Disruption of hippocampal synaptic transmission and long-term potentiation by psychoactive synthetic cannabinoid ‘Spice’ compounds: comparison with Δ⁹-tetrahydrocannabinol

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ABSTRACT

There has been a marked increase in the availability of synthetic drugs designed to mimic the effects of marijuana. These cannabimimetic drugs, sold illicitly as ‘Spice’ and related products, are associated with serious medical complications in some users. In vitro studies suggest that synthetic cannabinoids in these preparations are potent agonists at central cannabinoid CB1 receptors (CB1Rs), but few investigations have delineated their cellular effects, particularly in comparison with the psychoactive component of marijuana, Δ⁹-tetrahydrocannabinol (Δ⁹-THC). We compared the ability of three widely abused synthetic cannabinoids and Δ⁹-THC to alter glutamate release and long-term potentiation in the mouse hippocampus. JWH-018 was the most potent inhibitor of hippocampal synaptic transmission (EC₅₀ ~15 nM), whereas its fluoropentyl derivative, AM2201, inhibited synaptic transmission with slightly lower potency (EC₅₀ ~60 nM). The newer synthetic cannabinoid, XLR-11, displayed much lower potency (EC₅₀ ~900 nM) that was similar to Δ⁹-THC (EC₅₀ ~700 nM). The effects of all compounds occurred via activation of CB1Rs, as demonstrated by reversal with the selective antagonist/inverse agonist AM251 or the neutral CB1R antagonist PIMSR1. Moreover, AM2201 was without effect in the hippocampus of transgenic mice lacking the CB1R. Hippocampal slices exposed to either synthetic cannabinoids or Δ⁹-THC exhibited significantly impaired long-term potentiation (LTP). We find that, compared with Δ⁹-THC, the first-generation cannabinoids found in Spice preparations display higher potency, whereas a recent synthetic cannabinoid is roughly equipotent with Δ⁹-THC. The disruption of synaptic function by these synthetic cannabinoids is likely to lead to profound impairments in cognitive and behavioral function.

Keywords Drug abuse, electrophysiology, marijuana.

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INTRODUCTION

The potential therapeutic use of marijuana and related cannabinoids has led to a strong interest in developing compounds that can selectively target cannabinoid receptors but lack abuse liability (Izzo et al. 2009; Bisogno & Di Marzo 2010). Ironically, the development of such compounds by research laboratories across the world has provided clandestine chemists the framework from which to identify and synthesize potent drugs that mimic some of the psychoactive effects of Δ⁹-tetrahydrocannabinol (Δ⁹-THC). Consequently, there has been a global surge in the nonmedical use of synthetic cannabimimetic substances, marketed as ‘herbal incense’ and commonly known as ‘Spice’ (Logan et al. 2012; Lewin et al. 2014). Unlike the comparatively modest psychoactive and euphoric effects of marijuana, the use of Spice and related compounds has resulted in reports of severe anxiety, tachycardia, seizures and hallucinations (Schneir et al. 2011; Harris & Brown 2013). Nearly all of the identified chemical
constituents of synthetic marijuana act as agonists at cannabinoid CB1 receptors (CB1Rs), and the psychoactive compounds in these preparations are frequently modified in response to legislative control imposed upon existing chemical structures (Vardakou et al. 2010; Seely et al. 2012). The structures of three of the most popular synthetic cannabinoids and their appearance in the National Forensic Laboratory Information System database are depicted in Fig. 1. The National Forensic Laboratory Information System data reflect the prevalence of particular synthetic cannabinoids that were confiscated by local, state and federal law enforcement. The naphthoylindole JWH-018 (Fig. 1b) appeared frequently in Spice products confiscated during the years 2010 and 2011 in the United States but was rapidly supplanted by its fluoropentyl analog AM2201 (Fig. 1c; Seely et al. 2013). In the first half of 2013, the tetramethylcyclopropyl indole XLR-11 (Fig. 1d) became much more prevalent than JWH-018 or AM2201 (U.S. Drug Enforcement Administration Office of Diversion 2013), and this trend continued in 2014. Given the greater use of these synthetic marijuana preparations to avoid drug screen detection for Δ^9-THC, and increasing public health concerns regarding these compounds, there is a need to more fully characterize the neurobiological actions of these synthetic cannabinoids (Seely et al. 2012; Castaneto et al. 2014).

Among the best characterized effects of cannabinoids is the inhibition of neurotransmitter release from axon terminals via CB1R activation (Alger 2002; Hoffman & Lupica 2013). The cognitive deficits reported following acute or repeated use of marijuana (Abel 1970; Bolla et al. 2002) may reflect the effects of Δ^9-THC at CB1Rs located on both glutamatergic and GABAergic axon terminals in the hippocampus (Hájos et al. 2000; Sullivan 2000; Hoffman et al. 2007; Puighermanal et al. 2009). Most physiological studies have utilized synthetic agonists, such as WIN55212-2 and CP55,940, to evaluate the central actions of cannabinoids (Hoffman & Lupica 2000; Robbe et al. 2001; Holderith et al. 2011), and relatively few studies have examined the effects of Δ^9-THC at central synapses in vitro, owing to its poor solubility and penetration into brain slices (Laaris et al. 2010; Hoffman & Lupica 2013). JWH-018 has been evaluated in cultured hippocampal neurons (Atwood & Lupica 2013). The cognitive deficits reported following Δ^9-THC at hippocampal glutamatergic synapses.

METHODS

Subjects

All studies utilized 4- to 6-week-old male wildtype C57BL6 mice or CB1^+/+ and CB1^-/- mice bred on a C57BL6 background. Prior to initiating experimental work, studies were approved by the NIDA IRP Animal Care and Use Committee, in accordance with NIH guidelines.

Hippocampal slice preparation

Mice were anesthetized with isoflurane and euthanized by decapitation. The brain was removed and placed in a beaker containing modified ice-cold artificial cerebrospinal fluid (aCSF) containing, in millimolar, N-methyl-D-glucamine, 93; KCl, 2.5; NaH2PO4, 1.2; NaHCO3, 30; HEPES, 20; glucose, 25; sodium pyruvate, 3; MgCl2, 10; CaCl2, 0.5; and ascorbic acid, 5. Brain tissue was blocked and glued to the stage of a vibrating tissue slicer (Leica VT1200S, Leica Biosystems Nussloch, Germany) and submerged in modified aCSF. Transverse hippocampal slices (280 μm) were obtained and stored in standard aCSF containing, in millimolar, NaCl, 126; KCl, 3; CaCl2, 2.4; MgCl2, 1.5; NaH2PO4, 1.2; NaHCO3, 26; and glucose, 11. Slices were warmed in aCSF for 20–25 minutes at 35°C immediately after cutting and then allowed to gradually equilibrate to room temperature.
temperature for at least 45 minutes prior to initiating recording. All solutions were oxygenated with 95% O2/5% CO2. We have previously found that endogenous adenosine can disrupt CB1R-mediated inhibition of glutamate release in the hippocampus (Hoffman et al. 2010). Therefore, the selective adenosine A1 receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 200 nM), was included in the aCSF throughout incubation and recordings. On average, three to four slices were used from a single animal per day. All drugs were tested in slices obtained from at least two subjects.

**Electrophysiological recording**

Brain slices containing the hippocampus were submerged in aCSF in a slice chamber (RC-26, Warner Instruments, Hamden, CT, USA) and continuously perfused with aCSF (2 ml/minute) using a peristaltic pump (Cole-Parmer Instruments, Vernon Hills, IL, USA). Bath temperature was maintained at 30–32°C using an in-line solution heater (TC324-C and SH27-B, Warner Instruments). Borosilicate glass electrodes (1.5 mm o.d. × 0.86 mm i.d., Sutter Instruments, Novato, CA, USA) were fabricated using a horizontal puller (P-97, Sutter Instruments) and filled with aCSF. Electrodes were connected to the headstage of an AC amplifier (Model 1800, A-M Systems, Sequim, WA, USA). Under stereomicroscopic visualization, a bipolar-stimulating electrode consisting of twisted formvar-insulated nichrome wire (50 μm; A-M Systems) connected to a constant current stimulus isolation unit (DS3, Digitimer) was positioned in hippocampal area CA3 to activate Schaffer collateral fibers using 0.1-millisecond pulses. The recording electrode was manually positioned in CA1 stratum radiatum using a micromanipulator and gradually lowered while monitoring the field excitatory postsynaptic potential (fEPSP) response. Once the optimal position was determined, stimulus intensity was varied to produce an input–output curve. Baseline responses were then set by adjusting the stimulus intensity to achieve 30–50% of the maximum response. In some experiments, paired pulse stimuli were delivered at an interval of 50 milliseconds. Stimulation, data acquisition and signal analyses were performed on-line using an A/D board (PCIe-6321, National Instruments, Austin, TX, USA) and WinLTP software (www.win-ltp.com; Anderson & Collingridge 2007). Responses were obtained every 30 seconds, and drugs were applied after obtaining at least 10 minutes of stable baseline recording. High-frequency stimulation (HFS) consisted of three consecutive 100-Hz, 1-second trains, delivered 10 seconds apart. A three-way valve was used to switch between control aCSF and drug-containing aCSF. In most cases, drugs were applied for 40–60 minutes. To maintain consistent exposure times to cannabinoid agonists in long-term potentiation (LTP) experiments, brain slices were pre-treated for 90 minutes in the holding chamber (Collins et al. 1994, 1995). Thereafter, recordings were performed in either standard aCSF or aCSF containing the CB1 agonist PIMSR1 ((5-(4-Chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-[(E)-piperidinoiminomethyl]-1H-pyrazole)). The perfusion apparatus was washed with ethanol for 10–15 minutes between recordings.

**Chemicals and drug solutions**

1-Pentyln-3-(1-naphthoyl)-indole (JWH-018), (1-(5-fluoropentyl)-1H-indol-3-yl)(naphthalen-1-yl)methanone (AM-2201) and (1-(5-fluoropentyl)-1H-indol-3-yl)(2,2,3,3-tetramethylcyclopropyl)methanone (XLR-11) were supplied as dry powders by NIDA Drug Supply Program (Rockville, MD, USA). Stock solutions (1–10 mM) were prepared in dimethyl sulfoxide and frozen at −20°C until thawed for experiments. Δ9-THC (200 mg/ml in EtOH) was provided through NIDA Drug Supply and was diluted to 10 mM in dimethyl sulfoxide. PIMSR1 was generously provided by Dr Herbert Selzman (Research Triangle International, Research Triangle Park, NC, USA). AM251 and DPCPX were purchased from Tocris (Minneapolis, MN, USA). All drugs were dissolved to their final concentration in aCSF prior to each experiment. All other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

**Data analysis and statistics**

Data are presented as mean ± standard error of the mean. Comparisons were made using t-tests or ANOVA where appropriate, with a critical value for statistical significance set at P < 0.05. A Holm–Šidák’s multiple comparisons test was used to measure the mean level of LTP between 55 and 60 minutes following HFS. Drug responses were defined as the change in the fEPSP slope at the time of the peak drug effect, typically 40 or 60 minutes after drug application. Prior experiments with cannabinoids have established that the relatively long drug onset time reflects partitioning of these highly lipophilic molecules into the brain slice. Responses were normalized to the baseline recording period. Dose–response curves were generated using a three-parameter, global curve fitting nonlinear regression algorithm in Prism (GraphPad Scientific, San Diego, CA, USA):

$$ Y = \frac{\text{Bottom} + (\text{Top} – \text{Bottom})/(1 + 10^\left(\frac{(\text{LogEC}_{50} – X)}{\text{slope}}\right))}{\text{Bottom} + (\text{Top} – \text{Bottom})/(1 + 10^\left(\frac{(\text{LogEC}_{50} – X)}{\text{slope}}\right))}, $$

where Bottom and Top represent the plateaus and EC50 represents the agonist concentration that produces a response halfway between the top and bottom of the curve. The bottom was constrained to a value of 0. A global curve fit was first used to determine whether the top
plateau differed among the family of curves (JWH-018, AM2201, Δ⁹-THC and XLR-11). An additional sum of squares F-test revealed that the maximum plateaus did not significantly differ (F₃,₈₂ = 1.203, P = 0.3139). In addition, a one-way ANOVA revealed no significant differences in the maximal inhibition produced by each compound (F₁,₁₇ = 0.7656, P = 0.5289). Thus, the Top was allowed to be shared as a single value for all data sets, and the EC₅₀ values were calculated.

RESULTS

Inhibition of glutamate release by Δ⁹-tetrahydrocannabinol

The cannabinoids found in synthetic marijuana preparations are presumably used as substitutes for the phytocannabinoid Δ⁹-THC because they may mimic some of its pharmacological properties in the brain. Therefore, we first defined the effects of Δ⁹-THC on excitatory synaptic transmission in the hippocampus in vitro. Glutamatergic fEPSPs, elicited by stimulation of Schaffer collateral axons, were recorded in area CA1 in transverse mouse hippocampal slices. Bath application of Δ⁹-THC caused a concentration-dependent reduction of fEPSP rising slope (Fig. 2a), and this was completely reversed by the neutral CB1 antagonist PIMSR1 (Hurst et al. 2006; Fig. 2b; n = 7, 99 ± 4% of control, P = 0.9534, paired two-tailed t-test). The maximal inhibition of fEPSPs was 39 ± 6% (n = 7), with an EC₅₀ of 707 nM (95% CI = 333–1053 nM). These effects of Δ⁹-THC are comparable with those obtained at GABAergic synapses in mouse hippocampus in our lab (EC₅₀ 1.2 μM, 43 ± 2% maximal inhibition; Laaris et al. 2010).

Inhibition of glutamate release by designer cannabinoids

Among the first generation of synthetic cannabinoid compounds isolated from confiscated synthetic marijuana products was JWH-018 (Uchiyama et al. 2010; Wiley et al. 2014b). This compound was previously shown to inhibit glutamate release at hippocampal synapses in culture (Atwood et al. 2010). Consistent with these results, JWH-018 inhibited fEPSPs in mouse brain slices (Fig. 3a), and its EC₅₀ (Fig. 3b; 14.0 nM; 95% CI = 6–35 nM) was remarkably similar to that observed in cultured hippocampal neurons (14.9 nM; Atwood et al. 2010). The maximal inhibition was 46 ± 7% (n = 5) at 100 nM, and this was fully reversed by PIMSR1 (Fig. 4c; n = 3, 97 ± 6% of control, P = 0.184, two-tailed paired t-test).

Following the placement of JWH-018 on the US Drug Enforcement Agency Schedule I list in March 2011 (Drug Enforcement Administration, USA, Department of Justice 2011), a second generation of synthetic cannabinoids began to appear in confiscated products. Among these, a fluoropropyl derivative of JWH-018 known as AM2201 that binds to the CB1R with low nanomolar affinity (Deng & Makriyannis 2005) was detected in increasing amounts (Fig. 1). We observed that AM2201 also potently inhibited fEPSPs, with an EC₅₀ slightly higher than JWH-018 (Fig. 3c,g; 59 nM; 95%
CI = 24–145 nM), and generated a maximal inhibition of 53 ± 7% at 200 nM (n = 6; Fig. 3d). Consistent with a presynaptic site of action, AM2201 significantly enhanced the paired pulse ratio in response to paired stimuli (Fig. 4b; n = 6, P = 0.01, two-tailed paired t-test). The effect of AM2201 was reversed by bath application of the CB1R antagonist/inverse agonist AM251 (Fig. 4a; n = 4, 118 ± 18% of control, P = 0.3825, two-tailed paired t-test), and it had no effect on glutamate release in slices obtained from CB1R knockout mice (Fig. 4b; n = 6, P < 0.001, t = 6.246, d.f. = 10, unpaired t-test versus control).

By the end of the year 2012, a new designer cannabinoid-like compound was increasingly isolated from confiscated synthetic marijuana products. This synthetic compound was structurally novel and referred to as XLR-11 [(1-(5-fluoropentyl)-1H-indol-3-yl)(2,2,3,3-tetramethylcyclopropyl)methanone] (Seely et al. 2013;...
The deleterious effects of cannabinoids on learning and memory are well established in humans (Abel 1970) and in animal models (Wise et al. 2009; Han et al. 2012). It is hypothesized that the ability of cannabinoid agonists to impair hippocampal long-term potentiation may play a role in the cognitive impairments produced by these drugs (Misner & Sullivan 1999; Hoffman et al. 2007; Abush & Akirav 2010; Basavarajappa & Subbanna 2014; Navakkode & Korte 2014). We therefore evaluated the effects of the synthetic cannabinoids on hippocampal LTP and compared these effects with those of Δ⁹-THC. In untreated, control slices ($n=16$), delivery of HFS (3 × 100-Hz trains) resulted in reliable, stable potentiation of fEPSP slopes (Fig. 5a and b; 143 ± 7% of baseline). In contrast, LTP was absent in slices incubated for 90 minutes in 200 nM JWH-018 (Fig. 5a and b; $n=11$, 90 ± 7% of baseline, $P=0.0003$ versus control). This ability of JWH-018 to block LTP was prevented in another group of slices when the agonist treatment was followed by application of the CB1R antagonist PIMSR1 (2 μM), beginning 30 minutes prior to HFS (Fig. 5a and b; $n=5$, 127 ± 19% of baseline, $P=0.3320$ versus control). As shown in Fig. 5b, slices incubated for 90 minutes with AM-2201 (200 nM; $n=11$), XLR-11 (1 μM; $n=14$) or Δ⁹-THC (1 μM; $n=13$) also showed significantly reduced LTP (AM2201, 105 ± 12% of baseline, $P=0.0076$ versus control; XLR-11, 103 ± 8% of baseline, $P=0.0035$ versus control; Δ⁹-THC, 118 ± 7% of baseline, $P=0.0416$ versus control). Together, these results suggest that, in addition to their ability to acutely depress glutamatergic transmission, the synthetic cannabinoids present in ‘Spice’ preparations also limit synaptic plasticity in the hippocampus.

**DISCUSSION**

The present study compares the effects of several synthetic cannabinoids recently identified in psychoactive Spice formulations, using a functional synaptic response
The high potency of JWH-018 and AM2201 in inhibiting excitatory transmission in cultured hippocampal neurons (Atwood et al. 2006) is also in agreement with a previous report obtained in drug discrimination assays (Baumann et al. 2014; Järbe & Gifford 2014; Wiley et al. 2014a). XLR-11 is structurally distinct from JWH-018 and AM2201, having been derived from a series of cyclopropylindoles synthesized for potential use as cannabinoid 2 receptor agonists (Frost et al. 2010). This compound, which is emerging as a major component of Spice-related substances (U.S. Drug Enforcement Administration Office of Diversion 2013), has been reported to bind to CB1Rs, with slightly greater affinity than Δ⁹-THC, and to also substitute for Δ⁹-THC in a mouse drug discrimination paradigm (Wiley et al. 2013). Our results suggest that XLR-11 is nearly equivalent to Δ⁹-THC in inhibiting glutamate release via CB1Rs but that both compounds are far less potent than the synthetic constituents found in earlier synthetic marijuana formulations. Based on these observations, it is tempting to speculate that the adverse physiological and psychological effects often associated with use of synthetic marijuana formulated with higher-affinity CB1R agonists (Winstock & Barratt 2013) may be driving the demand for a synthetic drug with properties closer to those of Δ⁹-THC. Correspondingly, the incidence of adverse reactions may be smaller with lower-affinity synthetic cannabinoids.

Despite the differences in potency among the compounds tested, we did not observe statistically significant differences in the maximum inhibition (efficacy) of glutamatergic synaptic transmission. This was surprising for Δ⁹-THC, because previous studies have suggested that it is a partial agonist at CB1Rs in G-protein activation assays (Sim et al. 1996; Burkey et al. 1997) and at inhibition of synaptic responses in cultured hippocampal neurons (Shen & Thayer 1999; Straiker & Mackie 2005). Interestingly, the study by Shen and Thayer reported that Δ⁹-THC suppressed excitatory transmission by ~60% (Shen & Thayer 1999), which is typically the maximum inhibition seen by cannabinoid agonists in vitro and is greater than the effect observed in the present study. We have previously demonstrated that Δ⁹-THC displays full agonist properties at CB1Rs on hippocampal GABAergic terminals (Laaris et al. 2010). However, because tonic adenosine levels limit Δ⁹-THC effects at hippocampal glutamatergic, but not GABAergic synapses

CB1R antagonists AM251 or PIMSR1, consistent with the expression of CB1Rs on hippocampal glutamate axon terminals (Kawamura et al. 2006). The potency of JWH-018 is also in agreement with a previous report obtained in cultured hippocampal neurons (Atwood et al. 2010). The high potency of JWH-018 and AM2201 in inhibiting the synaptic response is consistent with the high affinity of these compounds at CB1Rs previously reported in receptor binding assays (K_i = 9 and 1 nM, respectively; Carroll et al. 2012), as well as with the ability of low doses of these compounds to substitute for Δ⁹-THC in drug discrimination assays (Baumann et al. 2014; Järbe & Gifford 2014; Wiley et al. 2014a).
mechanism through which other CB1 agonists have been of these compounds at CB1Rs. Indeed, this is the general ing suppression of glutamatergic transmission by actions (Basavarajappa & Subbanna 2014; Collins in vivo both cannabinoid-mediated inhibition of synaptic plasticity. This is consistent with prior work demonstrating cannabinoids-mediated inhibition of synaptic plasticity both in vivo (Abush & Akirav 2010) and in vitro (Basavarajappa & Subbanna 2014; Collins et al. 1994; Misner & Sullivan 1999; Navakkode & Korte 2014). In our studies, slices were exposed to each agonist for 90 minutes, followed by evaluation of LTP in agonist-free aCSF. This was performed in order to standardize the exposure time to the agonists, similar to previous studies (Collins et al. 1994, 1995). The slow-onset time of these agonists to inhibit glutamatergic transmission, coupled with the limited washout of these lipophilic compounds, suggests that they were present in the tissue during the recordings. Consistent with this, PIMSR1 prevented the effect of JWH-018 on LTP when applied immediately following the 90-minute pre-treatment period and 30 minutes prior to HFS. Thus, the most parsimonious explanation of our data is that reduced LTP reflects ongoing suppression of glutamatergic transmission by actions of these compounds at CB1Rs. Indeed, this is the general mechanism through which other CB1 agonists have been demonstrated to block LTP (Collins et al. 1994, 1995; Misner & Sullivan 1999; Sullivan 2000). The ability of these and other Spice compounds to disrupt hippocampal LTP is likely to have important consequences for learning and memory (Pastalkova et al. 2006; Whitlock et al. 2006; Compton et al. 2012; Basavarajappa & Subbanna 2014). We have previously demonstrated that repeated exposure to Δ9-THC disrupts hippocampal LTP and alters signaling at both glutamatergic and GABAergic synapses (Hoffman et al. 2007). Although we have not yet examined the effects of these compounds on GABAergic axon terminals in the hippocampus, where CB1Rs are also widely expressed (Katona et al. 1999; Hoffman & Lupica 2000; Dudok et al. 2014), the expected inhibition of GABAergic function likely also contributes to disrupted network activity in the hippocampus, leading to deficits in cognitive function (Hajos et al. 2000; Puighermanal et al. 2009). In addition, it is possible that the in vivo effects of these compounds will reflect the activity of both the parent compound and its metabolites, several of which retain strong biological activity at CB1Rs (Brents et al. 2011; Fantegrossi et al. 2014). Overall, the present findings demonstrate that synthetic cannabinoids most often found in psychoactive synthetic marijuana products have profound effects on hippocampal neurotransmission and are much more potent than Δ9-THC at activating CB1Rs. This much higher affinity for CB1Rs by synthetic cannabinoids, as compared with Δ9-THC, is likely to prolong the period in which this receptor is activated, thereby extending the duration of behavioral and psychological effects of these drugs. As CB1Rs are ubiquitously expressed in the human brain and extensively engaged by these agonists, the notion that synthetic cannabinoids are benign versions of naturally occurring marijuana is not supported. Given the continued widespread use of these marijuana-like products, especially by young people, future studies are warranted to elucidate the acute and chronic effects of synthetic cannabinoids on mood, learning and memory, and other physiological processes across development.

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Disclosure/Conflict of Interest
The authors declare no conflict of interest.

Author contributions
CRL and AFH designed the experiments. MDL, JRK and CES performed the experiments and analyzed data. AFH, MHB and CRL wrote the manuscript. All authors critically reviewed the content and approved the final version of the manuscript.

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