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Research report

Differential expression and tissue distribution of parkin isoforms during mouse development

Duong P. Huynh, Maria Dy, Dung Nguyen, Tim-Rasmus Kiehl, Stefan M. Pulst*

Division of Neurology and Rose Moss Laboratory for Parkinson and Neurodegenerative Diseases, Burns and Allen Research Institute, Cedars-Sinai Medical Center, UCLA School of Medicine, 8700 Beverley Boulevard, Los Angeles, CA 90048, USA

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Abstract

Mutations of the *parkin* gene are a cause of autosomal recessive juvenile parkinsonism. Although the *parkin* gene has been isolated from mouse, rat, and human, little is known about its expression in neural and nonneural tissues during development. In this study, we used a polyclonal antibody to a peptide downstream of the parkin ubiquitin domain to investigate (1) the differential expression of parkin isoforms in protein extracts from fetal and adult mouse tissues, and (2) the distribution of parkin in mouse fetal tissues at different developmental stages and in adult CNS tissues. By Western blot analyses, at least three isoforms of parkin of 22, 50, and 55 kDa were differentially expressed in mouse tissues. The p22 and p50 isoforms were found in fetal and adult mouse CNS tissues, while the p55 isoform was found only in adult tissues. The p50 isoform is the predominant form in both fetal and adult tissues. Immunolocalization in mouse fetuses showed that parkin was expressed only after neuronal differentiation. Although parkin was localized throughout the cytoplasm, the highest level of parkin was found in the neurites of both fetal and adult neurons.

Theme: Disorders of the nervous system

Topic: Degenerative disease: Parkinson's

Keywords: parkin; Early onset autosomal recessive juvenile Parkinson disease; Parkin expression; Mouse embryo

1. Introduction

Parkinson disease (PD) is a progressive neurodegenerative disease that is caused by loss of pigmented cells in the substantia nigra. The formation of Lewy bodies accompanies the characteristic depletion of pigmented cells. Lewy bodies are cytoplasmic inclusions in pigmented neurons. α-synuclein and ubiquitin are major constituents of Lewy bodies [5,8,16,17,24,25].

Two genes have recently been implicated in the development of familial parkinsonism. Missense mutations in the α-synuclein gene are a cause of autosomal dominant PD [2,20,21]. Mutation in the *parkin* gene [14] are involved in familial, levodopa-responsive, early onset autosomal recessive juvenile parkinsonism (AR-JP). These mutations range from point mutations to exonic deletions resulting in truncated gene products or parkin molecules containing pathogenic amino acid substitutions [1,14]. In AR-JP, substantia nigra neurons lack Lewy bodies [11,12,18].

Parkin is a 51.6 kDa protein with 465 amino acid residues. The N-terminal region comprising 76 amino acid residues has high homology with ubiquitin, a protein involved in proteasomal dependent protein degradation. Hence, this domain was designated the ubiquitin-like domain. In addition, parkin has two RING-finger motifs. RING 1 lies between residues 238 and 293, while RING 2 lies between residues 418 and 449 [14,22]. Interestingly, most inactivating mutations occur in the RING finger domains suggesting that these domains are essential for parkin function.

Analysis of the mouse *parkin* cDNA showed that the

*Corresponding author. Tel.: +1-310-423-5166; fax: +1-310-423-0149.

E-mail address: pulst@cshs.org (S.M. Pulst).

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parkin gene expresses at least two splice variants [15]. One variant consists of an open reading frame with 1392 bp that encodes a full-length parkin of 464 amino acids with a calculated molecular weight of 51.6 kDa. The other variant has an open reading frame of 783 bp encoding a protein with 261 amino acid residues, and a calculated weight of 28 kDa. This second isoform lacks the C-terminal RING domains [15]. Furthermore, Western blot analysis of rat brain homogenates revealed two specific protein bands at 55 and 44 kDa. The p55 band represents the full-length parkin, and the p44 band represents an unidentified parkin variant [7].

Exactly how mutations within the *parkin* gene lead to the death of dopaminergic neurons is unclear. Recent results confirmed the expression of parkin in neuronal cytoplasm and neurites, and its absence in glial fibrillary acidic protein (GFAP)-positive astrocytes [3,4,9]. Parkin interacts with the E2 ubiquitin-conjugating enzyme, UbcH8 [22,27]. Parkin also apparently has an E3 ubiquitin–protein ligase activity that allows it to regulate the degradation of misfolded proteins through the proteasomal pathway [22]. The ubiquitin–protein ligase activity of parkin can protect cells from cellular stresses induced by misfolded proteins [10]. In addition, the RING-finger domains interact with a synaptic vesicle-associated protein, CDCrel-1, aiding in its ubiquitination and subsequent degradation [27], leading to the hypothesis that parkin may be involved in regulating synaptic function. Mutant parkins from AR-JP patients did not have ubiquitin–protein ligase activity [22,27].

Recent findings indicate that parkin is associated with actin filaments pointing to the interaction of the cytoskeleton and protein complexes in neurodegeneration [9,23]. In mouse and rat adult brain tissues, high levels of parkin were found in the brain stem, substantia nigra, and striatum [3,4,6,26]. A subpopulation of parkin immunopositive neurons exhibited nuclear distribution suggesting a role of parkin in gene expression [26].

2. Materials and methods

2.1. Antibodies

We raised antibodies against peptide parkA (amino acids (aa) 96–109). The peptide was conjugated to keyhole limpet hemocyanin (KLH carrier) and injected into two rabbits. Antisera containing high ELISA titer and specifically detecting a band at the predicted size for parkin were subjected to affinity purification using the parkA peptide sepharose column. The parkA epitope is conserved between mouse and human parkin. These antibodies were characterized using protein extracts from human brain and monkey kidney COS1 cells [9].

2.2. Protein extraction and Western blotting

Fresh tissues were obtained separately from three different 4 month old B6/D2F2 mice (derived from C57BL/6J and DBA/2J) mice (Jackson Laboratories). Tissues were resuspended in triple detergent buffer (100 mM Tris–HCl (pH 7.4), 1 mM EGTA, 1% NP40, 0.5% sodium dodecyl sulfate (SDS), 0.5% deoxycholic acid, 1 mM Pefabloc SC, 1 μg/ml Pepstatin A, 2 μg/ml aprotinin, 50 μg/ml leupeptin), and homogenized using a polytron homogenizer. The protein extracts were first centrifuged at $1000\times g$ (3100 rpm in a JA17 rotor) for 5 min, followed by centrifugation at $105\,000\times g$ (54 000 rpm in a TLN100 rotor) for 1 h, and storage at −80°C. Protein concentration was determined using the Bradford Protein Assay Kit (BioRad). Prior to loading onto polyacrylamide gels, proteins were concentrated using Amicon Microcon 10 filters (Millipore), or acetone precipitation. Fifty micrograms of protein was loaded per lane in a precast 4–20% gradient SDS–polyacrylamide mini-gel (BioRad) and electrophoresed at 100 V for 1–2 h. Proteins were transferred to nitrocellulose filter (Amersham). The filter was rinsed briefly with TBS (150 mM NaCl, 50 mM Tris–HCl, pH 8.0), and blocked for 1 h with 5% nonfat dry milk (BioRad) for rabbit custom-made primary antibodies. The filter was then incubated with the desired dilution of tested antibody for 1 h at room temperature. The primary antibody was detected with the ECL Western blotting detection system (Amersham) using anti-rabbit IgG antibodies conjugated with horseradish peroxidase.

2.3. Immunohistochemistry

Sagittal embryonic sections at different embryonic stages of three different litters of NIH-Swiss embryos were purchased from Novagen (Madison, WI). Immunohistological analyses of adult brain tissues were performed on 4–6-month-old B6/D2F2 mice. These mice were perfusion fixed with 4% paraformaldehyde, followed by paraffin embedding for both coronal and sagittal sectioning. Both the embryonic and adult sections were rehydrated by rinsing three times at 5-min intervals in xylene, 100% ethanol, 95% ethanol, and 70% ethanol. After deparaffinization, sections were treated with a protease cocktail, blocked with avidin/biotin, and 3% normal goat serum. Sections were then incubated with 10 μg/ml of affinity-purified parkA antibody overnight at 4°C. Primary antibody was detected using the Vector rabbit ABC elite Peroxidase kit (Vector, CA), enhanced by DAB enhancer, and visualized with diaminobenzidine (DAB, Biomeda, CA). Sections were counterstained with aqueous hematoxylin (Xymed, CA). Controls consisted of antibody preabsorbed with 100 μM of the respective peptide. All slides for direct comparison were processed in a single batch to minimize variability. Anatomical structures of adult mouse

brain were identified using Paxinos and Franklin's mouse brain atlas [19]. The embryonic structures were identified using Kaufman's atlas of mouse development [13].

3. Results

To study the distribution of the parkin gene products, we had previously raised antibodies against synthetic peptide epitopes downstream of the ubiquitin-like domain of parkin [9]. In this study, we used the parkA antibody to investigate the distribution of parkin isoforms in mouse fetal and adult tissues, because its epitope is conserved in mouse and human. This antibody (parkA) specifically recognized proteins of 50 and 22 kDa in human brain tissues and monkey kidney COS1 cells [9].

3.1. Differential expression of parkin isoforms in mouse fetal and adult tissues

To investigate the relative distribution of the parkin isoforms in mouse development, we immunoblotted protein extracts from mouse embryos at embryonic days 13 (E13), E14, E15, E16 and adult mouse total brain using parkA antibody (Fig. 1a). The parkA antibody detected an intense band at 50 kDa and a faint band at 22 kDa in both embryonic and adult CNS tissues. Interestingly, the p22 protein was not detected in protein extracts isolated from the bodies of E15 (Fig. 1a, lane 3) and E16 embryos (Fig. 1a, lane 5), suggesting that the p22 isoform was expressed predominantly in neural tissues. In addition to the p22 and p50 proteins, a 55 kDa protein was also detected only in the adult mouse total protein extract (Fig. 1a, lane 7, arrow). All protein bands were absorbed out with the parkA peptide epitope indicating the specificity of the parkA antibody (Fig. 1a).

To determine the differential localization of the parkin isoforms, Western blots of protein extracts from specific brain regions (adult mouse cerebral cortex/diencephalon, cerebellum, brain stem, spinal cord) and internal organs (heart, liver, spleen, pancreas, and kidney) were detected with the parkA antibody (Fig. 1b). Both the p50 and p55 parkin isoforms were differentially distributed in these tissues. For example, both the cerebral cortex/diencephalon/thalamus and spleen expressed only the p50 parkin isoform (Fig. 1b, lanes 1, 7), while the liver (Fig. 1b, lane 6) expressed only the p55 isoform. In contrast, the cerebellum, brain stem, spinal cord, and kidney expressed both the p50 and p55 isoforms (Fig. 1b, lanes 2–4,9). The p50 isoform was predominantly expressed in neural tissues (Fig. 1b, lanes 2–4), while the p55 isoform was evident in the kidney (Fig. 1b, lane 9). Although the p22 protein was detected in whole brain homogenate (Fig. 1a, lane 7), it was undetectable in the subregional brain extracts or in nonneural tissues. This demonstrates a low level of the p22 protein in these tissues. The heart and pancreas did not express any parkin isoform. Immunoblotting the same blot with a monoclonal antibody to β-actin showed that protein extracts from all tissues contained similar amount of proteins suggesting that the differential levels of parkin isoforms among different tissues reflected quantitative differences of the expression of the parkin isoforms (data not shown).

3.2. Expression of parkin in mouse embryonic tissues

Fig. 2A shows low-magnification views of paraffin sections of mouse embryos labeled with the parkA antibody. From E8 to E9, parkin was not detectable in embryonic tissues although extraembryonic tissues showed strong levels of parkA immunolabeling (Fig. 2). In late E10, faint labeling with the parkA antibody was observed in postmitotic neurons in the mantle layer of the developing nervous system. However, parkin remained undetectable in mitotic neuroepithelia of the ependymal layer and in non-CNS embryonic tissues.

At E11–E12, parkin labeling increased in the mantle layer. This increased parkin immunoreactivity was probably due to the growing density of postmitotic neurons. Furthermore, the marginal layer, which consists of migrating nerve fibers of postmitotic neurons, showed high levels of parkin immunoreactivity at the tips of the migrating nerve fibers (Fig. 2B, E14). No parkin immunoreactivity was detected in the heart, lung, liver, and intestine. Although there were undetectable levels of parkin in the cell bodies of the dorsal root ganglia neurons, the nerve fibers of these sensory neurons exhibited high levels of parkin (Fig. 3A, panel g). Indeed, parkin appeared to be more concentrated in the nerve fibers as neurons mature. Starting at E13–E14, the level of parkin abruptly increased severalfold in postmitotic neurons. This sudden surge of parkin immunoreactivity began to level off at E15–E16 (Fig. 2B).

At E15–E16, as more specialized neurons and nonneural cells are formed, the distribution of parkin also became more tissue specific (Fig. 3A). In the neocortical area (Fig. 3A, panel a), mitotic neuroepithelia in the ventricular layer were devoid of parkin immunoreactivity. In contrast, moderate levels of parkin were observed in the migrating postmitotic neurons in the intermediate and neopallial layers. In the diencephalon and other CNS regions, the highest level of parkin was observed in the neurites, while the weakest level of parkin was observed in the cell bodies (Fig. 3A, panel b). No nuclear labeling was found in these maturing neurons. In nonneural tissues, high levels of parkin were found in the muscle walls of the intestine (Fig. 3A, panels d,e), the blood vessels (Fig. 3A, panel f), and the dermis (Fig. 3A, panel h). Parkin was undetectable in liver cells and intestinal villi (Fig. 3A, panel d), lung (Fig.

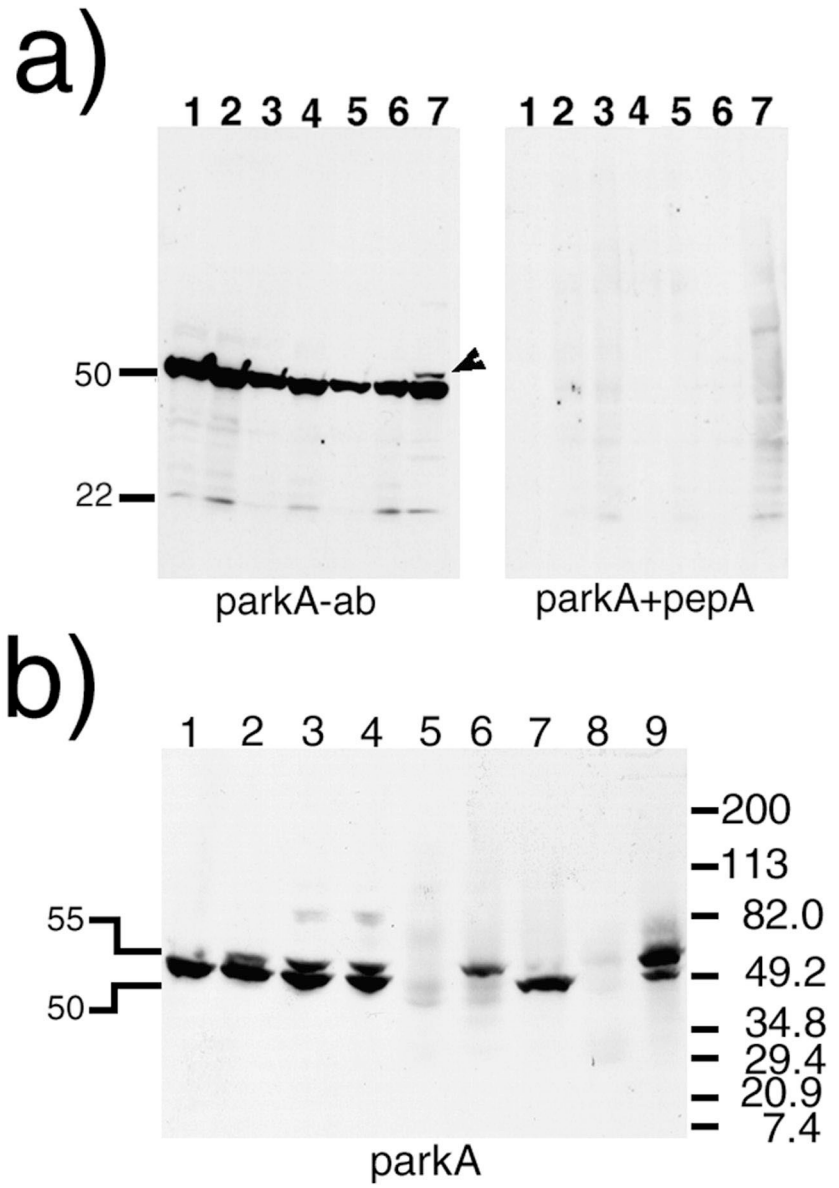


Fig. 1. Expression of parkin in mouse tissues. (a) Immunoblot of protein extracts from embryos at embryonic ages E13 (lane 1), E14 (lane 2), E15 body (lane 3), E15 head (lane 4), E16 body (lane 5), lane E16 head (lane 6), and whole adult mouse brain (lane 7). The parkA antibody detected an abundant protein with a MW of 50 kDa in both mouse embryo tissues and adult brain. The parkA antibody also detected a faint band at 22 kDa in the E13, E14, E15 and E16 heads, and the adult brain. The 55 kDa band was detected only in the adult mouse brain. All bands were absorbed out by addition of the parkA peptide to the antibody indicating the specificity of the parkA antibody. (b) Immunoblot of protein extracts from the cerebral cortex/diencephalon (lane 1), cerebellum (lane 2), brain stem (lane 3), spinal cord (lane 4), heart (lane 5), liver (lane 6), spleen (lane 7), pancreas (lane 8), and kidney (lane 9) with the park A antibody. The 50 kDa protein was detected strongly in all four major areas of the nervous system. The 55 kDa protein was detected in the cerebellum, brain stem, and spinal cord but it was absent in the cerebral cortex/diencephalon/thalamus area. In nonneural tissues, the liver expressed only the 55 kDa protein, the spleen expressed only the 50 kDa protein, while the kidney expressed a high level of the 55 kDa protein and a much lower level of the 50 kDa protein. The heart and pancreas did not express any parkin isoforms.

3A, panel f), heart (Fig. 3A, panel f), bone (Fig. 3A, panel f), and epidermis (Fig. 3A, panel h).

As neurons mature, axons and dendrites are formed, and some of these nerve fibers migrate great distances from the cell bodies to connect with their target tissues. As nerve fibers migrate away from the cell bodies, the level of parkin increased (Fig. 3A, panels c,g). The level of parkin increased dramatically at E14 and remained at this level through E16 and in adult mouse brain.

3.3. Expression of parkin in adult CNS tissues

Similar to the embryonic distribution, parkin was mainly localized in nerve fibers rather than in cell bodies. In the

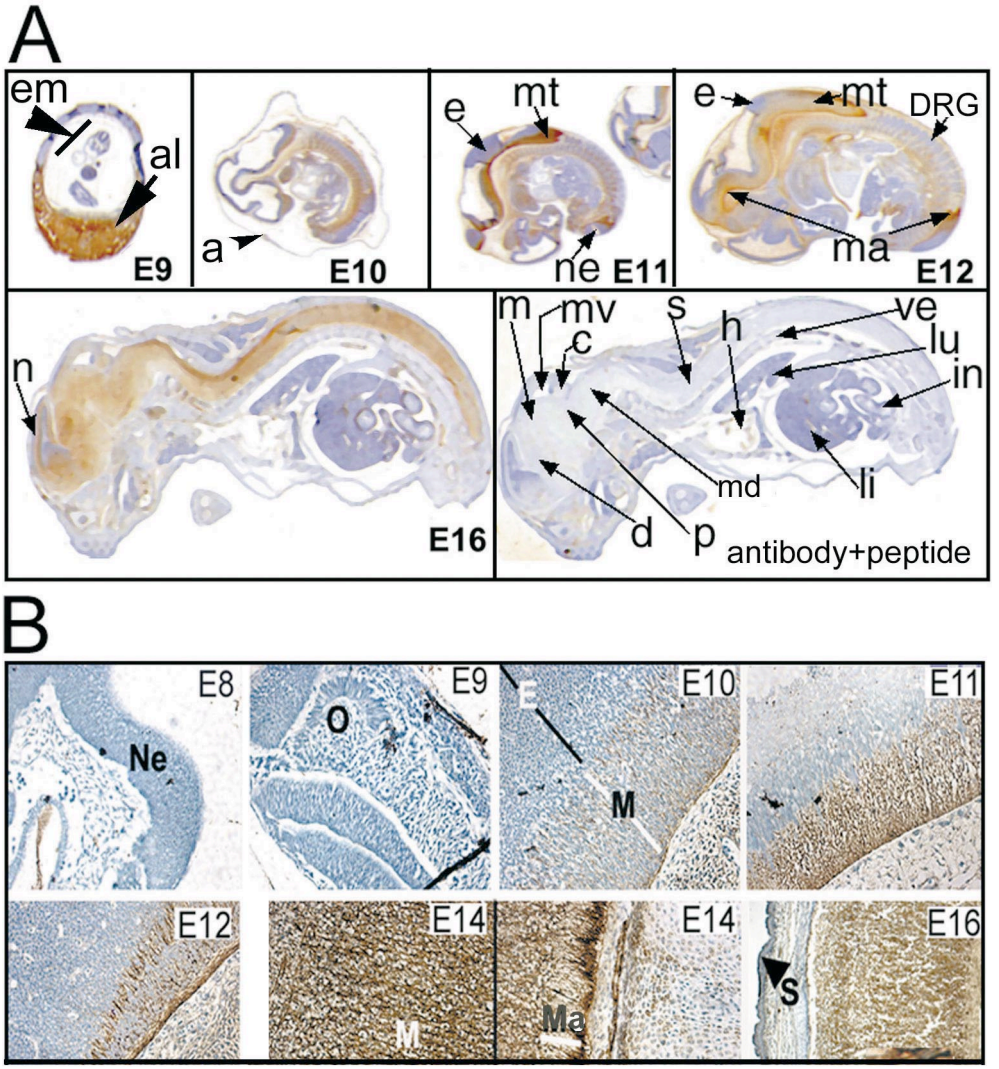
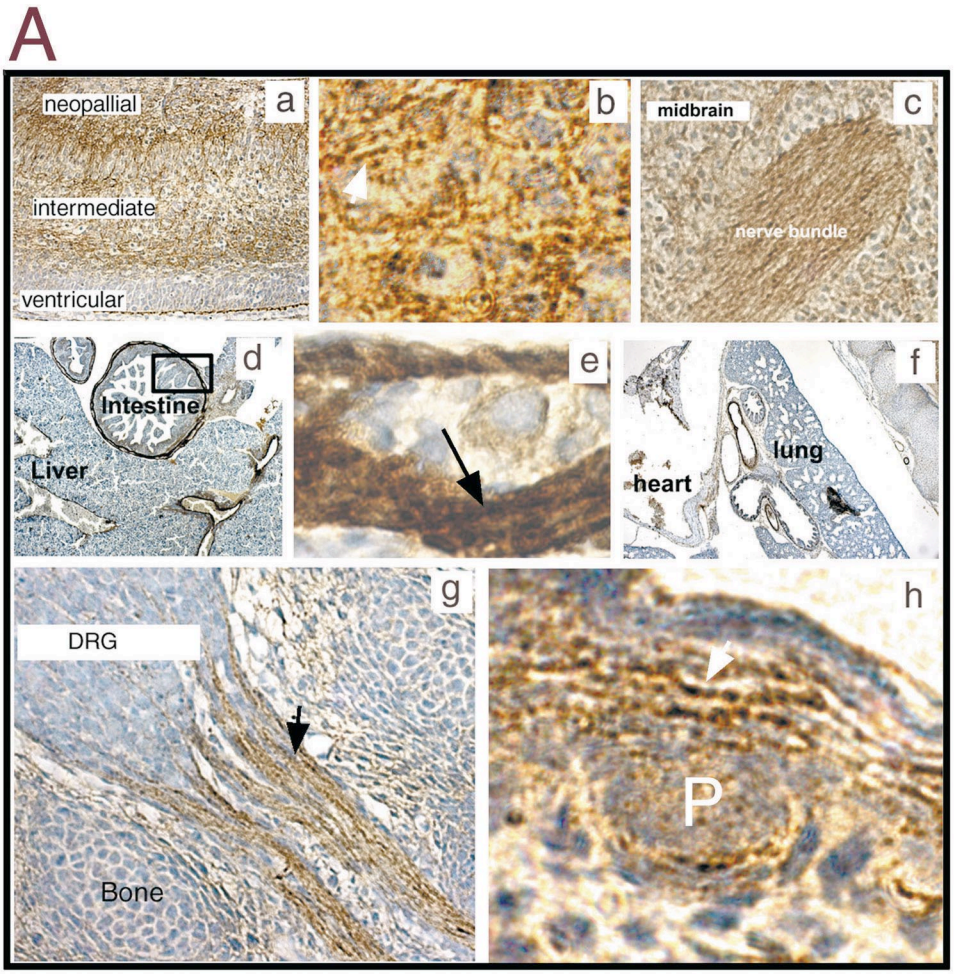


Fig. 2. Predominant expression of parkin in the developing nervous system. Sections of mouse embryos were labeled with 10 μg/ml affinity purified parkA antibody or with parkA antibody preabsorbed with 100 μM parkA peptide (lower right hand corner). Labeling was visualized with diaminobenzidene (DAB, brown color), and the nucleus was counterstained with hematoxylin (blue). (A) Whole mouse embryos showing prominent parkin labeling of the developing nervous system in embryonic days E10, E11, E12, and E16. However, no parkin immunoreactivity was found in embryonic day E8 (not shown) and E9. Magnification: 4× for E9–E12, 2× for E16. Arrows point to tissues using the following abbreviations. al, allantois; em, embryo; mt, mantle layer; a, amnion; e, ependymal layer; ne, neural tube; ma, marginal layer; DRG, dorsal root ganglion; n, neopallial layer; m, midbrain; h, heart; mv, mesencephalic vesicle; c, cerebellar primordium; p, pons; d, diencephalon; s, spinal cord; li, liver; ve, vein; lu, lung; in, intestine; md, medulla oblongata. (B) Developmental expression of parkin. Parkin is found only in postmitotic neurons in the mantle layer but not in proliferating neuroepithelia in embryonic days E8 through E16. Parkin is particularly concentrated in the migrating nerve fibers in both the mantle (M) and marginal (Ma-) layers but it is lacking in the neural tube (Ne) and mantle (M) layer. Magnification: 50× for all panels; O, optic; S, skin (black arrow). For E8 and E9, images were taken from the cranial neural tube, for E10–E4, images were taken within the spinal cord.

cerebral cortex, granule neurons in layer 2 were strongly immunoreactive while those neurons in other layers were moderately immunoreactive. In the hippocampus, cell bodies and mossy fibers of pyramidal neurons in all CA regions were parkin-immunoreactive (Fig. 3B, panels g–h). No parkin nuclear localization was observed in the hippocampus. Granule neurons of the dentate gyrus were weakly labeled by the parkA antibody.

In the basal ganglia, the three parts of the striatum — the caudate nucleus, putamen, and nucleus accumbens — were strongly labeled (Fig. 3B and Table 1). Parkin was found in the cytoplasm and in neuronal processes. However, nerve fiber bundles in the putamen lacked parkin immunoreactivity. In the output nuclei, the internal and ventral globus pallidus were labeled weakly. In comparison, the substantia nigra (Fig. 3B, panel f) exhibited labeling as strong as the putamen. The thalamus, which receives input from the globus pallidus and substantia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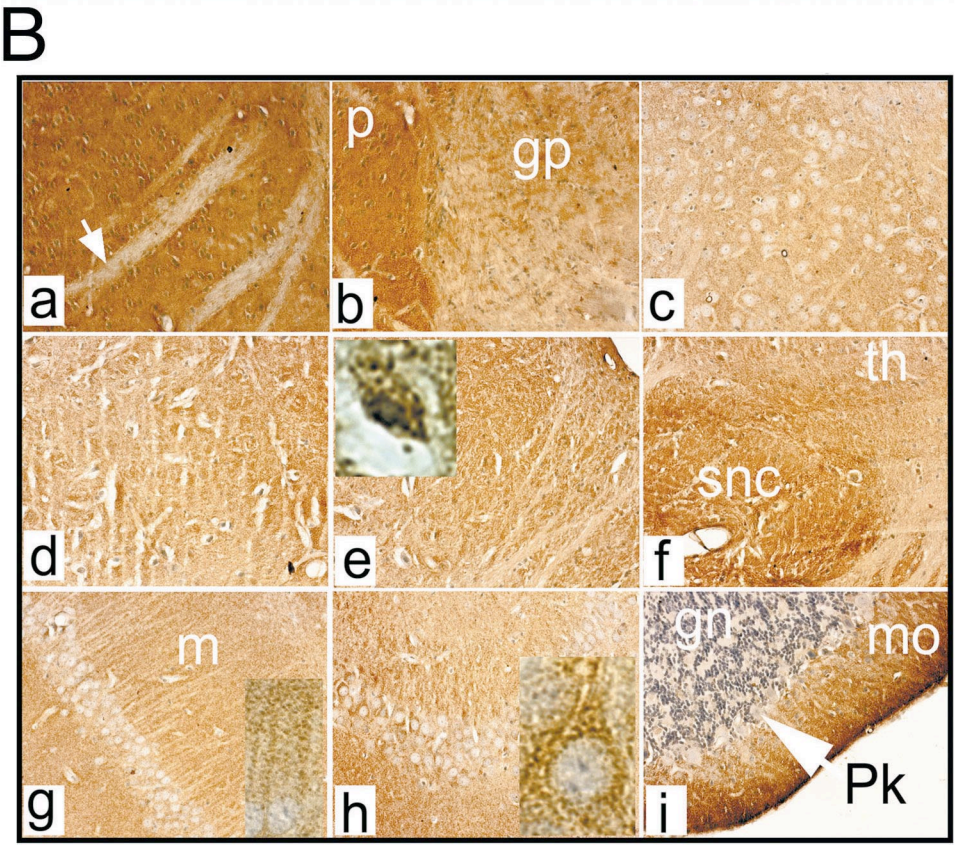
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nigra, was also strongly immunoreactive (Fig. 3B, panel d). In contrast, the hypothalamus and subthalamus were moderately labeled.

In the cerebellum, the molecular layer, which consists of nerve processes, showed strong parkin immunoreactivity, while the Purkinje cell body layer and granule neurons were faintly labeled (Fig. 3B, panel i). The white matter was also strongly labeled. In the medulla and pons nerve fiber bundles were strongly parkin immunoreactive, while cell bodies were weakly labeled.

4. Discussion

Mutations of the *parkin* gene cause autosomal recessive, juvenile Parkinsonism (AR-JP), a form of parkinsonism that does not have Lewy bodies. Recent evidence suggests that parkin probably functions as a ubiquitin–protein ligase to polymerize proteins targeted for degradation in the ubiquitin-dependent proteasomal pathway [10,22,27]. Immunohistochemical localization studies of human tissues also suggested that parkin was expressed in neuronal groups that are not primarily involved in the disease [9,23]. Although parkin was ubiquitously expressed in the nervous system, only a subset of neurons was affected by inactivating mutations of the *parkin* gene. These observations suggested that there are other factors involved in protecting nonaffected neurons from cell death in AR-JP. These factors may include specific parkin interactors or specific tissue distribution of parkin homologues or splice variants. This study investigated the distribution of parkin isoforms in mouse adult and embryonic tissues to determine tissue specific expression of the parkin isoforms by Western blot analyses of specific CNS regions and of nonneural tissues. Subcellular localization of parkin was further determined using immunohistochemical localization.

4.1. Biochemical analysis

Previous studies have found that parkin has at least three variants [7,9,15]. We found two proteins of 50 and 22 kDa in human brain protein extracts [9], while Horowitz et al. [7] detected two proteins of 50 and 44 kDa in rat brain homogenates. In this study, all three proteins were detected in whole adult mouse brain homogenates, as p22, p50, and p55 proteins (Fig. 1a). The reason we could resolve the p50 and p55 proteins clearly was that we used a 4–20% gradient polyacrylamide gel electrophoresis to resolve the

Table 1
Relative expression levels of parkin in different areas of mouse adult brains

Area/subregions	Parkin immunoreactivity
Basal ganglia	
1. Caudate nucleus	++++++
2. Putamen	++++++
3. Nucleus accumbens	+++
4. Internal globus pallidus	+++
5. Ventral pallidum	+++
Cerebral cortex	
Layer 1: molecular layer	++++++
Layer 2: external granule	++
Layer 3: pyramidal	++
Layer 4: internal granule	++
Layer 5/6: ganglionic fusiform layer	++
Diencephalon	
1. Thalamus	+++++
2. Hypothalamus	++
3. Subthalamus	++
4. Habenuclei	−/+
Cerebellum	
1. Molecular layer	+++++
2. Purkinje cell layer	+
3. Granule layer	+
4. White matter	+++++
Brainstem	
SN pars compacta	++++++
SN pars reticulata	++++++
Hippocampus	
1. Molecular layer	++++
2. CA1	++
3. CA2	++
4. CA3	+++
Mossy fibers	++++
Cell bodies	++

Brain slices from three adult mice were labeled simultaneously with affinity purified parkA antibody. The level of parkin was judged by comparing DAB deposits in specified CNS regions. Immunoreactivity intensity is described in each subregion as indicated by plus or minus signs. The negative (−) sign denotes undetectable parkin immunoreactivity, while the plus (+) sign denotes detectable parkin immunoreactivity.

Fig. 3. (A) Expression of parkin in selected tissues in mouse embryos. (a) E16 neocortex. Parkin is detected in the intermediate and neopallial zones of the developing neocortex. No parkin is detected in the proliferating ventricular zone. Insert shows a high magnification view of a migrating neuron. (b) Differential interference contrast (DIC) image of the intermediate zone in (a). (c) E16 midbrain section showing strong parkin labeling in nerve fibers. (d) E16 intestine and liver. No parkin is found in the hepatocytes of the liver, and intestinal villi epithelia. (e) DIC image of the intestinal villi showing high level of parkin in the muscularis externa of the intestine. (f) E16 lung and heart. Strong parkin immunoreactivity is detected in the blood vessels, but no parkin is detected in the lung or in cardiac muscle. (g) E12 dorsal root ganglion, parkin was found predominantly in nerve fibers (black arrow). (h) DIC image of the skin at E16 showing strong labeling in the dermis. P, Pacinian corpuscle. Magnification: 50× for a,c,d,f,g; 175× for b,e,h. (B) Expression of parkin in adult mouse brains. (a) Putamen; white arrow points to nerve bundle (b), putamen (p) and globus pallidus (gp); (c) thalamus, (d) lateral substantia nigra, (e) reticular substantia nigra; insert shows high magnification view of nigral neurons with perinuclear and punctated labeling; (f) substantia nigra compacta (snc), (g) CA1 region of the hippocampus (m, mossy fiber), (h) CA3 region of the hippocampus, (i) cerebellum. Inserts (in panels g and h) show high magnification views of pyramidal neurons with strong labeling in the neurites. gn, granule neurons; mo, molecular layer; Pk, Purkinje neurons. Magnification 50× for a–i; 175× for inserts.

mouse protein extracts. The p50 species is the most predominant form in CNS tissue, and it is likely the same protein as the p44 detected in rat brain described by Horowitz et al. [7]. Furthermore, the distribution of these proteins was tissue specific. Only the p50 isoform was found in the cerebral cortex/diencephalon, spleen, and mouse embryonic fractions, while both the p50 and p55 proteins were found in the cerebellum, brain stem, spinal cord, and kidney (Fig. 1). The p50 protein is the predominant isoform in the CNS. The absence of the p55 protein in fetal tissues suggests that this isoform may have specific functions in mature neurons.

The p55 protein may be a posttranslationally modified parkin or its splicing variant. Molecular cloning of the mouse *parkin* gene has identified two splicing variants [15]. One is the full-length mouse parkin homologue encoding a protein with a calculated molecular weight (MW) of 52 kDa, which is represented by the p50 band. The other is a truncating splice variant encoding a protein with a calculated MW of 28 kDa, which is probably represented by the p22 band. However, there is no known *parkin* mRNA transcript that encodes a protein with a MW of 55 kDa, although immunoblots of rat brain homogenate also identified two proteins with 44 and 50 kDa MW [7]. It is possible that both of these proteins correspond to our p50 and p55 proteins, and the discrepancy between our results was due to differences in methodologies. Therefore, the p55 protein may be derived from either an unknown parkin splice variant containing an additional exon, or from a posttranslationally altered parkin modified by addition of other molecular species. In either case, the observation that the p55 protein was detected in the mitochondrial fraction of a human neuroblastoma cell line [9] suggests that the p55 parkin isoform may have an unidentified mitochondrial function.

4.2. Restricted expression of the parkin gene during mouse development

The earliest parkin expression was detected in late E10 embryos. However, parkin labeling was restricted to postmitotic neurons in the mantle layer. No parkin was detected in mitotic neuroepithelial, mesenchymal, or other nonneural cells until later stages of development (Fig. 2A). At subsequent developmental stages, parkin was found in the peripheral nervous system such as the nerve fibers of the dorsal root ganglia (Fig. 3A, panel g). Although Western blots of adult tissues have detected parkin in the liver, spleen, and kidney (Fig. 1b), no parkin-immunoreactive labeling was found in these tissues up to E16. This suggests that parkin expression began after E16 in nonneural tissues. This may be related to expression of the p55 parkin isoform, which is present only in adult CNS and nonneural tissues. These data further support the hypothesis that the p50 parkin isoform may be the functionally most important isoform in mouse tissues. It will be of interest to compare the expression pattern of the *parkin* gene in different human nonneural tissues and CNS areas.

In the nervous system, two examples of rapid changes in parkin levels related to neuronal migration and differentiation were identified. First, postmitotic neurons in the mantle layer of the neural tube were strongly labeled, but proliferating neuroepithelia in the ependymal layer were devoid of parkin immunoreactivity (Fig. 2B). The second example involved parkin expression during the formation of the neocortex. Cells in the ventricular zone that were actively undergoing mitosis did not label with parkin. However, postmitotic migrating neurons in the intermediate layer and mature neurons in the neopallial layer were strongly immunoreactive (Fig. 3A, panel a).

As previously observed in human brain tissues [9], parkin labeling was detected in the nerve fibers of embryonic and adult mouse brain (Figs. 2 and 3). In the adult brain, high levels of parkin were observed in the molecular layers of the cerebral cortex and cerebellum, the nerve bundles of the substantia nigra, the thalamus, and brain stem. In the developing nervous system, high levels of parkin were found in the nerve fibers of the developing neocortex (Fig. 3A, panel a), the diencephalon (Fig. 3A, panel b), dorsal root ganglia (Fig. 3A, panel g), and the spinal cord (Fig. 2B). In the developing spinal cord, intense parkin immunoreactivity was seen in the outer border of the marginal layer (Fig. 2B, E14).

Recently, several findings have suggested that parkin might be associated with synaptic vesicles. First, parkin was found to interact with CDCrel-1, a synaptic vesicle-associated protein of the septin family. Parkin appears to regulate the level of this protein through the ubiquitin-dependent 26S proteasomal pathway [27]. Secondly, parkin was found colocalized with actin filaments [9], which are known to regulate synaptic vesicle endocytosis. Thirdly, using yeast two-hybrid assays, we have found that parkin also interacts with a member of the synaptotagmin family (Huynh and Pulst, in preparation). Both the synaptotagmin and septin family of proteins are known to function in synaptic vesicle transport, fusion, or recycling. The observation that parkin was abundantly localized in both fetal and adult neurites but weakly in the cytoplasm further supports these observations.

Although parkin was found to be expressed in many different neurons as previously reported [4,6,7,26], close examinations of the immunohistochemically labeled tissues showed great differences in parkin immunoreactive intensity among different areas of the brain, with strongest immunoreactivity reserved for the putamen and substantia nigra compacta (Fig. 3 and Table 1). Within the cerebellum and hippocampus, strong labeling was found in the nerve fibers of hippocampal and Purkinje neurons while the cell bodies of these neurons were weakly labeled (Fig. 3B, panels g,i). These observations place parkin in areas enriched with synaptic transmission activities. In addition, striatal nuclei exhibited different expression levels of

parkin. For example, the levels of parkin were highest in the input nuclei such as the caudate nucleus and putamen. In contrast, lower levels of parkin were found in the output nuclei, the internal globus pallidus and the ventral pallidum (Fig. 3B). Taken together, these observations show that parkin is particularly concentrated within the dopaminergic neural pathways, and suggest that parkin may be involved in regulating the level of dopamine release from dopaminergic neurons.

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