# Spatial Memory Impairment is Associated with Intraneural Amyloid-β Immunoreactivity and Dysfunctional Arc Expression in the Hippocampal-CA3 Region of a Transgenic Mouse Model of Alzheimer's Disease

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**Abstract**. Dysfunction of synaptic communication in cortical and hippocampal networks has been suggested as one of the neuropathological hallmarks of the early stages of Alzheimer's disease (AD). Also, several lines of evidence have linked disrupted levels of activity-regulated cytoskeletal associated protein (Arc), an immediate early gene product that plays a central role in synaptic plasticity, with AD "synaptopathy". The mapping of Arc expression patterns in brain networks has been extensively used as a marker of memory-relevant neuronal activity history. Here we evaluated basal and behavior-induced Arc expression in hippocampal networks of the 3xTg-AD mouse model of AD. The basal percentage of Arc-expressing cells in 10-month-old 3xTg-AD mice was higher than wild type in CA3 (4.88% versus 1.77%, respectively) but similar in CA1 (1.75%versus 2.75%). Noteworthy, this difference was not observed at 3 months of age. Furthermore, although a Morris water maze test probe induced a steep (~4-fold) increment in the percentage of Arc+ cells in the CA3 region of the 10-month-old wild-type group, no such increment was observed in age-matched 3xTg-AD, whereas the amount of Arc+ cells in CA1 increased in both groups. Further, we detected that CA3 neurons with amyloid-ß were much more likely to express Arc protein under basal conditions. We propose that in 3xTg-AD mice, intraneuronal amyloid-ß expression in CA3 could increase unspecific neuronal activation and subsequent Arc protein expression, which might impair further memory-stabilizing processes.

Keywords: Activity regulated cytoskeletal-associated protein, Alzheimer's disease, hippocampus, memory, neuroplasticity

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## INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive decline in cognitive functions and whose main risk factor is aging [1]. Evidence at pharmacological, biochem-ical, and behavioral levels suggests that a crucial step in the early pathogenesis of AD is the gen-eration of amyloid-ß peptide (Aß) from sequential proteolytic cleavage of amyloid-ß protein precursor (AßPP) [2]. Notably, in transgenic mice models that bear one or several human genes common to familiar AD, accumulation of Aß occurs concurrently with memory impairment [3, 4] and, as the disease pro-gresses, the presence of reactive microglia and the loss of neurons, white matter, and synapses begin to appear [5]. In AD models, hippocampal synaptic dysfunction was shown to correlate with cognitive impairment and proposed as an early event in the pathogenesis of AD in which Aß is believed to play a central role [6, 7]. The triple transgenic mouse model of AD (3xTg-AD) develop the behavioral and neu-ropathological hallmarks of AD in a temporal and region-specific manner [4, 8]. These mice display hip-pocampal long-term potentiation deficits that precede plaque and tangle formation [8]. In addition, just as what is observed in early stage human AD patients, the first cognitive symptoms in 3xTg-AD mice man-ifest as impaired retention—but spared learning—in medial temporal lobe-dependent declarative memory tasks [9–11]. Also, findings in 3xTg-AD mice and other AD models as well as cultured hippocampal neurons suggest that the onset of early AD-related symptoms are related to intraneuronal accumulation of Aß, before the appearance of neuritic plaques and neurofibrillary tangles [8, 9, 12–16].

The mapping of immediate early genes has proven to be a powerful tool to visualize experience-encoding networks of neurons that are critical for memory retention [17, 18]. One of these genes, Arc (also known as Arg3.1), is of particular interest because of its central role in synaptic plasticity and consolidation as well as the specificity of its expres-sion in putative memory-encoding neural networks [17]. Arc protein serves versatile functions at the synapse with established roles in long-term potenti-ation, long-term depression, and activity-dependent scaling of AMPA receptors [19, 20]. Furthermore, Arc knockout mice have impaired long-term memory, but spared learning in a variety of hippocampus-dependent and hippocampus-independent tasks [21]. Meanwhile, Arc knockouts also exhibit network hyper-excitability [22], while aberrantly high levels of Arc are associated with seizure-like activity in a mouse model of Angelman syndrome [23]. In addi-tion, recent evidence suggests that Arc plays a central role in AD "synaptopathy" [24]. For instance, Arc's association with presenilin-1 in early endosomes is necessary for activity-dependent Aß production [25] Also, it was recently shown that experience-driven cortical network responses, observed with neural activity mapping using Arc expression [17], were disrupted by the nearby presence of neuritic plaques [26]. However, information regarding how memory-encoding hippocampal networks are disrupted in transgenic AD models before the appearance for neu-ritic plaques, but when soluble oligomeric Aß as well as cognitive impairments are detected, remains scarce.

To further examine this issue, we mapped basal and behaviorally induced Arc protein expression in hippocampal neuronal networks of 10-month-old wild-type (WT) and 3xTg-AD (TG) mice at an age before the appearance of neuritic plaques in the hippocampus but after both intracellular Aß and long-term spatial memory deficits are detected. From our behavioral study, we found that under our settings, long-term memory of the Morris water maze (MWM) task was normal in WT, but impaired in TG mice, which, however, performed similar to the WT in the acquisition phase. Analysis of Arc protein expression in the hippocampus shows that a greater proportion of neurons are active under basal conditions in the CA3 but not in the CA1 region of TG mice. Also, spatial memory retrieval induced a marked increment in Arc-expressing cells in the CA3 region of WT mice but failed to do so in the TG. No such difference was observed in CA1 where a significant increase in the proportion or Arc-expressing cells was observed after MWM retrieval in both genotypes. Importantly, the percentage of CA3 neurons with Arc protein was positively correlated with performance in WT but not in TG mice. Finally, we found that Aß-expressing neurons in the CA3 region of 3xTg-AD mice are much more likely to express increased basal Arc than Aß-negative neurons.

## MATERIALS AND METHODS

All experiments were conducted in agreement with the Bioethics committee of the Institute of Neurobiol-ogy, UNAM. Thirteen 10-month-old male 3xTg-AD mice and 19 control age-matched male B6129SF2/J mice derived from breeding pairs kindly provided by Dr. F. LaFerla (University of California, Irvine, CA) were used for these experiments. An additional 8 mice (4 for each genotype) of 3 months of age were also used. Mice included in the analysis were 10-month-old unless otherwise specified. The 3xTg-AD mouse model of AD has been described elsewhere [27]. Genotyping was carried out as described previ-ously [11]. All mice were housed 2–4 per cage with access to food and water ad libitum in an inverted 12 h:12h light: Dark cycle. MWMexperiments were per-formed during the dark phase. The water tank's diam-eter was 1 m, the escape platform was 8.2 × 8.2cm and the water temperature was kept at 22 ± 1◦C. Once the animal reached the platform, it was allowed to remain there for 20 s, gently dried and put in a resting cage until the beginning of the next trial. Ani-mals were guided to the platform if they failed to reach it within 60 s.

Testing was done with the plat-form removed, 72 h after the mouse had reached the task acquisition criterion, which was when the aver-age latency to platform of a session's four trials was ≤20 s. All training trials and tests were monitored with a Sony DCR TRV280 camera connected to a computer equipped with Smart v2.5 software.

One hour after the test trial, animals were deeply anesthetized with an overdose of sodium pentobar-bital and perfused ice-cold 0.1 M phosphate buffer followed by ice-cold buffered 4% paraformaldehyde. Brains were cryoprotected with incremental sucrose gradients, until a concentration of 30% sucrose in 0.1 M phosphate-buffered saline was reached. Thirty-micron slices spanning the dorsal hippocampus (~AP –1.46 to AP –2.30 mm to Bregma) of the left hemi-sphere were then obtained with a cryostat maintained at –18ºC. Detection of Arc was performed with 1:500 affinity-purified rabbit antiactivity-regulated cytoskeletal associated protein (Synaptic Systems) in TSA blocking solution (Perkin Elmer). Signal was amplified using ABC kit (Vector Laborato-ries) and fluorescence signal detected with CY3 kit (Perkin Elmer). Sections were then counterstained with Hoechst (Life Technologies) and mounted with Vectashield (Vector Laboratories). For simultane-ous detection of Arc protein and Aß peptides, right hemispheres of the brains of 3xTg-AD animals assessed for basal Arc protein expression in the previous experiments were analyzed. Thirty ßm-thick coronal sections were obtained as described above, rinsed and incubated in 89% formic acid for 8 min and rinsed with water. Incubation with primary antibody included combination of primary Arc antibody and 1:500 mouse-anti-ß-Amyloid clone Bam10 (Sigma-Aldrich) in blocking solution. The secondary antibody cocktail consisted of Alexa-488 coupled goat anti-mouse and Alexa-555 coupled goat anti-rabbit (Invitrogen) both at a concentration of 1:500.

Images used in the analyses were obtained with a Zeiss 510 META confocal microscope. For each mouse, 3 to 5 Z-stacks (1 µm per plane) of the CA3 region were obtained with a 20x/0.50N.A. objective, with zoom set at 1.0, for both Arc and Arc/Aß cell-counting analysis. Z-stacks of the CA1 region were obtained with a 40x/1.3N.A. oil-immersion objec-tive, planes were 1 µm-thick and zoom was set at 0.7. High magnification photomicrographs presented in Fig. 3E-H were obtained with a 63x/1.3N.A. oil-immersion objective and planes were 0.5 µm thick. Multiphotonic Coherent-XR, multiple line Argon and DPSS channels were used for the detection of nuclei, Aß and Arc, respectively. For the analysis of Arc expression in 10-month-old mice, the first section to be captured for each slide was invariably from a WT mouse that did not perform MWM experi-ments (WTc, see below) in order to establish the laser intensity, gain and offset parameters, which were kept constant for the rest of the capturing session in the Arc (Cy3) channel. The rest of the sections present on a given slide were previously assigned a code to avoid bias. The other three groups are identified as WT: Wild-type mice that performed MWM; TG: 3xTg-AD mice that performed MWM; and TGc: Control 3xTg-AD that did not perform MWM.

Putative neuronal nuclei were counted [28] and classified as Arc+ or Arc- based on previously described criteria [29] with slight modifications. The sample size for the analysis of Arc expression in the CA3 region for each group was as follows: WTc: 1083.4 ± 91 analyzed cells/subject, $n$ = 7; WT: 946.9 ± 58 cells/subject, $n$ = 10; TGc: 1024.8 ± 100 cells/subject, $n$ = 5; TG: 980.2 ± 81 cells/subject, $n$ = 6. For the analysis of Arc expression in the CA1 region in 10-month-old animals, the number of ana-lyzed cells per subject in each group was 697.4 ± 105 for WTc, 570.3 ± 64 for WT, 544.8 ± 66 for TGc, and 608.3 ± 86 for TG ($n \geq 4$). For the quantifica-tion of cells co-expressing Arc and Aß, the same parameters were used, except that cells were fur-ther classified as Aß+ or Aß-. Arc and Aß channels were analyzed separately to prevent bias and regions of interest that overlapped between the two chan-nels were counted. For this analysis, a minimum of 3 z-stacks per subject were analyzed from the right hemispheres of TGc animals ($n$ = 5) used in the previous experiments (864.2 ± 181.94 cells/subject). All image analysis was performed with ImageJ soft-ware (NIH). All statistical analysis was performed using Statview (Abacus Concepts Inc.). Behavioral analysis was carried out using repeated measures ANOVA with "genotype" and "day" as factors for the training phase and unpaired Student t tests for the test trial, except for the time spent in specific quadrants, which was compared using paired t tests. Comparison of Arc expression was carried out using Two-way ANOVA with "genotype" and "MWM" as factors and Fisher post-hoc tests were used where appropriate. Comparison of Aß-positive and Aß-negative expressing cells was done using Student t test. Statistical significance was accepted when $p < 0.05$.

## RESULTS

We first evaluated the performance of 3xTg-AD and WT mice during acquisition and retrieval of the MWM task under our settings and at 10 months of age. During acquisition, animals showed a marked improvement from one day to another regardless of genotype (Repeated measure two-way ANOVA, Main effect of "Day", $F(2,18) = 15.44$, $p < 0.0001$)(Fig. 1A). Most animals, both WT and TG, had reached criterion by the third day of training and all of them had reached it on the fifth day.

On test trial, however, WT mice spent significantly more time in the target quadrant versus the opposite ($t_{(11)} = 3.47$, $p < 0.01$), whereas the swimming time spent by TG was similar in both quadrants ($t_{(7)} = 0.79$, $p = 0.46$). Also, the number of crossings to the target was sig-nificantly higher in WT than TG group ($t_{(18)} = 2.32$, $p < 0.05$) (Fig. 1B). Furthermore, the average dis-tance to the target was significantly lower in WT mice compared to TG ($t_{(18)} = 2.13$, $p < 0.05$) (Fig. 1C). Finally, swimming speed was similar in both groups ($t_{(18)} = 1.5$, $p = 0.15$) (Fig. D). These data show that under our settings, the acquisition of the MWM task was normal in 10-month old 3xTg-AD mice but long-term memory expression was impaired.

We next sought to evaluate the proportion of neu-rons that expressed Arc protein after this MWM test trial. For this analysis, two additional control groups that consisted of littermates of 10-month-old WT and TG mice that did not perform the MWM task—referred to as WTc and TGc, please see above—were included in order to compare memory retrieval-induced Arc protein expression with basal Arc protein levels, for both genotypes. Please note that MWM groups will be referred to as WT and TG as in Fig. 1. We first found that the percentage of Arc-expressing cells in CA3 significantly differed between groups (Two-way ANOVA, $F(3,21) = 9.67$, $p < 0.0005$) (Fig. 2A). Most strikingly, whereas the test trial induced a marked increase in the percentage of Arc protein expressing neurons in WT ($1.77 \pm 0.21$ versus $6.94 \pm 0.75$, $p < 0.0001$ versus WTc), it failed to do so in TG mice ($4.88 \pm 1.30$ versus $3.92 \pm 0.53$, $p = 0.43$). Notably also, the proportion of Arc+ cells was significantly larger in the TGc group compared to WTc ($4.88 \pm 1.30$ versus $1.77 \pm 0.21$, $p < 0.05$). Noteworthy, 3xTg-AD mice at 3 months of age, that is, before the appearance of long-term memory deficits in these animals [9], had a similar percentage of Arc-expressing cells in the CA3 region compared to age-matched wild-types ($t(6) = 0.7$, $p = 0.95$, Sup-plementary Figure 1). Also, the percentage of Arc+ cells after MWM test was significantly greater in the WT than in the TG group ($p < 0.01$) (Fig. 2D). On the other hand, comparison of Arc expression in the CA1 region revealed significant differences between groups ($F(3,13) = 6.69$, $p < 0.01$). However post hoc comparisons revealed that basal Arc protein expressing cells in CA1 did not differ between WTc and TGc ($2.75 \pm 0.54$ versus $1.75 \pm 0.19$, respec-tively, $p = 0.72$) and animals of both genotypes showed increased Arc expressing cells upon MWM test ($2.75 \pm 0.54$ versus $10.92 \pm 2.59$, $p < 0.01$ for WTc versus WT and $1.75 \pm 0.19$ versus $8.01 \pm 2.43$, $p < 0.05$ for TGc versus TG, Fig. 2D-E). We finally sought to evaluate whether there was a relation between memory performance during MWM test and the percentage of analyzed cells expressing Arc one hour after the test. Indeed, a significant corre-lation was observed between the percentage of Arc expressing cells in CA3 and the Average Distance to Target parameter of MWM performance (see Fig. 1)(Spearman's Rho = –0.71, $p < 0.001$, Fig. 2C). Specif-ically, analyzing the WT and TG clusters separately revealed that the percentage of CA3 cells expressing Arc increased in function of decreased average dis-tance to target in the CA3 region of WT but not TG mice (Spearman's Rho = –0.66, $p < 0.05$ and Spear-man's Rho = –0.43, $p = 0.34$, respectively, Fig. 2C). Meanwhile, no such correlation was observed for the CA1 region though a slight tendency was observed (Spearman's Rho = –0.57, $p = 0.13$). Together, these data strongly suggest that CA3 Arc protein expression after MWM test in WT animals is induced by spatial memory retrieval and not by swimming or navigation per se. While further analysis may elucidate if this is also the case for CA1, our results clearly suggest that the relation between performance and Arc expression during retrieval is more robust in CA3.

Intraneuronal Aß can alter synaptic function, which may underlie aberrant network excitability in AD models [12, 16]. Furthermore Aß levels are known to be upregulated by synaptic activity [30–32] and many receptors and signaling pathways involved Aß generation are also known to induce Arc expression [33]. This could explain why under basal conditions, we observed a greater percentage of Arc+ cells the CA3 region of 3xTg-AD mice compared to wild-types. Therefore, in order to test whether a link existed between Aß accumulation and Arc expression at the cellular level, we compared the percentage of Arc+ cells amongst those that showed immunoreactivity for Aß (Aß+) versus those that did not (Aß–). Indeed, we observed that the likelihood of CA3 Aß+ cells to express Arc was dramatically

increased; 30% of Aß+ cells also had Arc protein compared to 2% of Aß-- cells ($t(4) = 9.8$, $p < 0.0001$, Fig. 3A-D). This finding indicates that neurons with abundant intracellular Aß (see Fig. 3E-H) are more likely to express Arc under basal conditions and suggests that intraneuronal Aß underlies the dysfunc-tional CA3 Arc protein expression observed in TG mice.

## DISCUSSION

Under our experimental settings, middle-aged 3xTg-AD mice had spared acquisition, but impaired long-term memory expression of a spatial memory task. Interestingly, in age-matched WT mice, which showed adequate long-term memory for the MWM task, the test trial induced a robust increase of the percentage of Arc-expressing cells in both CA1 and CA3 subfields. This is in line with evidence showing that strong Arc protein accumulation occurs during spatial memory retrieval, irrespective of novelty or whether additional information is present [17, 34] and has been suggested to be required even during consol-idated tasks execution or highly familiar environment exploration in order to enhance memory persistence and precision [35]. In 3xTg-AD mice, however, the test trial induced a significant increase in the pop-ulation of Arc-expressing cells in CA1, but not in CA3, in which Arc levels remained similar to that of caged controls. This finding therefore suggests that impaired recruitment of CA3 neurons may under-lie spatial memory retention deficits observed in this AD mouse model. In fact in rodents, long-term struc-tural plasticity mechanisms have been shown to be involved in CA3 during long-term spatial memory formation [36, 37]. Interestingly, animals with CA3 lesions show impaired retrieval but spared acquisi-tion of a MWM task [38]. Furthermore, the recall of a remote spatial memory produced a robust task-specific Arc mRNA induction in the dorsal CA3 region and entorhinal cortex, but not in the CA1 area, consistent with a more transient role of CA1 in spatial memory consolidation [39, 40]. Retrieval of consolidated MWM was also shown to produce a dramatic increase in the levels of pCREB (a tran-scription activator involved in activity-dependent Arc expression [41]) in the dorsal CA3 region [42]. In our study, we observed a positive correlation between the percentage of Arc protein-expressing cells in CA3 and memory performance in WT animals, strongly arguing against the possibility that the observed Arc response could be due to stress or physical activ-ity during swimming. Since we did not observe such correlation in TG mice in which retrieval/long-term memory formation was clearly impaired, our findings are also consistent with the idea that the Arc-expressing cells observed after spatial memory retrieval represent specific reactivation of a spatial map, rather than exploration per se. Interestingly also, an earlier work found that Arc mRNA lev-els in hippocampus homogenates after a six-trial training session of MWM were positively correlated with performance during the last three learning trials [43]. Moreover, they showed that performance in the hippocampus-independent, cued version of the task did not correlate with hippocampal Arc mRNA levels. Here, we provide new evidence at the single-cell level that the magnitude of Arc-expressing CA3 population after retrieval is positively correlated with memory performance.

Our data also show that a higher percentage of CA3 cells express Arc protein in 10-month-old TG mice than in age-matched WTs under basal conditions. This difference was not observed in 3-month-old mice (Supplementary Figure 1), an age when no spatial memory retention impairments are observed in male 3xTg-AD [9]. To the extent that Arc protein is a marker of neural activity, this finding implies that a higher percentage of CA3 cells are usually active in 10-month-old 3xTg-AD mice. Importantly, aber-rant excitability in the CA3 region has consistently been observed during both normal and pathological cognitive aging [44]. For example, in vivo elec-trophysiological recordings have shown that higher percentage of CA3 neurons have been observed with high firing rates in aged rats compared to their younger counterparts. Crucially, these neurons were less flexible in that they failed to modify their firing rate when the animal was made to explore a novel environment [45]. More recently, increased den-tate gyrus/CA3 activity observed in human patients with age-related mild cognitive impairment was shown to be normalized by the administration of the anticonvulsant drug levetiracetam, a treatment that concomitantly improved their performance in a visual memory task [46]. Here, our observation of an increased population of Arc-expressing neurons at basal state in 3xTg-AD mice could imply that in these animals, the CA3 network is in a constant satu-rated state that impairs adequate recruitment of neural populations when information needs to be encoded or retrieved. Further activity-induced, Arc protein-dependent stabilization of synaptic weight in these additional cells could arguably contribute to shift the CA3 region to a more rigid basal state.

During the onset of AD, neuronal circuit hyperactivity, observed in large-scale cortical and hippocampal networks, might underlie early neu-ronal dysfunction and behavioral impairments [47]. Importantly, neuronal circuit hyperactivity does not seem to require the presence of neuritic plaques.

For example, it was recently shown using *in vivo* $Ca^{2+}$ imaging that in mice overexpressing mutated AßPPswe and PS1G384A, there was a higher pro-portion of hyperactive neurons than in their WT counterpart. This difference was observed at an age in which no plaques are detected but soluble Aß is present, suggesting a role for soluble Aß in neuronal hyperactivity [48]. In agreement, they found that inhibiting ß-secretase activity, an enzyme involved in soluble Aß production, restored normal neuronal functioning. Importantly, they also showed that direct application of Aß in hippocampal neurons from wild type mice could also induce hyperactivity [48].

Here, we document that expressing Aß increases the likelihood of a neuron to express Arc as well. Given the high degree of overlapping between the signaling cascades required for Arc protein and Aß expression [33], one could expect to observe an even greater percentage of Aß-expressing cells to express Arc protein as well than the ~30% we observed. However, while Arc protein accumulation is a very transient phenomenon that occurs in a sparse pop-ulation of neurons [35], intraneuronal Aß is much more stable [49], so even though at the moment of sacrifice ~70% of Aß-expressing neurons were Arc-negative, it is likely that those cells had been more active and expressed Arc more frequently that the Aß-negative ones. Indeed, a recent study showed that externally applied Aß increases intrinsic excitability in primary hippocampal neurons by a mechanism that depended on Aß internalization and subsequent K+ channels phosphorylation [16]. Meanwhile, recent lines of evidence have established a clear link between Arc protein and the molecular mechanisms underly-ing synaptic dysfunction in AD [24]. For example, in hippocampal neurons, treatment with Aß diffusible ligands induced a rapid and persistent increase in den-dritic Arc protein that correlated with alterations in spine morphology [50]. In addition, Arc was shown to be required for activity-dependent generation of Aß in recycling endosomes through an interaction with presenilin-1 [25]. Finally, deletion of Arc in a trans-genic mouse model of AD reduces both soluble Aß levels and plaque load [25]. Our results showing an increased likelihood of Arc's presence in Aß+ cells suggest that intracellular Aß may increase the prob-ability of a neuron to be randomly activated, which could further produce aberrant activity-dependent synaptic modifications. Hyperactivity of networks in the 3xTg-AD and other mouse models of AD have been well documented but the mechanisms underlying this phenomenon are poorly understood.

However, hyperactive cells will likely produce more Arc protein and given the direct role of Arc in Aß pro-duction this could produce a feed-forward mcrease in Aß production that would speed-up neuropatho-logical events. Additionally, cytoskeletal integrity and intracellular trafficking are known to be altered in AD and are crucial for precise localization of Arc essential for its function [19, 12]. Given the cen-tral role of Arc in neural plasticity, we hypothesize that overproduction—and perhaps impaired intracel-lular trafficking—of Arc protein in a specific subset of cells could have a dramatic role in synaptic com-munication and plasticity at the early steps of AD. Thus, further studies examining the link between intracellular Aß and Arc protein function should help disentangle the molecular and cellular mechanisms underlying episodic memory deficits during the early phases of AD. In addition, our data provide further evidence in support of the role of disrupted hippocam-pal excitability in memory retrieval deficits occurring in early stage AD-like pathology.

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## SUPPLEMENTARY MATERIAL

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