Role of motoneuron-derived neurotrophin 3 in survival and axonal projection of sensory neurons during neural circuit formation

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SUMMARY
Sensory neurons possess the central and peripheral branches and they form unique spinal neural circuits with motoneurons during development. Peripheral branches of sensory axons fasciculate with the motor axons that extend toward the peripheral muscles from the central nervous system (CNS), whereas the central branches of proprioceptive sensory neurons directly innervate motoneurons. Although anatomically well documented, the molecular mechanism underlying sensory-motor interaction during neural circuit formation is not fully understood. To investigate the role of motoneuron on sensory neuron development, we analyzed sensory neuron phenotypes in the dorsal root ganglia (DRG) of Olig2 knockout (KO) mouse embryos, which lack motoneurons. We found an increased number of apoptotic cells in the DRG of Olig2 KO embryos at embryonic day (E) 10.5. Furthermore, abnormal axonal projections of the motoneuron-derived factor that regulates sensory neuron development, we focused on neurotrophin 3 (Ntf3; NT-3), because Ntf3 and its receptors (Trk) are strongly expressed in motoneurons and sensory neurons, respectively. The significance of motoneuron-derived Ntf3 was analyzed using Ntf3 conditional knockout (cKO) embryos, in which we observed increased apoptosis and abnormal projection of the central branch innervating motoneuron, the phenotypes being apparently comparable with that of Olig2 KO embryos. Taken together, we show that the motoneuron is a functional source of Ntf3 and motoneuron-derived Ntf3 is an essential pre-target neurotrophin for survival and axonal projection of sensory neurons.

KEY WORDS: Motor and sensory circuits, Neurotrophin 3, Ntf3, NT-3, Apoptosis, Axonal projection, Pre-target neurotrophin, Proprioceptive neuron

INTRODUCTION
Neural circuit formation requires multiple steps: cell type specification, migration, axonal projection to their appropriate targets, formation of functional synapses and elimination of excess cells and synapses. Neural crest-derived sensory neuron precursors originate within the tips of neural folds and migrate ventrally to form the dorsal root ganglion (DRG). Sensory neurons extend two axon branches: the peripheral branch and the central branch. Different types of sensory neurons are specialized for different perceptual modalities. For example, proprioceptive sensory neurons extend axons to alpha-motoneurons in the ventral horn, and provide information about muscle length and tension to the spinal cord. This sensory-motor circuit provides an excellent system with which to study neural circuit formation. The developmental molecular interaction between motoneurons and sensory neurons has just begun to be elucidated (Gallarda et al., 2008; Wang et al., 2011). For example, Semaphorin 3e and its high-affinity receptor plexin D1 (Plxnd1), which are expressed by selected motoneuron pools and proprioceptive sensory neurons, respectively, are crucial determinants of synaptic specificity in sensory-motor circuits (Pecho-Vrieseling et al., 2009). It is known that early-born neurons regulate late-born neurons during development of some neuronal circuits, such as the olfactory pathway (Takeuchi et al., 2010) and the thalamocortical pathway (McConnell et al., 1989). As motoneuron development precedes sensory neuron development (Wentworth, 1984; Landmesser and Honig, 1986; Wang et al., 2011), it is conceivable that motoneurons have substantial roles in sensory neuron development.

In order to investigate the role of motoneurons on early sensory neuron development, we analyzed sensory neuron phenotypes in the DRG of Olig2 knockout (KO) embryos, which have no motoneuron in the spinal cord. Olig2 is a basic helix-loop-helix (bHLH) transcription factor that is essential for motoneuron and oligodendrocyte development (Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002). Here, we report abnormal phenotypes in the sensory neurons of Olig2 KO mice: first, the decreased number of sensory neurons and the increased number of apoptotic cells in the DRG; and second, the abnormal axonal projection of central and peripheral branches of DRG neurons. The neurotrophic factor neurotrophin 3 (Ntf3; previously NT-3) is specifically and strongly expressed in the motoneuron column of the embryonic spinal cord (Ernfors and Persson, 1991). As Ntf3 is not expressed in the ventral spinal cord of Olig2 KO embryos, we
investigated the role of motoneuron-derived Ntf3 in suppression of apoptosis and in proper axonal projection of sensory neurons during development.

Ntf3 is one of the well-known neurotrophic factors (Chao, 2003), which has been shown to exert a strong survival activity on DRG neurons (ElShamy and Ernfors, 1996; Farihas et al., 1994; Liebl et al., 1997; Tojo et al., 1995) and it acts as a chemottractant for DRG axons (Genc et al., 2004; Tucker et al., 2001). Main sources of Ntf3 are thought to be the target of innervation or the mesenchyme (Ernfors, 2001; Patapoutian et al., 1999). Although Ntf3 has been suggested to act as a pre-target neurotrophin, its detailed source and function have not yet been elucidated (Gaese et al., 1994; Howe and Mobley, 2005). Here, we generated and analyzed Ntf3 conditional KO (cko) mice, in which the Ntf3 gene is deleted in the ventral spinal cord. In these mice, we observed increased cell death of sensory neurons and abnormal axonal projection of central branches, the phenotypes being comparable with those of Olig2 KO mice. Our data indicate that motoneuron is a functional source of Ntf3 and that motoneuron-derived Ntf3 is involved in early sensory-motor interaction by acting as a pre-target neurotrophin for early sensory neurons. Taken together, this study shows the importance of a motoneuron-derived factor in the sensory-motor circuit formation.

MATERIALS AND METHODS

Mice

We used an Olig2-CreER mouse (Takebayashi et al., 2002), an Olig2-Cre knock-in mouse (H.T., unpublished) and a Pc36 transgenic mouse, which have ubiquitous Cre activity (Ding et al., 2005); a flox Ntf3 mouse (Bates et al., 1999; obtained from Jackson Laboratory; stock number 003541); and a Z/EG reporter mouse (Novak et al., 2000). The Olig2 mice used in this study were generated using an Olig2-CreER allele. PCR genotyping of Olig2, Cre and Z/EG alleles was performed as described previously (Masahira et al., 2006; Takebayashi et al., 2002; Tsuchita et al., 1999). The Ntf3 null allele was generated by mating a flox Ntf3 mouse line with a Pc36 Cre driver line. PCR primers (Ntf3_null_S2, 5'-TCA GGG GCT CGG CCA GGA AA-3'; Ntf3_null AS3, 5'-AGT TCT TTG GGG GAG GGG GCA-3') were designed to detect the recombined flox Ntf3 allele, the Ntf3 null allele and the wild-type Ntf3 allele, which yielded 2.5 kb, 1.3 kb and 2.3 kb products, respectively, using KOD FX Neo Taq (TOYOBO) (supplementary material Fig. S5). PCR genotyping for detection of the wild-type Ntf3 allele (375 bp band) and the mutant alleles (either flox Ntf3 allele or Ntf3 null allele, 325 bp band) was performed according to the protocol from Jackson Laboratory using following primers (olMR1336, 5’-GAC AAG CCA TCA GAA GAG CAG C-3; olMR1337, 5’-GAA TGG AGA GAG TGT CTC GCC-3’) and Quick Taq HS (TOYOBO). We maintained Olig2-CreER mice, Olig2-Cre mice and Z/EG mice in the ICR background (Japan SLC). For embryo staging, the day of detection of vaginal plug was considered to be E0.5. All procedures were approved by the Animal Research Committees at Kumamoto University and National Institute for Physiological Sciences.

Chick and in ovo electroporation

Fertilized hens’ eggs were purchased locally and incubated at 38°C. Approximately 1 μl of the plasmid DNA (1 μg/μl: pCAG-GFP and pCAG-DsRed, or pCAG-Ntf3-GFP and pCAG-DsRed) was microinjected into the chick neural tube at E3 [Hamburger and Hamilton (HH) stage 18-20] (Hamburger and Hamilton, 1951) with a glass capillary and then electroporated using an electroporator (CUY-21, NEPA GENE; 25 V, 30 msecounds on, 100 msecounds off, six pulses) and a platinum electrode (CUY610P4-1, NEPA GENE). After 48 hours of electroporation (at E5, HH stage 25-26), the electroporated embryos were fixed overnight using 4% paraformaldehyde (PFA) to carry out immunohistochemical analyses. The electroporated areas were identified by DsRed fluorescence.

Immunohistochemistry was performed as described previously (Takebayashi et al., 2008). Cryosections (20 μm thick) were stained with following primary antibodies: rabbit anti-cleaved caspase 3 (1:500, Cell Signaling Technology), rabbit anti-TrkA (1:2000; a gift from Dr Reichardt, UCSF, CA, USA), rabbit anti-TrkC (1:200, Santa Cruz Biotechnology), rabbit anti-Olig2 (1:200, IBL; 1:500, Chemicon), rat anti-Ki67 (1:20, TEC-3, DakoCytomation), mouse anti-bIII-tubulin (1:1000; Tuji, Covance), mouse anti-HB9 (1:20, MNR2, DSHB, mouse anti-Neurofilament M (1:20, 1C8; Watanabe et al., 2006) and chicken anti-peripherin (1:1000; Aves Labs). For fluorescence immunostaining, species-specific antibodies conjugated to Alexa 488 or Alexa 594 (1:2000; Invitrogen/Molecular Probes) were applied and nuclei were counterstained with Hoechst 33342 (1 μg/ml; Sigma). For diaminobenzidine (DAB) staining, biotinylated species-specific goat antibodies (1:400; Millipore), or a biotinylated donkey anti-chick antibody (1:100; Jackson ImmunoResearch) was applied and then processed with the ABC method (ABC Elite kit, Vector Laboratories) following the manufacturer’s instructions. The images were captured using an Olympus microscope and digital camera system (BX51 and DP70, or BX53 and DP72, Olympus).

In situ hybridization

In situ hybridization was performed as described previously (Takebayashi et al., 2008) using following riboprobes: rat TrkA (Ntrk1, Matsumoto et al., 2001), mouse TrkB (Ntrk2, Singh et al., 1997), rat TrkC (Ntrk3, Matsumoto et al., 2001), mouse Ngf (Yoshida et al., 2001), mouse Bdnf (Singh et al., 1997), mouse Ntf3 (GenBank Accession Number NM_008742, nt 280-987), mouse Runx1 (GenBank Accession Number NM_001111023, nt 2724-3861), mouse Runx3 (GenBank Accession Number NM_019732, nt 944-1938) and mouse Olig2 (Takebayashi et al., 2000). Digoxigenin (DIG)-labeled riboprobes were synthesized from the plasmids using in vitro transcription. Cryosections (20 μm) were fixed in 4% PFA, treated with Proteinase K (1 μg/ml), acetylated and then hybridized with DIG-labeled riboprobes overnight at 65°C. Then, sections were washed, blocked with blocking buffer for 1 hour at room temperature and incubated overnight at 4°C with alkaline phosphatase-conjugated anti-DIG antibody (Roche) in blocking buffer. Alkaline phosphatase was visualized by nitro-blue tetrazolium chloride (NBT, Roche) and 5-bromo-4-chloro-3-indolyphosphate (BCIP, Roche) according to the manufacturer’s instructions.

Statistical analysis

Quantification of TrkB-positive, TrkC-positive, Runx3-positive, cleaved caspase 3-positive and Ki67-positive cells was performed in E10.5, E13.5 and E18.5 transverse sections at thoracic levels. Cells with positive staining were manually counted on 10-20 sections from three or four embryos for each condition. Statistical significance was determined using Student’s t-test.

RESULTS

Sensory cell death and reduced cell number in the DRG of Olig2 KO embryos

To investigate the role of motoneurons on sensory neuron development, DRG phenotypes were analyzed in Olig2 KO embryos, which have no motoneuron in the spinal cord (supplementary material Fig. S1). The specification of DRG neurons occurs at a later stage (after E11.5), which lead to the subtype-specific expression of each Trk receptor (supplementary material Fig. 4D-F) (Marmigère and Ernfors, 2007). As most DRG neuron progenitors express all Trk receptors at E10.5, we performed in situ hybridization at E13.5 and E18.5 (Marmigère and Ernfors, 2007) in order to analyze the number of nociceptive (TrkA positive; Ntrk1 – Mouse Genome Informatics), mecanoreceptive (TrkB positive; Ntrk2 – Mouse Genome Informatics) and proprioceptive (TrkC positive; Ntrk3 – Mouse Genome Informatics) sensory neurons. Although quantification of nociceptive (TrkA-positive) neurons was difficult owing to the presence of a large number of positive cells, or in the DRG, no remarkable change was observed between...
heterozygotes and Olig2 KO mice (Fig. 1A-D). The number of mechanoreceptive (TrkB-positive) neurons in the DRG of Olig2 KO mice was decreased significantly at E13.5 (P<0.01), but the change was not significant at E18.5 (Fig. 1E-I). Furthermore, the number of TrkC-positive and Runx3-positive proprioceptive neurons was decreased at E13.5 (Fig. 1J-K, N-P, S). We observed similar phenomena at E18.5 (Fig. 1L-N), but only the decrease of Runx3-positive proprioceptive neurons was statistically significant (Fig. 1Q-R). Therefore, in Olig2 KO embryos, at least the number of mechanoreceptive and proprioceptive neurons was reduced at E13.5. This abnormal population of sensory neurons observed in Olig2 KO embryos results from a cell non-autonomous effect of Olig2 because no Olig2 expression is observed in the developing DRG of wild-type embryos at E9.5 and E10.5 (supplementary material Fig. S2).

We next analyzed cell death and cell proliferation to investigate why DRG neurons are decreased in Olig2 KO mice. An increased number of cleaved caspase 3-positive apoptotic cells was observed in the DRG of Olig2 KO mice at E10.5 (Fig. 2A,B,E) (Takebayashi et al., 2008). At E13.5, the difference between heterozygotes and Olig2 KO mice was not statistically significant (Fig. 2C-E). On the other hand, the number of Ki67-positive proliferative cells was not changed significantly in the DRG either at E10.5 or at E13.5 (Fig. 2F-J). These data indicate that the decrease in the number of DRG neuron is due to the increased cell death, but not due to the decreased proliferation.

Abnormal axonal projection of sensory neurons in the Olig2 KO mice

We next analyzed axonal projections of DRG neurons in Olig2 KO mice using βIII-tubulin immunostaining. In Olig2 KO mice, there are no motoneuron axons because of the lack of motoneurons (Fig. 3A-D). In addition, an abnormal axonal projection of the peripheral branches of sensory neurons was observed both at E10.5 and at E12.5 in those mutants (Fig. 3A-D). This phenotype was confirmed by whole-mount immunohistochemistry using a neurofilament M antibody at E10.5, and the results showed an irregular sensory axonal projection and fasciculation (supplementary material Fig. S3). Next, we performed immunostaining for neurofilament M or peripherin at E13.5. The Ia afferents, which constitute the central branches of proprioceptive neurons, are projected to motoneurons in the heterozygous spinal cord (Fig. 3E,G,L, arrows) (Ozaki and Snider, 1997). In Olig2 KO mice, the axonal projection of Ia afferents did not reach the ventral spinal cord (Fig. 3F,H,L, arrows).

Motoneuron-derived Ntf3 is an important factor for sensory neuron development

We hypothesized that motoneuron-derived neurotrophic factors are important for survival of sensory neurons, because the most prominent phenotype of the E10.5 Olig2 KO spinal cord is motoneuron deficiency. Among the neurotrophic factors, we...
focused on Ntf3, because it is specifically and strongly expressed in the motor column within the ventral spinal cord of heterozygote embryos at E10.5 and E13.5 (Fig. 4A,C) (Ernfors and Persson, 1991; Fariñas et al., 1996) and Ntf3 expression in the Olig2 KO spinal cord is below detection level (Fig. 4B,D). We also performed intensive expression analyses of two other neurotrophic factors (Ngf and Bdnf) in motoneurons and their receptors (TrkA, TrkB and TrkC) in the DRG. We confirmed that the expression of Ngf and Bdnf is below detection levels at E10.5 (supplementary material Fig. S4A,B). The Ntf3 expression in motoneurons became weaker during development, whereas the expression outside the spinal cord, such as in muscle and in skin, becomes stronger (Fig. 4A-D, arrowheads). The expression patterns of Ntf3 in the motoneuron and TrkC receptor in the DRG (supplementary material Fig. S4F) suggested that motoneuron-derived Ntf3 had an important role in the sensory neuron development at the early stage (neurogenetic stage, ~E10) and that mesenchyme- or target-derived Ntf3 takes over the role at the late stage (gliogenic stage, ~E13). To investigate the role of motoneuron-derived Ntf3, we generated Ntf3 cKO mice by crossing Olig2-Cre; Ntf3 null male mice and flox Ntf3 female mice (supplementary material Fig. S5). As expected, no Ntf3 expression was observed in the motor column of the Olig2-Cre; Ntf3 cKO mice (Fig. 5A,B). Interestingly, the number of cleaved caspase 3-positive cells was dramatically increased in the DRG of Ntf3 cKO mice at E10.5, compared with that of control embryos (Fig. 5C-E). The number of both TrkC-positive and Runx3-positive proprioceptive neurons was decreased in the DRG of Ntf3 cKO mice at E13.5 (Fig. 5F-J). Peripheral branches seemed to be normal in Ntf3 cKO mice at E10.5, as observed by βIII-tubulin immunohistochemistry (supplementary material Fig. S6). The immunostaining for neurofilament M and peripherin showed that the Ia afferents did not reach motoneurons (Fig. 5K-R). These data demonstrate that motoneuron-derived Ntf3 is essential for sensory neuron survival and proper innervation of Ia afferents.

Ntf3 secretion from motoneurons

Finally, we addressed the mode of Ntf3 secretion from motoneurons. As Ntf3 is believed to be a local neurotrophin (ElShamy and Ernfors, 1996) and sensory neuron axons have close contact with motoneuron axons in the spinal nerve at E10.5, transaxonal secretion is one suggested mechanism. To confirm the localization of Ntf3-GFP protein in the motoneuron axons in vivo, we next performed in ovo electroporation into chick embryonic spinal cord at E3 (HH stage 18-20) using DsRed as a control to indicate cytoplasmic localization. Indeed, we observed Ntf3-GFP localization in the motoneuron axons in vivo at E5 (HH stage 25-26, Fig. 6D-F). We also noticed that Ntf3-GFP signals were
observed in the DRG and the mesenchyme surrounding the spinal cord (Fig. 6D,F arrowheads; supplementary material Fig. S7B), whereas GFP signals were observed only intracellularly in the control experiment (Fig. 6A-C; supplementary material Fig. S7A), suggesting Ntf3-GFP secretion from electroporated cells in the neural tube. These data suggest that the motoneuron-secreted Ntf3 reaches the DRG not only from motor axons, but also from the margin of the spinal cord.

**DISCUSSION**

In this study, we analyzed the DRG phenotype of motoneuron-deficient Olig2 KO mice to investigate the role of motoneurons on sensory neuron development, and demonstrated increased apoptosis and abnormal projection of peripheral and central branches of sensory axons. The neurotrophic factor Ntf3 is specifically and strongly expressed in the motoneuron column of the embryonic spinal cord (Fig. 4A,C) (Ernfors and Persson, 1991). As Ntf3 expression was not observed in the ventral spinal cord of Olig2 KO mice, we analyzed the role of motoneuron-derived Ntf3 on sensory neuron development by generating Ntf3 cKO mice. We further demonstrated that the motoneuron-derived Ntf3 is essential for survival of proprioceptive neurons in the DRG. This study implies motoneuron as a functional source of Ntf3, a pre-target neurotrophin for sensory neurons.

**Effects of motoneuron deficiency on sensory neuron development**

In the DRG of Olig2 KO embryos, increased apoptosis was observed only at E10.5 but not at E13.5 (Fig. 2A-E), suggesting motoneuron-derived neurotrophin(s) had an important role at the early stage. Consistently, we observed decreased expression of Ntf3 in motoneurons, and increased expression in the mesenchyme and target organs at E13.5. As DRG apoptosis peaks around embryonic stage E12-E14 (Sun et al., 2008), it is difficult to detect any subtle increase in apoptosis caused by the motoneuron deficiency in Olig2
KO embryos at E13.5. For proper axonal projection of peripheral branches, not only the secretion of soluble factors from motoneurons, but also the contact-mediated mechanism between motor axons and sensory axons (Landmesser and Honig, 1986) may play important role. Disruption of (any or both of) these mechanisms may account for the abnormal axonal projection of the peripheral branch of sensory neurons in Olig2 KO mice. It is possible that abnormal peripheral branch projection results in abnormal central branch projection (Patel et al., 2003). In addition, because Ia afferents of proprioceptive neurons make synapses with motoneurons, such synapses cannot be formed in the Olig2 KO embryos, which have no motoneurons. It appears that multiple mechanisms may exist through which a motoneuron defect could affect the projection of sensory neurons. We observed that Ia afferents did not project towards ventral motoneurons in the Ntf3 KO mice (Fig. 5). KO mice (20.3±8.3 in Ntf3 KO; 12.4±3.6 in Ntf3 cKO). There are two possibilities to explain this phenomenon: first, the neurotrophic factors other than Ntf3 may have roles to prevent apoptosis; and second, the transaxonal transmission is missing in motoneuron-deficient Olig2 KO mice.

Our data from Ntf3 cKO embryos indicate that the motoneuron-derived Ntf3 acts as a pre-target neurotrophin to prevent apoptosis at the early stage of neural circuit formation in vivo (Fig. 7). As sensory neurons locate very close to motoneurons at this early stage (Fig. 7), it is conceivable that the motoneuron-derived Ntf3 can act as a local signal to prevent apoptosis of sensory neurons. As the number of apoptotic cells in Ntf3 KO embryos is higher than that of Ntf3 cKO embryos (20.3±8.3 in Ntf3 KO; 12.4±3.6 in Ntf3 cKO, Fig. 5E), Ntf3 from tissues other than motoneurons may also have some trophic functions. Another possible role of the motoneuron-derived Ntf3 at the early stage is to support outgrowth or attraction of the peripheral branch (Fig. 7), because NT-3 has role in sensory axonal projection, as shown previously by gain-of-function and loss-of-function experiments in vivo (Lefcort et al., 1996; Tucker et al., 2001). The reason why there is no obvious defect in peripheral branch in the Ntf3 cKO mice might be attributed to the presence of many repulsive cues around the DRG (Keynes et al., 1997; Masuda et al., 2008); that is, sensory axons may finally find their route and fasciculate with motoneuron axons without attractive cues. After fasciculation, it is possible that Ntf3 is secreted from motor axons as a transaxonal signaling factor, analogous to the secretion of Sema3a and Sema3f from developing axons (Moret et al., 2007; Takeuchi et al., 2010). At E15, central branches of sensory neurons enter spinal cord from the dorsal root entry zone after the waiting period (Ozaki and Snider, 1997; Watanabe et al., 2006), and, then, proprioceptive axons directly innervate motoneurons. We observed that 1a afferents did project towards ventral motoneurons in the Ntf3 cKO mice (Fig. 5L,N,P,R). This is attributable to the loss of TrkB-positive neurons during DRG development in Ntf3 cKO embryos (Fig. 5).

Neurotrophins including Ntf3 have multiple roles in not only developing brains but also adult brains and pathological brains. Neurotrophins are secreted in an activity-dependent manner from mature neurons to regulate synaptic plasticity (Je et al., 2005; Vicario-Abejon et al., 2002). Exogenous Ntf3 expression in target nucleus facilitates axonal regeneration and synapse formation after the spinal cord injury (Alto et al., 2009; Kadoya et al., 2009) and phenotypes, we also generated Ntf3 cKO embryos using Olig2-Cre driver mice to investigate the role of motoneuron-derived Ntf3 on sensory neuron development. The number of apoptotic cells in the E10.5 DRG is higher in Olig2 KO mice (49.7±11.4; Fig. 2) than in Ntf3 KO mice (20.3±8.3). There are two possibilities to explain this phenomenon: first, the neurotrophic factors other than Ntf3 may have roles to prevent apoptosis; and second, the transaxonal transmission is missing in motoneuron-deficient Olig2 KO mice.

Role of motoneuron-derived Ntf3 on spinal circuit formation
Because of the specific expression of Ntf3 in motoneurons at the early stage and both Ntf3 KO mice (ElShamy and Ernfors, 1996) and Olig2 KO mice (this study) show some comparable...
such findings indicate that Ntf3 can be a candidate factor for promoting neuronal regeneration after injury. Accordingly, understanding the mechanism of neurotrophin secretion and function during development is crucially important for the field of CNS regeneration. Our study implies the possibility of using Ntf3 as a pre-target neurotrophin to facilitate the axonal regeneration.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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