus or minus SEM.

In addition to committing fewer reference and working memory errors, Shank1 knockouts' first arm choice was correct more often than wildtypes' first arm choice (Figure 3).

Decline and recovery of knockout performance in retention and reversal trials

After eighty-four radial maze trials, mice were given a twenty-eight day break from the radial maze. Four trials were performed each day for the two days following the twenty-eight day break.

On the first day after the break, knockout mice committed as many errors as wildtypes (Figure 4). The difference between genotypes in trials 88-92 was not significant ($t=0.41$, $df=27$, $p=0.69$; two-tailed t-test). On the second day after the break, the number of knockout reference memory errors had returned to previous levels. Knockout performance in trials 93-96 was again significantly better than wildtype performance ($t=2.15$, $df=27$, $p=0.04$; two-tailed t-test assuming unequal variances).

Following the retention trials, reversal training began. For reversal training, mice who had formerly found a pellet

in arms 1 and 6 now found a pellet in arms 2 and 5, and vice versa. In

the first four reversal trials, both groups made many more reference memory errors (+/+ : 3.63 errors, +/- : 4.09 errors) than they had in the last four acquisition trials (+/+ : 2.02 errors, +/- : 0.96 errors). By the last block of four reversal trials, knockouts were again committing significantly fewer reference memory errors than their wildtype littermates ($t=3.03$, $df=23$, $p=0.006$; two-tailed t-test assuming unequal variance). Knockout performance in the last four reversal trials was not significantly different than knockout performance in the last four acquisition trials ($t=0.80$, $df=26$, $p=0.43$; two-tailed t-test assuming unequal variance).

Knockout performance does not improve with intensive training

The third cohort of mice was trained on a more intensive protocol, in which the number of trials per day was escalated more rapidly than in the standard protocol and a two-day weekend break period was given after each five-day training period.

There was no significant difference between the number of reference errors committed by knockouts in cohort three and those committed by knockouts in cohorts 1 and 2 ($F(1,28)=0.003$, $p=0.96$; one-way ANOVA). Wildtype mice, however, improved markedly with the intensive training protocol. Throughout the intensive training period, the number of reference memory errors committed by knockout and wildtype mice were indistinguishable (Figure 5). Thus, there was no significant difference in reference memory errors made by knockouts and wildtypes in cohort 3 ($F(1,28)=0.08$, $p=0.78$; one-way ANOVA).

Shank1^{-/-} mice show reduced activity in the open field

Shank1^{-/-} mice appear grossly normal, with body weight and fur appearance similar to their wildtype littermates (data not shown). These mice cannot be distinguished from wildtypes by physical appearance or by social characteristics.

Despite their normal appearance, Shank1^{-/-} mice are much less active than wildtype mice in the open field test (Figure 6). Activity in the open field test is measured by the number of times the subject crosses a beam projected from one of several photocells embedded in the walls of the testing apparatus. Shank1 knockouts show little interest in exploring the open field; they are significantly less active over the 30-minute testing period than wildtypes ($F(1,58)=40.5$, $p=3.4 \times 10^{-8}$).

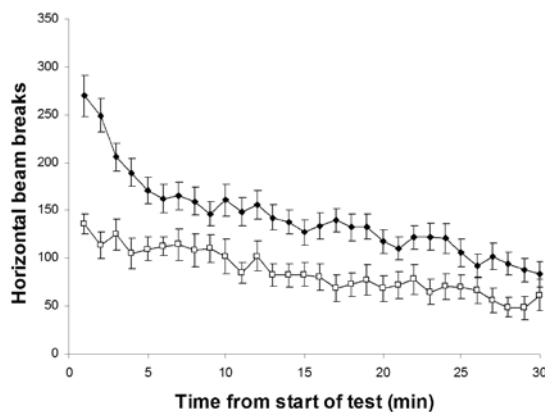


Figure 6. Horizontal activity of wildtype (◆) and knockout (□) mice in the open field test. Digipro software (AccuScan, Columbus, Ohio) determines horizontal activity by measuring horizontal beam breaks; results were recorded after each minute of testing. Data is pooled among three cohorts. Data points represent the group means plus or minus standard error of the mean (SEM).

Poor motor coordination in Shank1 knockouts

Both the wire hang test and the rotarod test assessed the gross motor skills of knockout mice compared with wildtype littermates. In the wire hang test, Shank1^{-/-} mice were less proficient than their wildtype counterparts (Figure 7). Knockout mice were able to grip the cage top for an average of 36.3 seconds, while wildtypes stayed on for an average of 55.4 seconds ($t=3.18$, $df=22$, $p=0.004$; two-tailed t-test assuming unequal variance). Motor coordination was also impaired for knockouts in the rotarod test (Figure 8). By

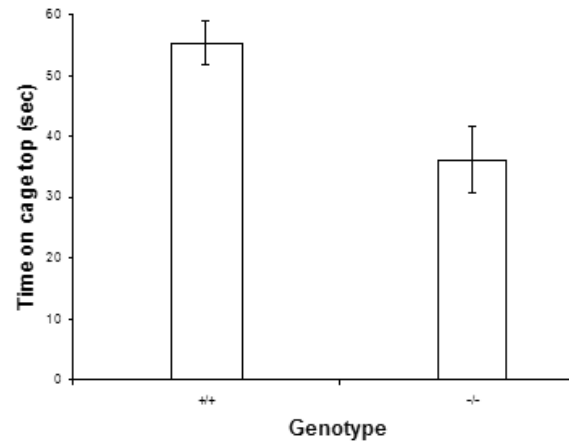


Figure 7. Decreased ability of Shank1^{-/-} mice in the wire hang test. Mice were suspended from an inverted wire cage top for a maximum period of 60 seconds; testing ended when the mouse fell from the cage top into the cage below. Data is from one representative cohort, and bars represent the group means plus or minus SEM.

the final trial, almost all wildtypes could stay on the rod for the full 300 seconds, while only a few knockouts could do so. Knockout mice stayed on the rotating rod for an average of 173 seconds over all trials; wildtype mice remained on the rod for an average of 242 seconds. Over all trials, knockouts were significantly less adept at the rotarod test than their wildtype littermates ($t=4.60$, $df=26$, $p=9.58 \times 10^{-5}$; two-tailed t-test assuming unequal variance).

Discussion

Mice deficient in Shank1, a scaffold protein in the post-synaptic density, show a remarkable gain in the ability to learn the eight-arm radial maze compared to their wildtype littermates. These mice also display motor impairment compared to wildtypes on a variety of motor tests.

Reduced exploration in Shank1^{-/-} mice

Most rodents, including the Shank1 wildtypes, initially show great interest in exploring a novel environment, which

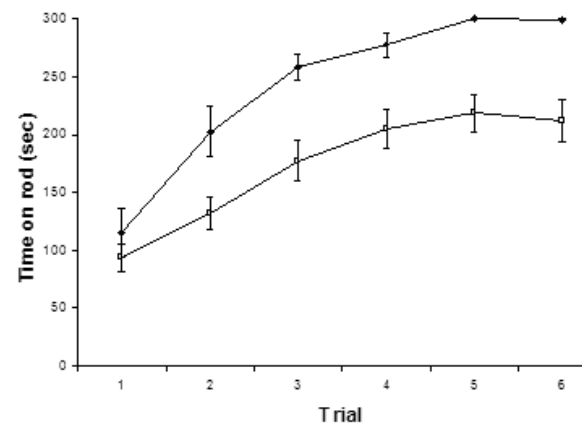


Figure 8. Decreased motor skill of Shank1^{-/-} mice in the rotarod test. Both wildtype (◆) and knockout (□) mice were placed on a rotating rod and tested for 300 seconds. Testing ended after the mouse fell off the rod, or after 300 seconds. Data points are the pooled group means plus or minus SEM.

wanes over time (Crawley, 2000). Shank1 knockouts, in contrast, are reluctant to explore novel environments such as an open field (Figure 6). This reduced activity in Shank1 knockouts was not unique to the open field test; knockouts were hesitant to explore the radial maze during training periods, and an object exploration test was abandoned because knockouts refused to explore the given objects (data not shown).

Two possible explanations for these data exist. First, Shank1 knockouts could be hypoactive in general, or their motor deficits could be so severe that they experience difficulty in normal movement. Although the mice were not observed systematically for activity in their home environments, such as in a social interaction test, they exhibited no clear hypoactivity when in a familiar environment.

Second, and most likely, Shank1^{-/-} hypoactivity in unfamiliar contexts could be a result of altered activity in the amygdala. The amygdala is a region of the brain involved in emotion,

particularly fear (Davis, 1997). Shank1 is abundant in the amygdala, where it serves as a scaffold linking NMDA, AMPA, and mGluR receptors (Sheng and Kim, 2000). Interestingly, altering NMDA receptors appears to affect the performance of experimental animals in fear-conditioning paradigms, but the linkage has not been fully explained (Rogan et al., 1997). Presumably, the deletion of Shank in amygdala of knockouts causes knockouts to be more fearful in unfamiliar contexts; however, further research is necessary to resolve this issue.

Poor locomotor activity in Shank1^{-/-} mice

Shank1 knockouts showed impairment on many general tests of motor ability, including the rotarod test and the wire hang test. This general motor impairment is probably due to disruption of the cerebellum, a brain structure chiefly concerned with motor coordination (Ghez and Thach, 2000).

Shank1 is also richly expressed in the cerebellum (Lim et al., 1999). Motor deficits seen in Shank1 knockouts are similar to those in human subjects with cerebellar lesions, who have difficulty with coordinating movements and with producing even force with opposing limbs (Ghez and Thach, 2000). Shank1 knockouts appear to have difficulty producing even force with all four of their feet, particularly in the wire hang test; knockouts often grip the cage top with only three feet. Using only three feet reduces the mouse's ability to stay on the cage top, because it causes an unusual shift in center of gravity, which further destabilizes the mouse and causes it to fall.

Improved performance in the eight-arm radial maze

Mammalian reverse genetics has enabled the creation of lines of mice deficient in various brain proteins. These mouse lines have proved invaluable for studying learning and memory. In contrast to the Shank1 knockout, knockouts in the major lines discussed in the literature are either unaffected by the deletion or are impaired in learning and memory tasks (Chen and Tonegawa, 1997). Rarely, if ever, has knocking out a gene in the brain been shown to facilitate learning.

Shank1 is a "master" scaffold protein, linking intermediate scaffolds such as PSD95 and GKAP, which in turn attach to NMDA, AMPA, and mGluR receptors. Because Shank1

ultimately links so many proteins, it is an important site of regulation. Through its creation of large protein complexes, Shank1 seems to promote stability of dendritic spines (Kim and Sheng, 2004).

The dendritic spines of knockouts lack large-scale scaffolding through Shank1, and it is reasonable to assume they might be more pliable than dendritic spines of wildtypes; that is, the spines in knockout neurons are more adept at change. This increased plasticity may cause Shank1 knockouts to learn the radial maze faster than wildtypes (Figure 2), but also to forget the maze more rapidly after a four-week break (Figure 4).

Shank1 knockouts fail to improve their maze performance when trained on an intensive protocol. This result is difficult to interpret, especially because wildtype performance improves so drastically on the intensive protocol. It is certainly possible that knockout performance, already averaging less than one reference memory error per trial, could not have been improved further. However, it is also possible that wildtype dendritic spines can mimic a knockout state by undergoing intensive training; in this way, intensive training on a learning task could forcibly rearrange protein complexes, imitating Shank1 deletion. Further studies on Shank1 and other scaffold proteins in the postsynaptic density may enable investigators to draw better informed conclusions on this perplexing result.

In contrast to results observed in other learning and memory knockouts, Shank1 deletion has a positive effect on the ability of a mouse to learn the eight-arm radial maze. Because Shank1 has not been previously studied *in vivo*, this analysis provides a critical set of information regarding the role of Shank1 and related scaffold proteins in synaptic function.



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