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Antioxidant Cu/Zn SOD: Expression in postnatal brain progenitor cells

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Abstract

Precursor cells have been shown to be affected by oxidative stress, in vivo and vitro, but little is known about the expression of antioxidant mechanisms in neuronal/glial differentiation. We have characterized the expression of Cu/Zn superoxide dismutase (Cu/Zn SOD), one of the main antioxidant proteins involved in the breakdown of superoxide, in the immature rat dorsolateral subventricular zone (SVZ), rostral migratory stream (RMS) and hippocampal subgranular zone (SGZ). Progenitor cells were identified immunohistochemically on cryostat sections by 5′Bromodeoxyuridine (BrdU) incorporation and expressing cells were further characterized using double labeling for progenitor markers. In the SVZ, only a subpopulation of BrdU+ cells, mostly found in the medial SVZ, expressed Cu/Zn SOD. These cells were mostly nestin+ and some were also vimentin+. In contrast, in the lateral SVZ few Cu/Zn SOD+/BrdU+ cells were found. These were primarily nestin+, vimentin−, showed some PSA-NCAM expression, but only a few were NG2+. In the RMS and SGZ virtually all BrdU+ progenitors were Cu/Zn SOD+ and expressed nestin and vimentin. Some RMS cells were also PSA-NCAM+. These findings show a heterogeneous expression of Cu/Zn SOD in restricted cell types in the germinative zones and suggest a role for antioxidant Cu/Zn SOD in progenitor cells of the immature rat brain.

Keywords: Stem/progenitor cell; Cu/Zn superoxide dismutase; Subventricular zone; Rostral migratory stream; Subgranular zone; Antioxidant enzyme

Under normal physiological conditions, cellular levels of the reactive oxygen species superoxide (O_2^-) are kept low by several antioxidant mechanisms including the enzyme copper/zinc superoxide dismutase (Cu/Zn SOD) [26], which catalyzes the dismutation of O_2^- to hydrogen peroxide (H_2O_2) in the cytosol of various cell types.

In the immature rat brain, we have previously described widespread expression of Cu/Zn SOD in neurons; although, no Cu/Zn SOD immunoreactivity is seen in glial cells [31]. Moreover, scattered Cu/Zn SOD positive cells, negative for glial fibrillary acidic protein (GFAP) and tomato lectin (microglia/macrophage marker) staining, were observed in germinative zones of the control postnatal brain (unpublished results).

Progenitor cells are especially abundant in the immature brain [2,5,12,35]. Although the functional role of these cells in the germinative areas is still not clear, it is obvious that understanding their cell fate decision mechanisms is crucial in order to manipulate these cells to proliferate, differentiate, migrate and integrate into damaged tissue. It has already been established that neural precursors and neural stem cells are sensitive to various types of damage, including irradiation, ischemia, and trauma [9,17,21,33] and that they are affected by oxidative stress [19,20,23,32,33,36]. However, very little is known about the presence of antioxidant mechanisms in progenitor populations.

Accordingly, the aim of this study was to characterize the expression of Cu/Zn SOD, one of the most important cellular mechanisms in coping with oxidative stress, and the phenotype of Cu/Zn SOD expressing cells in the germinative zones of the immature rat brain.

Sixteen Long-Evans black-hooded rat pups aged 9, 12 and 16, and 23 postnatal days (P9, P12, P16, and P23) were used. These ages of study were chosen according to previous results showing maximal progenitor cell number in germinative zones at P12 [5]. Experimental animal work was conducted

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Table 1
Semi-quantification of double labeled Cu/Zn SOD+ cells in the MSVZ, LSVZ, RMS and SGZ

	BrdU/nestin+ precursors expressing Cu/Zn SOD	Cu/Zn SOD/nestin	Cu/Zn SOD/vimentin	Cu/Zn SOD/PSA-NCAM	Cu/Zn SOD/NG2
MSVZ	+++	++++	+++	+/−	+/−
LSVZ	+	++++	+	++	++
RMS	++++	++++	++++	+++	+/−
SGZ	++++	++++	++++	+/−	–

In the SVZ subpopulations of BrdU+/nestin+ precursors expressed Cu/Zn SOD; the majority were located in the MSVZ and few were found in the LSVZ. In the MSVZ the Cu/Zn SOD+ cells showed BrdU, nestin, and vimentin expression, but little PSA-NCAM and NG2 expression. In the LSVZ, the Cu/Zn SOD+ cells showed BrdU and nestin, little vimentin, but more PSA-NCAM and NG2 expression than the MSVZ. In the RMS and SGZ BrdU+/nestin+ precursors were always Cu/Zn SOD+. In the RMS, these cells were nestin, vimentin, and PSA-NCAM+, but rarely NG2+ whereas in the SGZ, they were only nestin+ and vimentin+, rarely PSA-NCAM+ and never showed NG2. MSVZ: medial dorsolateral subventricular zone; LSVZ: lateral subventricular zone; RMS: rostral migratory stream; SGZ: subgranular zone.

according to Spanish regulations, in agreement with European Union directives. Experimental procedures were approved by the ethical commission of the Autonomous University of Barcelona.

Intraperitoneal injection of 5′Bromodeoxyuridine (BrdU, 50 mg/kg; Sigma Chemical, St. Louis, MO, USA) diluted in TB (0.05M Trizma base, pH 7.4) was used to label actively proliferating cells every 2 h for 10 h before sacrifice. Rats were sacrificed by intracardial perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and immersed in the same fixative for 2 h and cryoprotected in a 30% sucrose solution in 0.1 M phosphate buffer. Brains were then frozen with dry CO_2 and 30 μm thick series of parallel coronal sections were obtained using a cryostat (Leitz).

To prove Cu/Zn SOD expression in progenitor cells double immunofluorescence labeling for BrdU and Cu/Zn SOD was performed on free-floating cryostat sections. For BrdU labeling, sections were incubated in HCl for 10 min at 4 °C and then 30 min at 37 °C for DNA denaturation. Subsequently, sections were rinsed and incubated for 1 h at room temperature (RT) in blocking buffer (BB) then overnight at 4 °C and for 1 h at RT in a primary mouse anti-BrdU antibody (1:80, M0744, DAKO, Denmark). After washing, sections were incubated for 1 h at RT in a secondary Cy2-conjugated goat anti-mouse antibody (1:1000, PA42002, Amersham, UK). Sections were washed and incubated for 1 h at RT in BB then overnight at 4 °C and for 1 h at RT in a primary sheep polyclonal anti-Cu/Zn SOD antibody (1:300, 574597, Calbiochem, Germany), rinsed and incubated for 1 h at RT in a secondary Cy3-conjugated anti-sheep antibody (1:150, AP147C, Chemicon, CA, USA).

To identify the phenotype of Cu/Zn SOD expressing progenitor cells double labeling for BrdU or Cu/Zn SOD combined with either nestin, vimentin, polysialiated cell adhesion molecule (PSA-NCAM) or NG2 proteoglycan (NG2) was then performed. Sections were incubated in either mouse monoclonal anti-nestin antibody (1:1000, MAB 353, Chemicon, CA, USA), mouse monoclonal anti-vimentin antibody (1:1000, M0725, DAKO, Denmark), mouse monoclonal anti-PSA-NCAM antibody (1:1000, MAB5324, Chemicon, CA, USA), or a rabbit polyclonal anti-NG2 antibody (1:500, AB5320, Chemicon CA, USA). After washing, nestin and vimentin sections were incubated in a Cy2-conjugated goat anti-mouse secondary antibody (1:1000, PA42002, Amersham, UK), NG2 sections were incubated in a Cy2-conjugated anti-rabbit secondary antibody (1:1000, PA42004, Amersham, UK), and PSA-NCAM sections were first incubated in a biotinylated anti-mouse IgM antibody (1:250, E0465, DAKO, Denmark) and then in Cy2-conjugated streptavidin (1:1000, PA42001, Amersham, UK). Selected sections were incubated for 5 min in a 0.00125 μg/ml solution of 4,6-diamino-2-phenylindole (DAPI) in TBS. Double stained sections were analyzed using a LEICA TCS SP2 AOBS confocal microscope.

As described in detail in a previous study [5], the number of BrdU+ cells in the dorsolateral subventricular zone (SVZ), RMS and SGZ of the immature rat brain peaked at P12, diminished slightly at P16 and decreased significantly by P23. Most BrdU+ cells in the three germinative zones colocalized with nestin+ filaments, indicative of progenitor cells.

The analysis of Cu/Zn SOD and BrdU double labeling showed that all Cu/Zn SOD+ cells showed BrdU labeling in the germinative zones. However, only a subpopulation of BrdU+ cells expressed Cu/Zn SOD in the SVZ (Table 1; Fig. 1b and c), in contrast to the constant colocalization of Cu/Zn SOD in BrdU+ cells of the SGZ and RMS (Table 1; Fig. 1p–s). In general, BrdU+ and Cu/Zn SOD+ double labeled cells in the germinative zones appeared as small and round-shaped, displaying typical progenitor cell morphology. The pattern of Cu/Zn SOD expression was consistent at all four ages analyzed: P9, P12, P16 and P23 but the quantity of BrdU+ cells expressing Cu/Zn SOD decreased by P23, paralleling the general decrease in BrdU+ cells previously reported.

nestin (arrow, t), vimentin (arrows, u), and PSA-NCAM (arrow, v), whereas no colocalization with NG2 was seen (w). In the SGZ, nestin (arrow, x) and vimentin (arrow, y) staining colocalized in Cu/Zn SOD+ progenitor cells, whereas little PSA-NCAM (arrow, z) and no NG2 was seen (aa). In photos b–k and p–aa, Cu/Zn SOD staining is shown in red and all other markers in green. In photos l–o BrdU staining is shown in red and all other markers in green. High magnification photos c, e, g, i, and k, correspond to photos b, d, f, h, and j, respectively. v: ventricle; L: lateral dorsolateral subventricular zone (LSVZ); M: medial dorslateral subventricular zone (MSVZ); sgz: subgranular zone; gcl: granular cell layer.

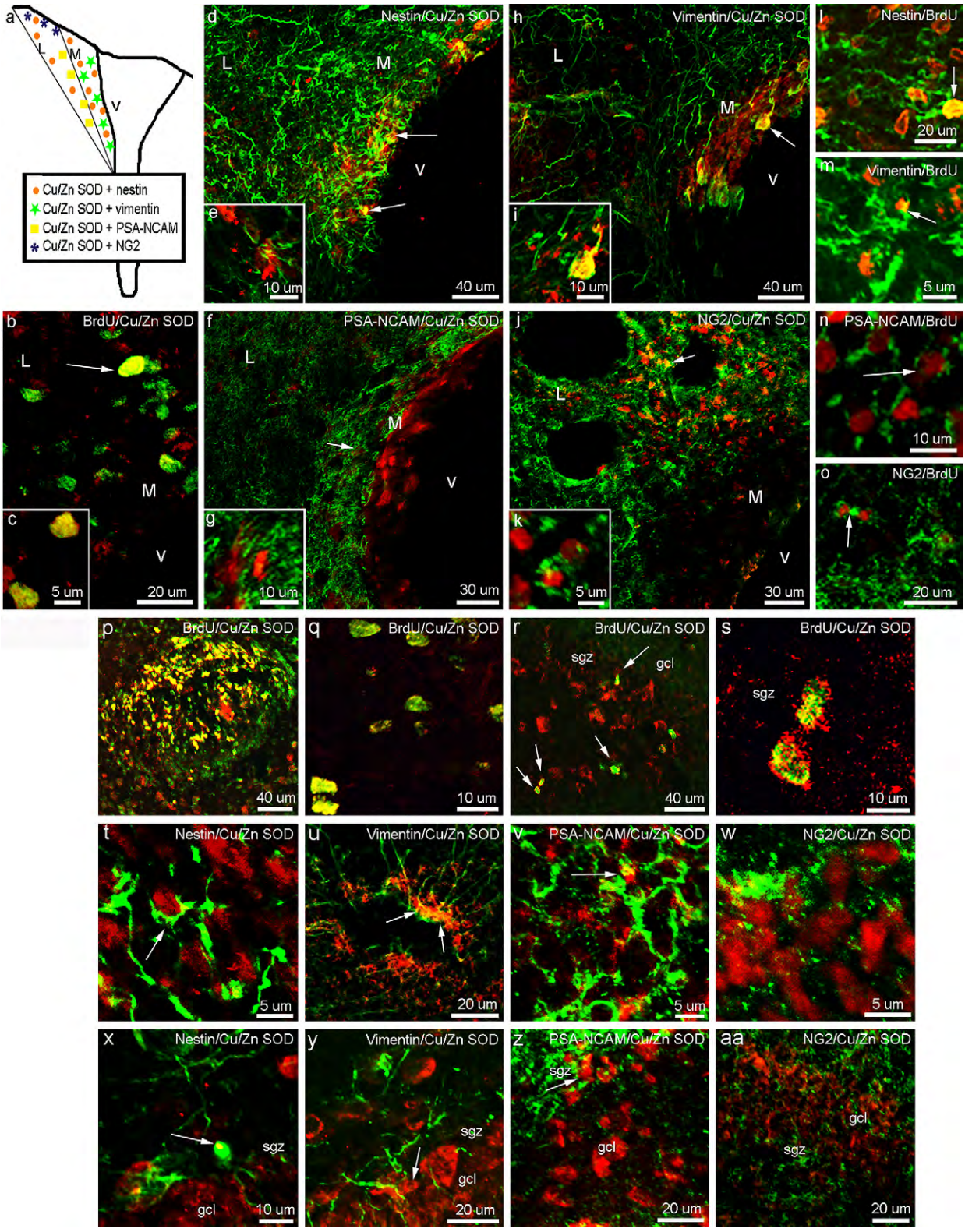

Fig. 1. The characterization of subtypes and distribution of Cu/Zn SOD+ cells in the SVZ, RMS and SGZ. Confocal microscope studies of coronal brain sections after double-labeled immunofluorescence combinations showed that Cu/Zn SOD was expressed in various subpopulations of progenitor cells located in either the lateral SVZ (L) or medial SVZ (M) (a). The majority of Cu/Zn SOD+/BrdU+ precursor cells were located in the medial SVZ (a; arrow, b; c) and were nestin+ (arrows, d; e; arrow, l). These cells also showed high vimentin expression (arrow, h; i; arrow, m) but almost no Cu/Zn SOD+/PSA-NCAM+ (f, g; arrow, n) or Cu/Zn SOD+/NG2+ (j) cells were seen. In the lateral SVZ cells were Cu/Zn SOD+/nestin+ (a, d) but very little Cu/Zn SOD/vimentin colocalization was seen (a, h). PSA-NCAM+ cells were Cu/Zn SOD+ in the area of the lateral SVZ bordering the medial SVZ (a; arrow, f), whereas NG2+/Cu/Zn SOD+ cells were located in the area of the lateral SVZ closest to the corpus callosum (a; arrow, j; k shows cells at the corpus callosum border; arrow, o). In the RMS (p; high magnification, q) and SGZ (arrows, r; high magnification, s) double labeling showed that virtually all BrdU+ precursor cells coexpressed Cu/Zn SOD. In the RMS Cu/Zn SOD+ cells colocalized with

In the SVZ, where only a subpopulation of BrdU+ cells expressed Cu/Zn SOD, double labeled cells were not homogeneously distributed (Fig. 1a). According to the anatomical division of the postnatal SVZ by Romanko et al. [33,34], Cu/Zn SOD+/BrdU+ cells were more abundant in the medial dorsolateral SVZ (MSVZ) (Fig. 1a and b), whereas fewer cells were seen in the lateral dorsolateral SVZ (LSVZ). In the MSVZ, Cu/Zn SOD+/BrdU+ cells, expressed nestin (Fig. 1d, e, l) and the majority of them also expressed vimentin (Fig. 1h, i, m). Almost no BrdU+ cells or Cu/Zn SOD+ cells showed PSA-NCAM (Fig. 1f, g, n) or NG2 double labeling (Fig. 1j, k, o) in this area. In the LSVZ, the few Cu/Zn SOD expressing cells showed nestin labeling (Fig. 1a and d). However, while most BrdU+ cells were vimentin+, very few Cu/Zn SOD+ cells showed vimentin expression (Fig. 1a and h), correlating with small number of BrdU+/Cu/Zn SOD+ cells in the LSVZ. A number of LSVZ Cu/Zn SOD+ cells adjacent to the MSVZ showed both BrdU and PSA-NCAM double labeling (Fig. 1a, f). Finally, a great number Cu/Zn SOD+ cells located in the MSVZ bordering the corpus callosum, showed both BrdU and NG2 labeling (Fig. 1a, j, k, o).

In the RMS, Cu/Zn SOD+ cells showed nestin (Fig. 1t), vimentin (Fig. 1u) and PSA-NCAM double labeling (Fig. 1v), whereas NG2 expression was limited to a few cells on the border of the migratory stream (Fig. 1w). In the SGZ, Cu/Zn SOD+ cells expressed both nestin (Fig. 1x) and vimentin (Fig. 1y). However, few of these cells were PSA-NCAM+ and no NG2+ cells were seen (Fig. 1z, aa).

These findings report the expression of Cu/Zn SOD for the first time in cell progenitor populations of the immature rat brain. Cu/Zn SOD expression was found consistently in BrdU labeled cells in the SGZ and RMS. The differential expression of Cu/Zn SOD in the SVZ supports many studies that describe the heterogeneity of progenitor pools in the germinative areas. Within the subventricular zone, a mix of multipotent, bipotent and lineage restricted progenitors has been described (for review see [18,25]). Subsets of radial glial cells have shown to be neuronal progenitors [8,10,24,29].

Our results suggest that Cu/Zn SOD may be important for early stages of all neural precursors and restricted to cells of the neuronal lineage as progenitors reach more differentiated stages. The restricted expression of Cu/Zn SOD in SVZ progenitor cells in contrast to the widespread expression of Cu/Zn SOD in progenitors located in the RMS and SGZ, could be attributed to the fact that Cu/Zn SOD is only expressed in certain populations of progenitor cells at certain stages in the maturation process. In the MSVZ, where most neural stem cells reside [33,34], Cu/Zn SOD labeling was seen in progenitors expressing the intermediate filaments nestin and vimentin, markers of shared early cell phenotypes; whereas in the LSVZ, where neural precursors are found [33,34], Cu/Zn SOD expression was mainly found in PSA-NCAM+ cells, suggesting Cu/Zn SOD expression in early precursors of the MSVZ that eventually differentiate into migrating neuroblasts of the LSVZ. Additionally, NG2 expression in some BrdU+ and Cu/Zn SOD+ cells was also seen at the border of the SVZ, possibly indicating the presence of an antioxidant system in an early oligodendrocyte progenitor that is deactivated upon differentiation and maturation, as NG2+ cells in the corpus callosum are both BrdU and Cu/Zn SOD negative. In agreement, in the RMS, Cu/Zn SOD expression was seen in early precursors (nestin+ and vimentin+) and migrating neuroblasts (PSA-NCAM+), again implying an antioxidant capacity in cells of the neuronal lineage. These patterns of Cu/Zn SOD correlate with the high constitutive expression found in mature neurons and the lack of Cu/Zn SOD expression in differentiated astrocytes and oligodendrocytes in the immature brain [31] and the adult brain [7,16,22,27,30,38,40].

Developmentally, Cu/Zn SOD levels are reported to peak around the second week of postnatal life and decline to reach adult levels by the first month [1,3]. Developmental changes in the concentration of this enzyme could form part of a protective strategy to combat increasing concentrations of oxygen when the fetus moves from a relatively hypoxic environment, in utero, to a hyperoxic environment after delivery [15]. It has been suggested that the SVZ is a relatively hypoxic environment [28], implying that at various stages of differentiation, progenitor cells would need certain antioxidant systems to ensure cell survival as they migrate out of the hypoxic SVZ, to other brain areas. In parallel, the differential gene expression in early neuronal precursors, could be linked to electrical excitability and so, increased endogenous energy requirements and overall oxidative stress. In progenitors destined to a neuronal fate, neuronal biochemical systems, such as Cu/Zn SOD expression, could be important to ensure their survival and differentiation capability.

Additionally, several studies suggest a role for reactive oxygen species (ROS) in a number of signaling pathways that may be involved in the differentiation and maturation of precursor cells, such as the ROS-dependent activation of neurotrophin receptors [11] and neurite extension in PC12 cells [13,14,37]. Accordingly, a recent in vitro study has been able to differentiate populations of progenitor cells, double labeled with BrdU and nestin, versus newborn neurons based on their low or high ROS content (H_2O_2 and O_2^-), respectively [39], implying that ROS and their associated antioxidant pathways could be used to distinguish different states of maturation in the progression from precursor cells to mature neurons.

Finally, O_2^- radicals are important mediators of oxidative damage after various types of brain injury and are highly implicated in cell death. H_2O_2, a product of the superoxide dismutation reaction catalyzed by SOD, is constantly generated during cellular metabolism and can activate several downstream signaling pathways involved in cell survival or apoptosis during oxidant insults in various cell types including neural progenitor cells [4,6]. In vivo studies of precursor cell death after radiation, chemotherapeutic drugs and hypoxic/ischemic injury in the immature brain have demonstrated the differential vulnerability of precursor cell populations in the SVZ [28,33,34]; populations of precursor cells with high proliferation rates in the LSVZ are more vulnerable to radiation, chemotherapy and hypoxia/ischemia, whereas neural stem cells with lower proliferating rates in the MSVZ are more resistant. Our results suggest that an increased antioxidant capacity in precursors of the MSVZ (with greater Cu/Zn SOD expression), could contribute to their

increased capacity to cope with cell death than LSVZ precursors (with restricted expression of Cu/Zn SOD).

In summary, the expression of Cu/Zn SOD in progenitor cells shown in this study may suggest Cu/Zn SOD as: (i) a fundamental part of the differential gene expression pattern associated with neuronal fate; (ii) a mechanism to prevent oxidative stress-induced death early in postnatal life in order to be able to form stable progenitor pools that remain in adulthood and/or; (iii) a mechanism used by neuronal progenitors in order to cope with increased energy requirements.

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