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IMMUNOHISTOCHEMICAL ANALYSIS OF HUNTINGTIN-ASSOCIATED PROTEIN 1 IN ADULT RAT SPINAL CORD AND ITS REGIONAL RELATIONSHIP WITH ANDROGEN RECEPTOR

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Abstract—Huntingtin-associated protein 1 (HAP1) is a neuronal interactor with causatively polyglutamine (polyQ)-expanded huntingtin in Huntington's disease and also associated with pathologically polyQ-expanded androgen receptor (AR) in spinobulbar muscular atrophy (SBMA), being considered as a protective factor against neurodegenerative apoptosis. In normal brains, it is abundantly expressed particularly in the limbic-hypothalamic regions that tend to be spared from neurodegeneration, whereas the areas with little HAP1 expression, including the striatum, thalamus, cerebral neocortex and cerebellum, are targets in several neurodegenerative diseases. While the spinal cord is another major neurodegenerative target, HAP1-immunoreactive (ir) structures have yet to be determined there. In the current study, HAP1 expression was immunohistochemically evaluated in light and electron microscopy through the cervical, thoracic, lumbar, and sacral spinal cords of the adult male rat. Our results showed that HAP1 is specifically expressed in neurons through the spinal segments and that more than 90% of neurons expressed HAP1 in lamina I–II, lamina X, and autonomic preganglionic regions. Double-immunostaining for HAP1 and AR demonstrated that more than 80% of neurons expressed both in laminae I-II and X. In contrast, HAP1 was specifically lacking in the lamina IX motoneurons with or without AR expression. The present study first demonstrated that HAP1 is abundantly expressed in spinal neurons of the somatosensory, viscerosensory, and autonomic regions but absent in somatomotor neurons, suggesting that the spinal motoneurons are, due to lack of putative HAP1 protectivity, more vulnerable to stresses in neurodegenerative diseases than other HAP1-expressing neurons probably involved in spinal sensory and autonomic functions.

Key words: huntingtin-associated protein 1, stigmoid body, motoneurons, neurodegenerative diseases, neuroprotection, immunohistochemistry.

INTRODUCTION

Huntingtin-associated protein 1 (HAP1) was first identified as a polyglutamine (polyQ) length-dependent interactor of huntingtin (Htt) in the brain, the gene product responsible for Huntington's disease (Li et al., 1995). Transfection of HAP1 cDNA into cultured cells can induce occurrence of a distinct cytoplasmic inclusion, the stigmoid body (STB) (Li et al., 1998a; Nagano et al., 1999; Takeshita et al., 2006; Fujinaga et al., 2007). The STB was originally identified as a spherical-to-ovoid, non-membrane-bound neuronal inclusion (0.5–3 μm in diameter) with a granular, fuzzy texture and moderate-to-low electron density in rat brains (Shinoda et al., 1992, 1993). In normal rodent brains, HAP1 is localized to the STB (Gutekunst et al., 1998; Li et al., 1998a; Fujinaga et al., 2007, 2009; Islam et al., 2012), and the STB formation is suppressed in HAP1 (+/−) hetero-mice (Li et al., 2003), suggestive of HAP1 as an essential component and a determinant marker of the STBs (Fujinaga et al., 2007). HAP1 is abundantly expressed in the rat and mouse limbic regions, shown to be particularly concentrated in the preoptic-hypothalamic areas and medial amygdala, by Western blotting (Li et al., 1998a,b; Fujinaga et al., 2007; Sheng et al., 2008), reverse transcription polymerase chain reaction (RT-PCR) (Li et al., 2000), immunohistochemistry (Gutekunst et al., 1998; Fujinaga et al., 2007, 2009; Islam et al., 2012), and *in situ* hybridization (Li et al., 1996; Page et al., 1998; Dragatsis et al., 2000; Fujinaga

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Abbreviations: ALS, amyotrophic lateral sclerosis; AR, androgen receptor; BDNF, brain-derived neurotrophic factor; CGRP, calcitonin gene-related peptide; ChAT, choline acetyltransferase; DAB, diaminobenzidine; DLN, dorsal nucleus; ENK, enkephalin; GFAP, glial fibrillary acidic protein; HAP1, huntingtin-associated protein 1; Htt, Huntingtin; Iba-1, ionized calcium-binding adapter molecule 1; ICL, intercalated nucleus; IML, intermediolateral nucleus; ir, immunoreactive; KO, knockout; LDCom, lumbar dorsal commissural nucleus; LMN, lower motor neurons; LSPN, lumbosacral parasympathetic preganglionic neurons; MND, motor neuron diseases; NDS, normal donkey serum; NeuN, neuronal nuclei; nNOS, neuronal nitric oxide synthase; Olig2, oligodendrocyte linage transcription factor 2; PB, phosphate buffer; PBS, phosphate-buffered saline; polyQ, polyglutamine; RDLN, retrodorsal nucleus; RT-PCR, reverse transcription polymerase chain reaction; SBMA, spinobulbar muscular atrophy; SCA, spinocerebellar ataxia; SDCom, sacral dorsal commissural nucleus; SDS, sodium dodecyl sulfate; SMA, spinal muscular atrophy; SNB, spinal nucleus of bulbocavernosus; SP, substance P; STB, stigmoid body; TBST, tris-buffered saline with 0.1% Tween; UMN, upper motor neurons.

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et al., 2004). Studies using *Hap1*-knockout (KO) mice have indicated that HAP1 plays an important role in hypothalamic functions, including the maintenance of neuronal survival (Li et al., 2003), regulation of food intake and body weight, and control of the locomotor activity (Chan et al., 2002; Dragatsis et al., 2004; Sheng et al., 2008; Lin et al., 2010).

Intriguingly, the areas with little STB/HAP1, including the striatum, thalamus, cerebral neocortex and cerebellum, are neurodegenerative targets in Huntington's disease, while the STB/HAP1-abundant regions tend to be spared from neurodegeneration. In addition, apoptosis or neurodegeneration is facilitated in the hypothalamus of *Hap1*-KO mice (Li et al., 2003). Thus, we have previously hypothesized that the STB/HAP1 serves as a protective factor against apoptosis induced by polyQ-expanded Htt mutants (Kamei et al., 2001; Koga et al., 2002; Fujinaga et al., 2004) rather than as a toxic enhancer of Htt mutants (Li et al., 1996; Gutekunst et al., 1998).

The STB/HAP1 has also been demonstrated to be co-expressed with steroid receptors (Nagano and Shinoda, 1994; Islam et al., 2012), interacting with them and regulating their nuclear translocation (Takeshita et al., 2006; Fujinaga et al., 2011), thus suggestive of its critical effects on the hormonal functions. Above all, the STB/HAP1 can also bind to AR in a polyQ-length dependent manner, more strongly sequester AR mutants with more expanded-polyQ derived from the spinal and bulbar muscular atrophy (SBMA) (Takeshita et al., 2006), and suppresses the SBMA-mutant-AR-induced apoptosis via inhibition of its nuclear translocation from cytoplasm (Takeshita et al., 2006). In addition, the STB/HAP1 can also interact with other polyQ-disease-related gene products, including ataxin-3 in spinocerebellar ataxia (SCA) type 3 (Takeshita et al., 2011) and TATA-binding protein in SCA type 17 (Prigge and Schmidt, 2007), and suppresses their nuclear translocation. Furthermore, it can interact with Abelson helper integration site 1 (Sheng et al., 2008), mutations which lead to Joubert syndrome (Dixon-Salazar et al., 2004; Ferland et al., 2004) and schizophrenia (Amann-Zalcenstein et al., 2006; Ingason et al., 2007). The line of data strongly suggests that the "STB/HAP1 protection hypothesis" could be applied more widely to neurodegenerative diseases other than Huntington's disease. The STB/HAP1 expression could raise the threshold of vulnerability for neurodegeneration and might render more beneficial stability to neurons (Fujinaga et al., 2004; Takeshita et al., 2006, 2011).

The spinal cord is another major degenerative target in the neurological disorders, particularly in the spinal motoneuron diseases (Rezania and Roos, 2013). Only limited data regarding HAP1 expression, however, have been reported in the spinal cord by Western blotting (Li et al., 1998a), RT-PCR (Li et al., 2000) and *in situ* hybridization (Dragatsis et al., 2000). Owing to lack of immunohistochemical analysis, the localization and distribution of HAP1 in the spinal cord have yet to be clarified. In the current study, we set out to ensure the spinal HAP1 expression by Western blotting, to identify the HAP1-expressing cells immunohistochemically in light and electron microscopy, and to clarify its detailed distribution through the cervical, thoracic, lumbar, and sacral spinal cords of the adult male rat. Finally, we attempted to clarify the regional relationship between HAP1 and AR in the context of SBMA pathogenesis.

EXPERIMENTAL PROCEDURES

Characterization of primary antibodies

The details of the primary antibodies used in these experiments are listed in Table 1. A rabbit polyclonal anti-HAP1 antibody (R12) was produced in our laboratory by immunization with glutathione-S-transferase-fused rat HAP1$^{70–433}$ (Table 1). The specificity of this antibody has been confirmed in our previous study (Fujinaga et al., 2007). We have also used a goat polyclonal anti-HAP1 antibody (R19) that was raised against a peptide mapped to the C-terminus of HAP1 of rat origin (manufacturer's technical information; see also Table 1). The specificity of this antibody has been validated for rat brain in our previous study (Islam et al., 2012). In the present study we have also characterized the specificity of this antibody in the spinal cord by Western blotting and immunohistochemistry (See results, Fig. 1).

Other commercially obtained antibodies used in this study were mostly chosen from the Antibody Registry of the Neuroscience Information Framework and their specificity was demonstrated previously by others (Table 1).

Animals

Thirty Wistar male rats (10 weeks old) were acquired from Japan SLC Inc. (Shizuoka, Japan). They were housed under controlled temperature and provided food and water *ad libitum*. Experimental protocols used in this study were approved by the Committee on the Ethics of Animal Experimentation at Yamaguchi University School of Medicine and conducted according to the guidelines for Animal Research of Yamaguchi University School of Medicine and the Law (No. 105) and Notification (No. 6) of the Japanese Government, and all efforts were made to minimize the number of rats used and their suffering.

Tissue preparation

For immunohistochemistry, the rats were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) under anesthesia with pentobarbital sodium (60–80 mg/kg, intraperitoneal injection). The spinal cords were exposed by laminectomy and dissected further until all of the spinal nerves could be identified. The spinal cord segments were separated by a series of transverse cuts and post-fixed for 24 h in the same fixative used for perfusion, soaked in cold 0.1 M PB containing 30% sucrose for one week, frozen in powdered dry ice, and then sectioned at a thickness of 30 μm on a cryostat.