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Early expression of D_3 dopamine receptors in murine embryonic development

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Abstract In order to determine whether the D_2 and D_3 dopamine receptors may have a role in prenatal development, we have studied the mRNA expression and distribution of these receptors during murine embryonic development. Using RT-PCR on RNA from embryos taken at progressive stages of development, we have shown that the D_3 receptor is expressed significantly earlier than the D_2 receptor, being detectable at day 9.5 post-conception (p.c.), compared with day 13.5 p.c. for the D_2 subtype. We have also examined the mRNA distribution of the two receptors by whole mount in situ hybridisation. In agreement with the PCR assays, the D_3 receptor was expressed earlier than the D_2 sybtype. D_3 receptor transcripts were first detected at day 9.5 p.c. in the ventral aspect of the anterior neural tube, whereas D_2 receptor transcripts first appeared a day later. By day 10.5–11.5 p.c. both D_3 and D_2 receptor transcripts were present in the developing forebrain, and later also in the branchial arches and along the prospective vertebral column. The early appearance of the D_3 subtype in murine development and its predominance over the D_2 subtype suggest that the D_3 receptor may have a functional role in prenatal development.

Key words: D_3 dopamine receptor; Embryo; Prenatal development; Whole mount in situ hybridization; Alternative splicing; G protein-coupled receptor

1. Introduction

The existence of multiple dopamine receptors was for many years postulated to underlie the complex behavioural and biochemical properties associated with dopaminergic neutrotransmission and dopamine receptor activation [1]. The identification and cloning of a number of dopamine receptor genes in the last few years has enabled a more detailed analysis of the functional roles played by the respective dopamine receptors than was previously possible. Two categories of dopamine receptor are currently recognised, termed D1 and D2[1], the latter in particular being associated with a number of neuropathological conditions (reviewed in [2]). While it is generally accepted that the cloned D_2, D_3 and D_4 subtypes are of the D2 type, their differential roles in brain function remain to be determined [3,4]. A further level of diversity is found in the D2 subclass by the presence of alternative splicing in the D_2 and D_3 subtypes, generating long and short isoforms of each receptor, termed D_{2L}, D_{2S}, D_{3L} and D_{3S} [5–8], but the significance of alternative splicing to receptor function is still not clear.

Studies examining the expression of D2-type receptors have shown that in the adult brain, the three subtypes exhibit distinct but overlapping distribution. The D_2 subtype is the most abundant of the three, being expressed most highly in the striatum and olfactory tubercle, and showing higher mRNA levels than either the D_3 or D_4 subtypes in all tissues where they are co-expressed. The D_3 and D_4 receptors are most highly expressed in limbic tissue [6,9,10], the D_3 sybtype being found as both an autoreceptor and a post-synaptic receptor.

The expression of dopamine receptors in pre- and post-natal development has previously been studied using autoradiography and radioligand binding on embryonic rat brain slices [11,12]. These have shown D2-type receptors to be expressed before the formation of synaptic connections in cortical and subcortical regions, but concomitantly with the expression of tyrosine hydroxylase, which serves as a marker for the presence of the neurotransmitter. This approach, however, observes the receptors at the protein level, and does not permit distinction between the different receptor subtypes or their alternatively spliced isoforms, which are all capable of binding the ligands used. Sutidies on whole brains from rat embryos have employed PCR to examine the expression of D2-type receptors, and have shown the D_2 receptor to be present at rat embryonic day 14 [13] and the D_3 receptor at rat embryonic day 11 [14], but did not examine the distribution of these receptors in the embryos.

The use of foetal implants for the treatment of neurodegenerative disorders such as Parkinson's disease, has created significant interest into the distribution and function of dopamine receptors in embryonic tissue. In particular, the isolation of a number of different dopamine receptor subtypes has generated a need to understand the respective roles these subtypes may play in the developing embryo. In this study, we have used PCR analysis and in situ hybridisation to examine and compare the expression and distribution of the D_2 and D_3 receptors in murine prenatal development. We have found that the D_3 subtype is expressed significantly earlier than the D_2 subtype, and suggest that at early stages of embryonic development it may represent the dominant D2-type receptor.

2. Materials and methods

2.1. Materials

All molecular biology reagents, unless stated otherwise, were purchased from Boehringer Mannheim (Mannheim, Germany). PCR on cDNA was performed using *Taq* polymerase, Promega. Oligonucleotides and PCR primers were prepared by the laboratory of oligonucleotide synthesis. [γ-^{32}P]dATP (3000 Ci/mmol) and [^{35}S]UTP (>1000 Ci/mmol) were purchased from Amersham (Aylesbury, England), and nitrocellulose membranes were purchased from Schleicher and Schuell, GmbH (Dassel, Germany). All other reagents, unless stated otherwise, were purchased from Sigma Chemical Co. (St. Louis, MO).

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[1]To avoid confusion, D2 shall be used to signify the subclass of D2-type receptors, defined by pharmacological and biochemical characteristics at the protein level, and a subscript shall be used to represent the cloned receptor subtypes, e.g. D_2, D_3 and D_4.

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2.2. RT-PCR and Southern analysis

For RNA extraction, embryos were isolated and frozen immediately in liquid nitrogen. Total RNA was prepared from whole embryos using the guanidium thiocyanate-caesium chloride method [15]. cDNA was prepared and PCR performed essentially as described previously [6]. Briefly, 15 μg total RNA was used for the generation of cDNA using oligo dT (New England Biolabs) and AMV Reverse Transcriptase (Promega), and the resulting cDNA was resuspended in 20 μl; 5 μl of this was taken for amplification by PCR with either D_2-specific (5′-CCTTCACCATCTCTTGC-3′ and 5′-CCTTCTGCTGGGAGAGC-3′) or D_3-specific primers (5′-CCGTTGCTGAGTTTTCGAACC-3′ and 5′-CCAGGTTTCTGTCAGATGCC-3′). 35 μl of PCR products were electrophoresed on 1.2% agarose gels and transferred to nitrocellulose membranes (Schleicher & Schuell). Hybridisation was performed with D_2-specific (5′-TGACAGTCCTGCCAAACC-3′) and D_3-specific (5′-CGGAACTCCTTGAGCCCCACCATGGCACCCAAGC-3′) oligonucleotide probes, end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase, by standard procedures [16].

2.3. Preparation of probes for in situ hybridisation

D_2- and D_3-specific cDNA probes as detailed in section 3 were subcloned into the vector pBluescript and linearised using Asp-718 and *XbaI* respectively. cRNA probes were generated by in vitro transcription using T7 and T3 RNA polymerase for the D_2 and D_3 receptors respectively, including 10 mM digoxin-UTP (DIG-UTP) for whole mount in situ hybridisation and [^{35}S]UTP for paraffin section in situ hybridisation.

2.4. Whole mount in situ hybridisation

Whole mount in situ hybridisation was performed essentially according to Conlon and Herrmann [17], with minor modifications. Briefly, embryos were isolated and immediately fixed for 2 h in fresh 4% paraformaldehyde (PFA) and bleached in H_2O_2/methanol. Following rehydration, embryos were treated with 20 μg/ml proteinase K, rinsed twice in 2 mg/ml glycine, refixed for 20 min in 0.2% glutaraldehyde/4% PFA/PBS, and treated for 20 min in sodium in sodium borohydride. Embryos were then prehybridised for 1 h at 63°C in hybridisation buffer (50% formamide, 0.75 M NaCl, 1×PE (10 mM PIPES pH 6.8, 1 mM EDTA), 100 μg/ml tRNA, 0.05% heparin, 0.1% BSA and 1% SDS), before hybridisation overnight at 63°C in the same solution containing 0.5–2 μg/ml DIG-labelled probe. Embryos were washed prior to digestion with 100 μg/ml RNase A and 100 U/ml RNase T1 for 60 min at 37°C and were then further washed prior to incubation at room temperature in 2% levamisole in TBST (20 mM Tris, 140 mM NaCl, 2.7 mM KCl, 0.1% Tween 20), containing 10% heat-inactivated goat serum. The secondary antibody (anti-digoxigenin-AP, [alkaline phosphatase] Fab fragments [Boehringer Mannheim]) was diluted 1:5000 and preabsorbed with 2 mM levamisole, 1% heat-inactivated goat serum and heat inactivated embryo powder, in TBST, for 30 min at 4°C, prior to incubating overnight with the embryos at 4°C. Embryos were then extensively washed in 2 mM levamisole/TBST, and then in 2 mM levamisole in NTMT (100 mM NaCl, 100 mM Tris, pH 9.5, 50 mM $MgCl_2$, 0.1% Tween 20), before developing the colour reaction with nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (BCIP) (Boehringer Mannheim).

2.5. Paraffin section in situ hybridisation

Paraffin section in situ hybridisation was performed according to Wilkinson and Green (1990) [18], using cRNA probes for the D_2 and D_3 dopamine receptors labelled with [^{35}S]UTP.

3. Results

3.1. The D_3 receptor appears at an earlier developmental stage than the D_2 receptor

In order to examine whether the D_2 and D_3 receptors are expressed during embryonic development, PCR was used to amplify fragments of the D_2 and D_3 receptors from murine embryos at progressive stages of prenatal development. Total RNA was prepared from murine embryos of day 9.5 to day 17.5 post-conception (p.c.), and was used for the generation of cDNA. The cDNA templates for each developmental stage were used in PCR with D_2-specific or D_3-specific primers. These primers were based on sequences in each receptor's third cytoplasmic loop and were designed to amplify fragments of both short and long receptor isoforms which could be distinguished by agarose gel electrophoresis. Control PCR reactions using no template DNA produced no signal for either the D_2 or the D_3 receptors (data not shown). Fig. 1 shows that the D_2 receptor transcripts first appear at day 13.5 p.c., and show a gradual increase in the subsequent days. The D_3 receptor, by contrast, is detectable as early as day 9.5 p.c., and its mRNA levels peak at day 12.5 p.c. The long and short isoforms of the D_3 receptor show some slight variation in the pattern of their appearance, unlike those of the D_2 receptor which show an identical pattern. This differs quite markedly from the situation in the adult brain, in which the D_2 receptor is generally expressed at much higher levels than the D_3 subtype, and gives rise to the notion that the D_3 receptor subtype may serve as the dominant D2-type receptor in early developmental stages.

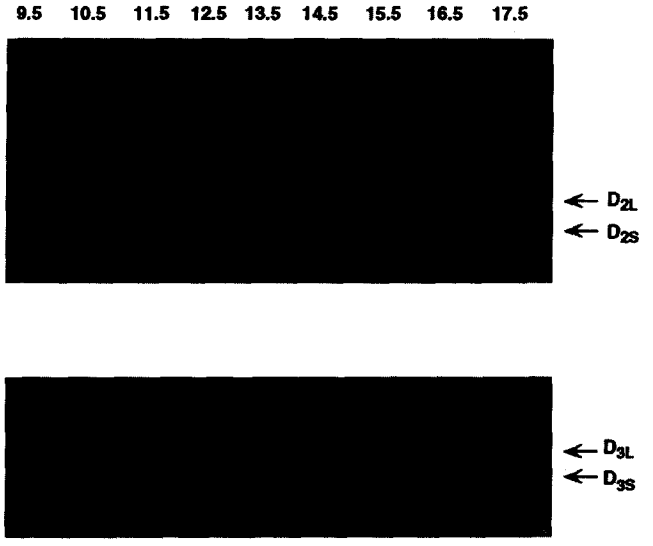

Fig. 1. Southern analysis of PCR performed on embryonic RNA taken from progressive stages of development. Hybridisation of electrophoresed PCR products was performed with D_2-specific (top) and D_3-specific (bottom) primers as described in section 2.

3.2. Distribution of D_2 and D_3 receptor transcripts

In order to examine the localisation of D_2 and D_3 receptor transcripts in murine embryos, DIG-labelled D_2-specific and D_3-specific cRNA probes were used in whole mount hybridisation. This technique permits the entire embryo rather than sections to be visualised, and involves detection by alkaline phosphatase-linked anti-DIG antibodies and generation of a colour signal.

No signal could be detected in 8.5 day embryos for either D_3 receptors (Fig. 2) or D_2 receptors (data not shown). At day 9.5 p.c. however D_3 receptor mRNA was present in or around the optic vesicle and at the ventral aspect of the diencephalon, whereas the D_2 receptor was still undetectable. The high specificity of the signal in whole mount in situ hybridization is documented by the low background in the 9.5 day old embryo hybridized with the D_2 probe. As shown in Fig. 3, at day 10.5 p.c., the D_3 receptor mRNA is present in the otic pit,

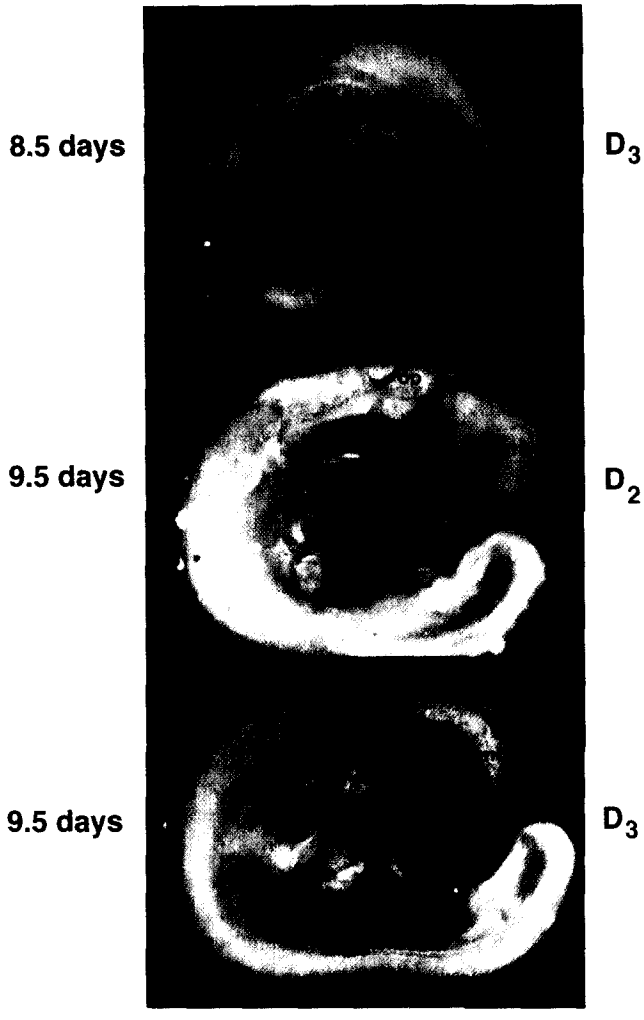

Fig. 2. Whole mount in situ hybridisation of 8.5 day and 9.5 day p.c. mouse embryos using D_2- and D_3-specific probes as indicated. The first appearance of D_3 receptor mRNA can be seen at day 9.5, compared with the absence of D_2 receptor at this stage. Specific signals for the D_3 receptor can be seen around the optic vesicle, and in the ventral aspect of the diencephalon. Abbreviations: fb, forebrain; hb, hind-brain; mb, mid-brain; op, otic pit; ov, optic vesicle. ←

and in the mid and forebrain, including the telencephalic vesicles. A sharp boundary of D_3 receptor expression is demonstrated along the border of the mid-brain and hind-brain, and signals begin to be detectable in the branchial arches and along the prospective vertebral column (Fig. 3, left). At the same developmental age similar, but considerably weaker D_2 receptor expression becomes detectable (Fig. 3, right). This supports the results from PCR shown in Fig. 1, which indicate that the D_3 receptor is expressed prior to the D_2 receptor during murine embryonic development.

To obtain additional evidence of the distribution of D_2 and D_3 receptor transcripts and to assess the identity of the cell layer where the receptor is expressed, histological in situ hybridisation was performed on paraffin sections of 11.5 day embryos, using the same cRNA probes as those above, labelled with [^{35}S]UTP. Fig. 4 demonstrates that both D_2 and D_3 receptor transcripts are present at this age along the external marginal layer of the telencephalic vesicles and, to a lesser degree, the hind-brain and spinal cord, also demonstrating that by day 11.5 p.c., both D_3 and D_2 receptor transcripts are present in the developing central nervous system.

4. Discussion

In this study, we have examined the expression of the D_2 and D_3 dopamine receptor subtypes in murine embryonic development, and have found that the D_3 receptor appears to be the dominant D2-type receptor in early stages of prenatal development. The appearance of the D_3 receptor mRNA prior to that of the D_2 receptor is evident from experiments described here using both PCR and in situ hybridisation. This earlier expression of the D_3 receptor appears also to occur in the rat, in which mRNA encoding the D_3 receptor is ex-

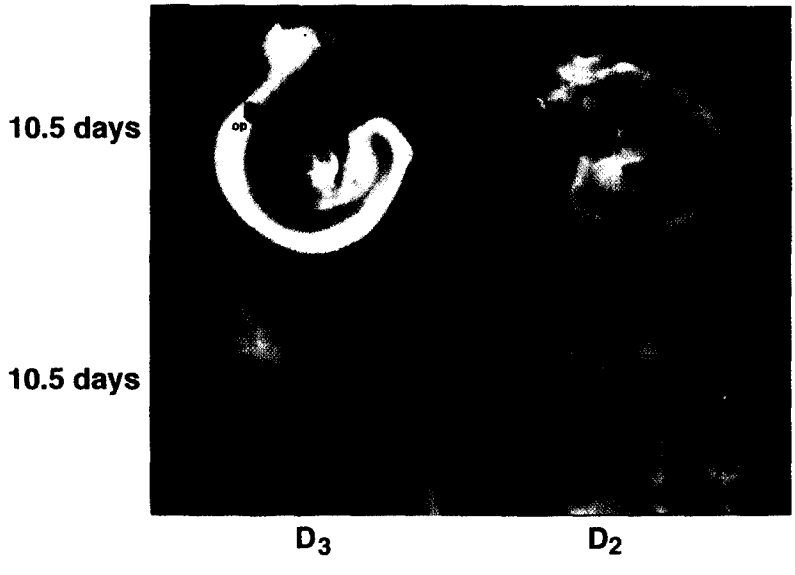

Fig. 3. Whole mount in situ hybridisation of 10.5 day p.c. mouse embryos using D_2- and D_3-specific probes as indicated. Increasing distribution of the D_3 receptor signal can be seen in the optic pit (top, left) and telencephalic vesicles (bottom, left). First appearance of the D_2 receptor occurs at day 10.5 in the otic pit (bottom, right). 10.5 day embryos are shown from more than one angle. Abbreviations: op, otic pit; tv, telencephalic vesicle.

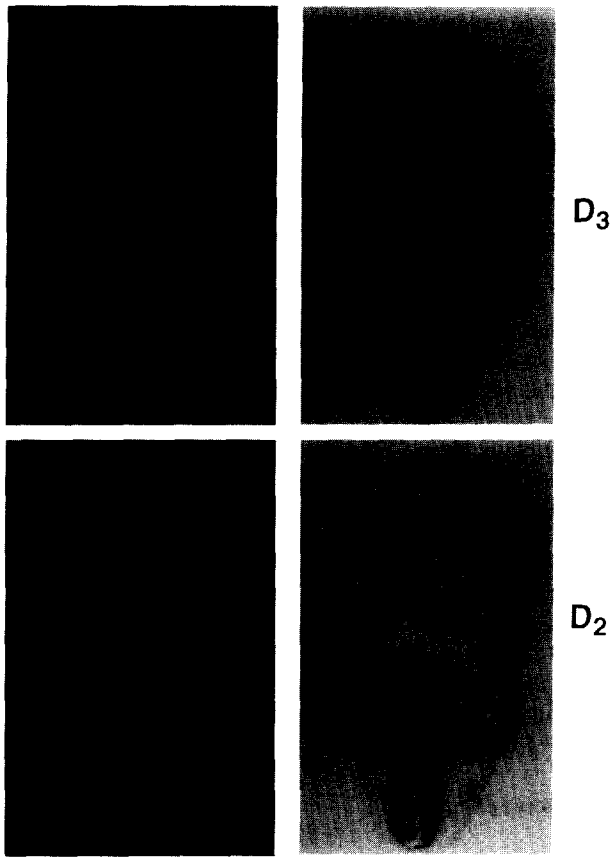

Fig. 4. Paraffin section in situ hybridisation 11.5 day p.c. mouse embryos using D_3-specific (top) or D_2-specific (bottom) antisense RNA probes, showing the presence of D_2 and D_3 receptor mRNA at day 11.5 in the developing nervous system. Abbreviations: de, diencephalon; hb, hind-brain; tv, telencephalic vesicle.

pressed, albeit at very low levels, at rat embryonic day 11 [19], while the earliest reported appearance of the D_2 subtype is at rat embryonic day 14 [20]. Comparison of the developmental stages in the rat and mouse shows that rat day 11 p.c. is equivalent to murine days 9.5–10 p.c., and rat day 14 p.c. relates approximately to murine days 12.5–13.5 p.c. Our data therefore place the first detectable expression of the murine D_2 receptor to an earlier stage than that reported for the rat by Mack et al. [20]. High sensitivity of localization techniques, as compared to averaging approaches, and other technical differences as well as the species difference may explain this discrepancy.

Earlier studies using ligand-binding autoradiography demonstrated the presence of D_2-type receptor protein in rat embryos at rat embryonic day 12 [12]. We were also able to detect the presence of D_2 and D_3 receptor protein in murine embryos, using whole mount immunohistochemistry with subtype-specific antibodies (data not shown), supporting the studies in the rat. Interestingly, the radioligand binding studies in the rat showed that by rat embryonic day 15, the D_2-type receptors expressed during this stage of prenatal development are functional and can operate signal transduction pathways.

Using the technique of whole mount in situ hybridisation, we have been able to analyse the distribution of the receptor transcripts at early developmental stages, and have shown that although there is a temporal difference between the expression of the D_2 and D_3 receptors, their patterns of distribution are quite similar. The presence of D_2 receptor mRNA at earlier stages of development (day 10.5 p.c.) than was detected by PCR is most probably due to the fact that the D_2 receptor signal is very weak at this stage and may therefore not be sufficiently abundant for detection by PCR under the conditions used. The distribution of D_3 receptor mRNA at early developmental stages is also highly restricted, appearing first in the optic and otic vesicles and in the diencephalon, which ultimately form the eye, ear and thalami, respectively. This expression pattern may indicate that dopaminergic receptors may be involved in the development of the porsencephalon and its derivatives the diencephalon the neuroretina and optic nerve as well as the auditory apparatus. The presence of D_2 and D_3 receptor mRNA in the adult thalamus has previously been shown using Northern analysis and in situ hybridisation [21,22].

It is worth noting that the areas richest in D_2 and D_3 receptors in the murine embryo, the optic and otic vesicles, develop into structures, the eye and the ear, which do not belong to the central nervous system. Significantly, the presence of D_2-type receptors in the adult retina has been demonstrated in several species [14] and in the goldfish retina, D_2-type receptors are found to be coupled to the regulation of dopamine release [23], implying an autoreceptor role in this tissue. Given the previous characterisation of the D_3 subtype as a possible autoreceptor [9], and the identification of D_3 receptor transcripts in the optic vesicle described here, it would be interesting to determine whether the D_3 may have a role in the developing or adult eye, and if so, whether that role may involve an autoreceptor function.

The results presented here indicate clearly that D_3 receptor mRNA is expressed in murine embryonic development significantly earlier than the D_2 receptor, and demonstrate that for both receptors, the long and short isoforms follow similar patterns of appearance. Although D_2 receptor mRNA can be detected in early embryos using whole mount in situ hybridisation, the ability of PCR to amplify D_3 receptor transcripts under conditions where the D_2 receptor was not amplified, suggests that the D_3 receptor is more abundant than the D_2 receptor at these stages. Thus it appears that at early stages of development, the D_3 receptor may be the dominant D_2-type receptor, in contrast to the adult, in which the D_2 receptor is expressed at considerably higher levels in all tissues where the two receptors are co-located. Whether the D_2 and D_3 receptors act as functional receptors or influence the developmental process in the early murine embryo remains to be determined. It is worth noting that these receptors can regulate neuronal outgrowth when expressed in a clonal mesencephalic cell line (MN9D) [24], suggesting that they are capable of modulating neuronal morphogenesis. It will be interesting to determine whether the D_3 receptor indeed functions as the principal D_2-type receptor in early murine embryos, and whether these receptors may be involved in the process of prenatal development.

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