Role of Adenosine and N-Methyl-D-aspartate Receptors in Mediating Haloperidol-Induced Gene Expression and Catalepsy

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Accepted for publication July 19, 1999

ABSTRACT

Acute blockade of dopamine D2 receptors by the typical antipsychotic drug haloperidol leads to alterations in neuronal gene expression and behavior. In the dorsolateral striatum, the levels of mRNA for the immediate-early gene c-fos and the neuropeptide gene neurotensin/neuromedin N (NT/N) are significantly increased by haloperidol. An acute behavioral response to haloperidol is catalepsy, considered to be a rodent correlate of some of the immediate extrapyramidal motor side effects seen in humans. Several lines of evidence suggest a link between neurotensin induction in the dorsolateral striatum and catalepsy. We hypothesize that both striatal gene induction and catalepsy elicited by haloperidol arise from the combined effect of excitatory adenosinergic and glutamatergic inputs acting at adenosine A2A and N-methyl-D-aspartate (NMDA) receptors, respectively. In agreement with our previous reports, adenosine antagonists reduced haloperidol-induced c-fos and neurotensin gene expression as well as catalepsy. In agreement with other reports, the noncompetitive NMDA receptor antagonist MK-801 also reduced gene expression and catalepsy in response to haloperidol. The competitive NMDA receptor antagonist LY235959 decreased haloperidol-induced catalepsy. We show here that blocking both A2A and NMDA receptors simultaneously in conjunction with haloperidol resulted in a combined effect on gene expression and behavior that was greater than that for block of either receptor alone. Both c-fos and NT/N mRNA levels were reduced, and catalepsy was completely abolished. These results indicate that the haloperidol-induced increases in c-fos and NT gene expression in the dorsolateral striatum and catalepsy are driven largely by adenosine and glutamatergic inputs acting at A2A and NMDA receptors.

Activation or inhibition of dopamine receptors in the striatum can lead to a vast array of gene and behavioral responses; however, the mechanisms that regulate these responses are still relatively unknown. Most antipsychotic drugs interfere to some extent with the actions of dopamine in the brain as antagonists of the dopamine D2 receptor family (Baldessarini, 1990). Haloperidol (HAL), a prototypic antipsychotic drug, is primarily a D2 receptor antagonist and can elicit extrapyramidal motor side effects (EPS) in humans. In rodents, an analogous response to HAL is the behavior catalepsy, which is defined as a reduced ability to initiate movements. Understanding the signal transduction pathways that underlie HAL-mediated alterations in gene expression as well as the behavior catalepsy will provide insights into the molecular mechanisms through which HAL causes EPS as well as exerts its therapeutic actions.

In rodents, HAL administration in vivo leads to an induction of the immediate-early gene c-fos in striatopallidal neurons and a subsequent induction of the gene for the neuropeptide neurotensin (Merchant and Dorsa, 1993). This effect is dependent on antagonism of D2 receptors because D2 agonists attenuate the effect of HAL (Miller, 1990). c-fos expression is colocalized with neurotensin/neuromedin N (NT/N) mRNA (Merchant and Miller, 1994b) and is necessary for the NT/N response (Merchant, 1994a). Neurotensin is a 13-amino acid peptide and has been suggested to be an endogenous neuroleptic. Injected intracerebroventricularly, it causes catalepsy (Nemeroff et al., 1983). A strong correlation can be made between the induction of NT/N mRNA in the dorsolateral striatum (DLSt) by antipsychotic drugs and their ability to cause EPS (Merchant and Dorsa, 1993). Thus, the induction of NT/N mRNA by HAL in the DLSt may play
a role in mediating cataleptic behavior and potentially the motor side effects seen in humans taking HAL.

The seven-transmembrane D2 receptor is negatively coupled to adenylyl cyclase via Gi, and endogenous dopamine acts to suppress the activity of adenylyl cyclase in the D2 receptor-containing medium spiny neurons (Missale et al., 1998). HAL presumably relieves this inhibitory effect of Gi, on adenylyl cyclase. Hence, one possible explanation for the induction of gene expression by HAL would be an “unmasking” of Gi-coupled inputs. Several Gi-coupled receptors are colocalized with D2 receptors on striatopallidal neurons, and activation of these receptors in the presence of HAL could lead to gene induction and behavior. The adenosine A2A receptor, for example, has a striatal expression pattern mimicking that of D2 receptors (Ferre et al., 1997), and selective pharmacological blockade of the A2A receptor has been shown to reduce HAL-induced c-fos immunoreactivity (Boegman and Vincent, 1996) and NT/N mRNA levels, as well as catalepsy (Ward and Dorsa, 1999). The antagonism between A2A and D2 receptors has been well described (Ferre et al., 1997; Fredholm et al., 1997) and is thought to arise either from competing effects of Gi and Go or from an actual intramembrane interaction of A2A and D2 receptors involving the formation of heterodimers (Fuxe et al., 1998). Interestingly, A2A antagonists only partially attenuate the effects of HAL, implicating inputs in addition to A2A receptor activation.

Recently, much attention has been given to the interaction between dopaminergic and glutamatergic pathways in the striatum and prefrontal cortex. The striatum receives a large glutamatergic input from cortical areas, with the DLST receiving primarily motor information (Gerfen and Wilson, 1996), and it is thought that dopamine serves to modulate this input. Striatopallidal neurons express a variety of glutamate receptors, including the N-methyl-D-aspartate (NMDA) receptor. NMDA antagonists have been shown to inhibit c-fos induction by amphetamine (Torres and Rivier, 1992), and NMDA itself can induce c-fos expression when injected directly into the striatum (Berretta et al., 1992). Less is known about the role of NMDA receptors in mediating the function of the D2 striatopallidal neurons. Studies have shown that NMDA receptor antagonists reduce the induction of c-fos protein by HAL in the striatum (Ziolkowska and Holtt, 1993; Boegman and Vincent, 1996).

In the present study, we tested the hypothesis that concomitant activation of both the A2A and NMDA receptors is necessary for maximal c-fos and NT/N mRNA induction by HAL, as well as for induction of the behavior catalepsy. The systemic treatment of rats with both A2A and NMDA receptor antagonists in conjunction with HAL resulted in a slight but insignificant decrease in c-fos mRNA levels compared with blocking either receptor alone, an additive decrease in the levels of HAL-induced NT/N mRNA, and an abolition of catalepsy. Our results suggest that the A2A and NMDA receptors activate a common pathway that leads to the parallel induction of a modulatory neuropeptide gene response and behavior.

Materials and Methods

Animals and Drug Treatments. Adult male Sprague-Dawley rats (250–300 g; B & K Universal, Edmonds, WA) were housed two per cage in a temperature-controlled environment with a 12-h light/dark cycle and were given free access to standard laboratory chow and water. The animals were allowed to habituate for 1 week before conducting the study. To determine the role of NMDA and adenosine receptors in mediating catalepsy and the induction of c-fos and NT/N mRNA in the DLST by HAL, the following drugs were used: HAL (1 mg/kg i.p.; SoloPak Laboratories, Inc., Franklin Park, IL), 8-(3-chloroaryl)-caffeine (CSC; Research Biochemicals, Inc., Natick, MA), (+)-MK-801 hydroxide maleate (0.1 mg/kg i.p.; Research Biochemicals, Inc.), and LY235959 (2.5 mg/kg i.p.; Torcis Cookson, St. Louis, MO). The doses of HAL and CSC used were based on previously reported work (Ward and Dorsa, 1999). A dose-response pilot was conducted to determine the minimum doses of MK-801 and LY235959 that significantly reduced HAL-induced catalepsy without noticeably altering basic motor functions when administered alone. MK-801 was dissolved in 0.9% saline, LY235959 was dissolved in water, and CSC was in a vehicle consisting of a 20:80 v/v mixture of Alkamuls EL-620 (Rhone-Poulenc, Cranbury, NJ) and PBS (Jacobson et al., 1993). The treatment groups of the first study using MK-801 were: 1) saline, 2) HAL, 3) CSC plus HAL, 4) MK-801 plus HAL, and 5) MK-801 plus CSC plus HAL. To maximize the effectiveness of the noncompetitive NMDA antagonist, MK-801, it was administered 20 min before the other drugs. The treatment groups of the second catalepsy study using LY235959 were: 1) saline, 2) HAL, 3) CSC plus HAL, 4) LY235959 plus HAL, and 5) LY235959 plus CSC plus HAL. To maximize the effectiveness of LY235959, it was administered 15 min before the other drugs.

Catalepsy Analysis. Fifty minutes after the administration of HAL or saline, catalepsy was measured using a standard bar test (Sanberg et al., 1988). Briefly, the rats' forepaws were placed on an elevated wooden rod (height from table, 10 cm; rod diameter, 2 cm), and the latency to removal of the forepaws from the rod was recorded. The test was repeated for each rat until the rats' forepaws remained on the bar for at least 10 s or for a maximum of three attempts. Catalepsy was measured for 5 min, and an animal was scored as cataleptic if both forepaws remained on the bar for at least 1 min. Of 85 rats tested for catalepsy in this study, only 6 had a latency to removal of the forepaws from the bar of more than 1 min but less than 5 min. In other words, the animals either were or were not cataleptic, with no significant distribution of latencies between 1 and 5 min. Therefore, the data are presented as the percentage of animals in a treatment group that are cataleptic. Behavioral analysis was carried out over a period of 5 days, and the order of treatments was cycled such that each treatment group was represented at least once per day as a control for potential between-day variabilities.

In Situ Hybridization Histochemistry. Previous work has shown that c-fos mRNA levels in the striatum 30 min after HAL injection but are still significantly elevated after 1 h (Merchant et al., 1992a). Likewise, NT/N mRNA levels peak at approximately 6 to 7 h after HAL but are significantly elevated as early as 1 h after HAL (Merchant and Dorsa, 1993). The cataleptic response to HAL peaks at approximately 1 h (Adams et al., 1997); hence, a 1-h time point was chosen to analyze both gene expression (c-fos and NT/N) as well as behavior. Thirty minutes after the administration of HAL or saline (immediately after the 5-min catalepsy test), rats were sacrificed by decapitation. The brains were quickly removed and placed on dry ice to freeze. Details of the in situ hybridization assay have been published previously (Merchant et al., 1992b; Merchant and Dorsa, 1993). Briefly, frozen brains were sectioned coronally on a cryostat and thaw mounted on Fisherbrand Superfrost microscope slides. Multiple sets of matched sections were cut from bregma 2.70 to 0.48 mm (Paxinos and Watson, 1986). Tissue sections were kept frozen at −80°C until processed as follows. Sections were thawed to room temperature, fixed in paraformaldehyde, acetylated, delipidated, and dehydrated. For a given riboprobe, a complete set of slides from all animals were processed simultaneously. To detect NT/N mRNA, an antisense RNA probe labeled with 35S-UTP was transcribed with T7 RNA polymerase from an EcoRI-linearized prNT4 template.
pared with HAL (junction with HAL reduced NT/N mRNA levels by 24% compared with HAL reduced NT/N mRNA levels by 37% compared with receptor antagonist MK-801 (0.1 mg/kg) in conjunction with 1.0 mg/kg MK-801 (0.1 mg/kg) plus HAL reduced levels of NT/N mRNA in the DLSt of the CSC plus MK-801 (0.1 mg/kg) group were significantly less than those of MK-801 alone (0.1 mg/kg) plus HAL. Interestingly, CSC failed to further reduce NT/N mRNA levels when administered in conjunction with 1.0 mg/kg MK-801 (data not shown).

To test for the specificity of action of CSC and MK-801, NT/N mRNA levels were also quantified in the lateral septum. There was no significant difference in mRNA levels in this region among the saline control, HAL, HAL plus MK-801 (0.1 mg/kg), HAL plus CSC, and HAL plus CSC plus MK-801 (0.1 mg/kg) groups (data not shown).

Effects of Adenosine and NMDA Receptor Antagonists on HAL-Induced c-fos mRNA Expression. The increase in NT/N mRNA in the DLSt by HAL is preceded by the induction of the immediate-early gene c-fos. We hypothesized that the signaling mechanisms by which A2A and NMDA receptor activation lead to NT/N mRNA induction and the behavior catalepsy in response to HAL occur via activation of c-fos. To test this hypothesis, we measured the effects of CSC and MK-801 on HAL-induced c-fos mRNA levels in the DLSt by in situ hybridization 1 h after either saline or HAL injection. As shown in Figs. 3 and 4, c-fos mRNA levels are...
increased 7-fold compared with saline controls in the DLSt. This is in agreement with previous work from our laboratory (Merchant and Dorsa, 1993). CSC (2 mg/kg) attenuates c-fos by 28% (P < .005), whereas MK-801 (0.1 mg/kg) does not significantly reduce c-fos mRNA compared with HAL treatment alone (P < .06). Previous work in our laboratory demonstrated that 2 mg/kg CSC failed to significantly reduce c-fos induction by HAL (Ward and Dorsa, 1999). The discrepancy in findings between our results probably reflects the difficulty in solubilization of CSC and hence the difficulty in administering identical doses of CSC from experiment to experiment. The simultaneous blockade of NMDA and A2A receptors in conjunction with HAL reduces c-fos induction by 42% compared with HAL alone (P < .0005). However, this is not significantly different from the level of c-fos mRNA in either the MK-801 (0.1 mg/kg) plus HAL or the CSC plus HAL treatment groups.

In a control experiment, varying doses of MK-801 were injected alone or in combination with HAL. In a concentration-dependent manner, MK-801 reduced the level of c-fos mRNA induction by HAL, whereas MK-801 alone had no effect on c-fos mRNA levels in the DLSt (data not shown). The highest dose of MK-801 used (1 mg/kg) reduced HAL-induced c-fos to the same level as that seen with CSC and MK-801 (0.1 mg/kg) in conjunction with HAL. CSC failed to further reduce c-fos levels with the high dose of MK-801 (data not shown).

**Effects of Adenosine and NMDA Receptor Antagonists on HAL-Induced Catalepsy.** Before analyzing the DLSt for NT/N and c-fos mRNA, catalepsy was measured. Two experiments were done with two separate groups of naïve rats. The first experiment used the noncompetitive NMDA receptor antagonist MK-801. Treatment groups included saline (n = 4), HAL (n = 14), HAL plus CSC (n = 12), HAL plus MK-801 (n = 11), and HAL plus CSC plus MK-801 (n = 8). The second experiment used the competitive NMDA receptor antagonist LY235959. Treatment groups included saline (n = 4), HAL (n = 7), HAL plus CSC (n = 5), HAL plus LY235959 (n = 6), and HAL plus CSC plus LY235959 (n = 7). Figure 5 shows the percentage of animals in each group that were cataleptic. Both CSC and MK-801 (0.1 mg/kg) reduced HAL-induced catalepsy approximately 50%. LY235959 (2.5 mg/kg) reduced catalepsy to 33%. Remarkably, the concomitant administration of CSC and MK-801 (0.1 mg/kg) or of CSC and LY235959 (2.5 mg/kg) completely abolished HAL-induced catalepsy. The behavior of the CSC plus MK-801
plus HAL animals was not distinguishable from that of saline-treated controls; however, the CSC plus LY235959 plus HAL animals had lower motor control, although in each case, they actively got down from the catalepsy bar. In this study, a control experiment was performed to assess the effect of increasing concentrations of MK-801 on HAL-induced catalepsy. The lowest dose tested (0.025 mg/kg) did not reduce catalepsy, whereas the highest dose tested (1.0 mg/kg) resulted in fewer than 20% of the animals being cataleptic (data not shown). However, this high dose of MK-801 (but not the other doses) resulted in an apparent sedation and loss of muscle tone in the animals.

Discussion

The results of the present study support our hypothesis that the induction of gene expression and the behavior catalepsy by HAL are dependent on the activation of both A2A and NMDA receptors in the striatum. By pharmacologically antagonizing the activation of A2A or NMDA receptors with low doses of either CSC or MK-801, respectively, the induction of c-fos and NT/N mRNAs by HAL was reduced. Likewise, the percentage of animals exhibiting catalepsy was reduced to 50% from almost 100%. The competitive NMDA receptor antagonist LY235959 also reduced HAL-induced catalepsy to 33 from 86% with HAL alone. The simultaneous

Fig. 4. Quantification of the effects of CSC and MK-801 on c-fos mRNA expression in the DLSt. ROD of the DLSt was obtained by digitizing autoradiographic sections using MCID. Two sequential coronal sections were analyzed per animal, and the columns represent the average ROD for a treatment group plus S.E.M. Number of animals per treatment group were 4 for saline (Veh), 10 for HAL (Hal; 1 mg/kg), 8 for HAL plus CSC (CSC; 2 mg/kg), 6 for HAL plus MK-801 (MK-801; 0.1 mg/kg), and 6 for HAL plus MK-801 plus CSC (MK-801 + CSC). **P < .005, ***P < .0005 compared with HAL-alone treatment (ANOVA followed by Fisher’s Protected Least Significant Difference).

Fig. 5. Effects of CSC and NMDA receptor antagonists on HAL-induced catalepsy. Rats were treated with CSC and either MK-801 or LY235959 alone or in combination with HAL (as described in Materials and Methods), and catalepsy was measured 50 min after HAL or saline injection. An animal was scored as cataleptic if its forepaws remained on the bar for at least 1 min. This figure represents the percentage of animals scored as cataleptic. A, experiment 1 using MK-801: saline (Veh; n = 4), HAL (Hal; n = 14), HAL plus CSC (CSC; n = 12), HAL plus MK-801 (MK-801; n = 11), or HAL plus CSC plus MK-801 (MK-801 + CSC; n = 8). B, experiment 2 using LY235959: saline (Veh; n = 4), HAL (Hal; n = 7), HAL plus CSC (CSC; n = 5), HAL plus LY235959 (LY; n = 6), or HAL plus CSC plus LY235959 (LY + CSC; n = 7).
administration of CSC and MK-801 resulted in further inhibition of HAL-induced NT/N gene induction, suggesting an additive effect. Both CSC plus MK-801 and CSC plus LY235959 administered in conjunction with HAL reduced the percentage of animals exhibiting catalepsy to 0%.

The A2A receptor is colocalized on the same neurons as the D2 receptor, and this population of striatal medium spiny neurons expresses the neuropeptide enkephalin. Previous work has demonstrated that A2A antagonists can reduce the activation of c-fos by HAL (Boegman and Vincent, 1996), as well as NT/N gene induction and catalepsy, although only partially (Ward and Dorsa, 1989). Adenosine is a hydrolysis product of ATP and can be formed both intracellularly and extracellularly. Basal concentrations of adenosine in the brain are thought to be between 30 and 300 nM. At this concentration, A2A receptors are able to be activated (Fredholm et al., 1997). In the presence of HAL, D2 receptors would be inhibited, and the ratio of Gs to Gi activation in the striatopallidal neurons would be increased because of this tonic presence of adenosine, thus enabling gene induction. However, previous work in our laboratory has demonstrated that increasing the dose of CSC does not significantly reduce NT/N mRNA induction by HAL more than the 2 mg/kg dose used in this study (Ward and Dorsa, 1999). This suggests that inputs in addition to A2A receptor activation are necessary for the gene response to HAL.

It is important to note that although high doses of the general adenosine receptor antagonist caffeine can induce c-fos expression in the striatum (Johansson et al., 1994), the dose of the more specific A2A antagonist used in this study does not alter striatal c-fos or NT/N mRNA expression when administered alone.

The specific A2A antagonist CSC reduces NT/N mRNA induction by no more than 50%; therefore, changes in cAMP levels via activation of A2A receptors by endogenous adenosine can account for only a portion of the NT/N mRNA induction. We hypothesized that activation of the NMDA receptor was responsible for a portion of the gene response to HAL as well. The NMDA receptor is an oligomer of receptor subunits that combine to form a cationic ionophore. The activation of NMDA receptors in the striatum would lead to an increase in intracellular calcium levels, which in turn could play a role in multiple signaling pathways, leading to increases in gene expression (for a review, see Ghosh and Greenberg, 1995). It has been shown that there are presynaptic D2 receptors on corticostriatal nerve terminals (Filloux et al., 1988), which would function in an inhibitory manner to reduce the efflux of glutamate. Hence, HAL treatment would relieve this presynaptic inhibition, thus leading to increases in glutamate release. There is evidence that chronic HAL treatment leads to increases in extracellular levels of glutamate in the striatum as well as an up-regulation of NMDA receptor 1 subunit levels (Fitzgerald et al., 1995).

The dose of MK-801 chosen in this study (0.1 mg/kg) had no obvious effects on the animals' behavior when administered alone and did not significantly reduce the level of c-fos mRNA induction when administered before HAL. However, the decrease in NT/N mRNA levels and the decrease in catalepsy demonstrate the requirement for the NMDA receptor in sustaining a HAL-induced response. Although it is possible that MK-801 exerts its effects indirectly (i.e., at sites not within the striatum), previous work has shown that the direct application of MK-801 into the striatum reduces HAL-induced catalepsy (Ozer et al., 1997). Increasing the dose of MK-801 to 1 mg/kg further attenuated NT/N mRNA levels compared with the 0.1 mg/kg dose, although not to basal levels. The residual NT/N activation implies that other mechanisms are also necessary to explain the full NT/N gene response to HAL. The L-type Ca2+ channel has been implicated in mediating the response of D2 neurons to endogenous dopamine inputs (Hernandez-Lopez et al., 1997), and perhaps the blockade of D2 receptors by HAL unmasks excitatory input through these voltage-sensitive Ca2+ channels.

Previous work from this laboratory has documented a requirement for the enzyme protein kinase A (PKA) in the striatal response to HAL (Adams et al., 1997). Mice that are homozygous for a targeted deletion of the RI/β subunit of the PKA holoenzyme have a 75% reduction in striatal PKA activity. These mice fail to induce both c-fos and NT/N mRNA and fail to become cataleptic in response to HAL; therefore, it is likely that the input from the NMDA receptor converges on the PKA signaling pathway, possibly via activation of a Ca2+-sensitive adenylyl cyclase. Recent work has demonstrated that in PC12 cells, Ca2+-induced gene transcription requires PKA activation for translocation of the extracellular signal-related protein kinase to the nucleus (Impy et al., 1998). The extracellular signal-related protein kinase family of kinases is known to be activated by Ca2+ (Bading and Greenberg, 1991). The results of this study suggest that Ca2+ increases via activation of the NMDA receptor and that cAMP increases via activation of the A2A receptor act additively to increase NT/N mRNA levels in the striatum.

Blockade of both A2A and NMDA receptors completely abolished HAL-induced catalepsy in these rats. It is important to note that the failure of the rats to become cataleptic could not be attributed to sickness or sedation but rather to blockade of the neuronal inputs initiating catalepsy. The time course of activation of catalepsy and NT/N mRNA induction by HAL is not consistent with the theory that the induction of NT/N mRNA causes catalepsy. Cataleptic behavior is detectable up to 3 or 4 h after HAL, but it peaks at approximately 1 h. NT/N induction is maximal at 7 h but is detectable at significant levels as early as 1 h. We hypothesize that HAL results in a release of previously synthesized stores of neurotensin, and the increase in gene transcription reflects a subsequent replenishment of stores. Although it has been shown that direct injections of neurotensin into the ventricles result in catalepsy, there is insufficient proof that HAL-induced catalepsy is a result of neurotensin release. It would be of value to determine the effects of a neurotensin antagonist on HAL-induced catalepsy.

MK-801 acts at the NMDA receptor ion channel as an open channel blocker. MK-801 has also been shown to have effects at other gated ion channels such as the nicotinic acetylcholine receptor. To strengthen the argument that the NMDA receptor is mediating the effects of HAL, the competitive NMDA receptor antagonist LY235959 was used. In a separate experiment, rats were treated with HAL plus either CSC or LY235959 or HAL plus both CSC and LY235959. Just as with MK-801, LY235959 in addition with CSC completely abolished catalepsy.

It is interesting that the induction of c-fos in the DLSt by HAL is not very sensitive to changes in A2A and NMDA receptor inputs, whereas NT/N induction is clearly regulated.
by both receptor types in an additive manner. Finally, the expression of catalepsy appears to be the most sensitive to changes in $A_{DA}$ and NMDA receptor activation, either in an additive or potentially synergistic manner. This suggests that the neuronal inputs required for catalepsy are fewer and more specific than those required for striatal $c$-fos induction. Also, it is possible that NT/N and catalepsy require a certain threshold of neuronal activation, as measured by $c$-fos. A slight reduction in $c$-fos expression would therefore result in a dip below the required threshold for neuropeptide expression and behavior.

In summary, the results shown here demonstrate the importance of two neurotransmitter systems, adenosine and glutamate, in modulating the effects of the antipsychotic drug HAL in the striatum. An important future question is whether these receptors are also important for gene responses and behaviors of the nucleus accumbens, which is thought to be more directly involved in the therapeutic aspects of antipsychotic drug treatment.

**References**


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