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γ-Diketone Central Neuropathy: Quantitative Analyses of Cytoskeletal Components in Myelinated Axons of the Rat Rubrospinal Tract

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Abstract

Loss of axon caliber is a primary component of γ-diketone neuropathy [LoPachin RM, DeCaprio AP. γ-Diketone central neuropathy: axon atrophy and the role of cytoskeletal protein adduction. Toxicol Appl Pharmacol 2004;199:20–34]. It is possible that this effect is mediated by changes in the density of cytoskeletal components and corresponding spatial relationships. To examine this possibility, morphometric methods were used to quantify the effects of 2,5-hexanedione (HD) intoxication on neurofilament–microtubule densities and nearest neighbor distances in myelinated rubrospinal axons. Rats were exposed to HD at one of two daily dose-rates (175 or 400 mg/kg per day, gavage) until a moderate level of neurotoxicity was achieved (99 or 21 days of intoxication, respectively) as determined by gait analysis and measurements of hindlimb grip strength. Results indicate that, regardless of dose-rate, HD intoxication did not cause changes in axonal neurofilament (NF) density, but did significantly increase microtubule (MT) density. No consistent alterations in interneurofilament or NF–MT distances were detected by ultrastructural morphometric analyses. These data suggest that the axon atrophy induced by HD was not mediated by major disruptions of stationary cytoskeletal organization. Recent biochemical studies of spinal cord from HD intoxicated rats showed that, although the NF protein content in the stationary cytoskeleton (polymer fraction) was not affected, the mobile subunit pool was depleted substantially [LoPachin RM, He D, Reid ML, Opanashuk LA. 2,5-Hexanedione-induced changes in the monomeric neurofilament protein content of rat spinal cord fractions. Toxicol Appl Pharmacol 2004;198:61–73]. The stability of the polymer fraction during HD intoxication is consistent with the absence of significant ultrastructural modifications noted in the present study. Together, these findings implicate loss of mobile NF proteins as the primary mechanism of axon atrophy.
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INTRODUCTION

Growing evidence now indicates loss of axon caliber as a primary pathophysiological process in the neuro-

toxicity associated with exposure to certain hexacarbons (*n*-hexane, methyl *n*-butyl ketone) or their common active γ-diketone metabolite, 2,5-hexanedione (reviewed in LoPachin and Lehning, 1997; LoPachin et al., 2000; LoPachin and DeCaprio, 2004). The molecular mechanism of γ-diketone atrophy is unknown, but is likely to involve disruption of axonal processes that determine axon caliber. In this

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regard, neurofilament (NF) content and corresponding spacing (interfilament distances) are important determinants of caliber (reviewed in Muma and Hoffman, 1993; Nixon, 1998a, 1998b) and, as such, are likely candidates for γ-diketone disruption. NF structure is maintained by dynamic turnover involving the interaction of mobile NF subunit proteins (monomeric or heterooligomeric subunits) with the stationary neurofilamentous polymer (Nixon, 1998a, 1998b; Shea, 2000). The association and dissociation of NF subunits within the polymer is presumably regulated by phosphorylation (Angelides et al., 1989; Okabe et al., 1993; Takeda et al., 1994). The factors that regulate NF spacing are less understood; i.e., some research suggests that interfilament distances are determined by the lengths of the C-terminal tail domains of the NF-M and NF-H subunits (e.g., Chen et al., 2000), whereas data from other studies suggest that NFs do not interact and instead are distributed randomly across the radial dimensions of the axon (Price et al., 1988). Nonetheless, based on the presumed determinants of caliber, γ-diketone-induced axon atrophy could be due to a reduction in NF content or to NF compaction (decreased interfilament distances; e.g., see studies by Elder et al., 1998; Hoffman et al., 1984; Uchida et al., 2004).

In a previous biochemical study (LoPachin et al., 2004), we used triton extraction procedures to determine the effects of HD on the mobile NF pool (triton-soluble) and corresponding stationary cytoskeletal polymer (triton-insoluble). The triton-insoluble polymer is the presumed biochemical correlate of the structural NFs observed in electron micrographs of axonal cross-sections (see Jung et al., 2000; Yabe et al., 2000). Results showed that in spinal cord of moderately affected HD intoxicated rats (400 mg/kg per day × 22 days or 175 mg/kg per day × 99 days), the NF protein content of the mobile pool was significantly reduced, whereas the triton-insoluble polymer was not altered. These data suggested that the prevalent axon atrophy at this neurological endpoint (LoPachin et al., 2003) was not mediated by changes in the stationary axonal cytoskeleton as might be expected. In the present study, this apparent contradiction was addressed using analytical morphometric techniques to quantify NF and microtubule densities in photomicrographs of myelinated axons from the descending rubrospinal tract of HD-intoxicated rats or their age-matched controls. In addition, we measured the ultrastructural spatial relationships among stationary cytoskeletal components. The mobile NF pool in axons is not visible in photomicrographs of fixed

nervous tissue and therefore could not be quantified in these studies.

METHODS

In the present study, spinal cord samples were derived from experimental rat groups used in an earlier morphometric investigation of HD-induced axon atrophy (LoPachin et al., 2003). In the following subsections we provide a brief overview of the corresponding intoxication paradigm, neurological evaluation and tissue fixation process; for more detailed methodological information see LoPachin et al. (2003).

Treatment of Animals

Adult male rats Sprague-Dawley, 250–275 g; Taconic Farms, Germantown, NY) were housed individually in polycarbonate boxes, and drinking water and Purina Rodent Laboratory Chow (Purina Mills, Inc., St. Louis, MO) were available ad libitum. HD was administered by gavage to randomly assigned groups of rats according to one of two daily dose-rates; 175 mg/kg per day × 99 days or 400 mg/kg per day × 21 days (Anthony et al., 1983a, 1983b; LoPachin et al., 2003; Spencer and Schaumburg, 1977a, 1977b). HD was dissolved in 0.9% saline and was administered at 3 ml/kg per day. Age-matched control rats received an equivalent volume of saline by daily gavage.

Neurological Testing

For each HD dose-rate, the onset and development of neurotoxicity were assessed by open field gait observations and measurements of grip strength and body weight (Lehning et al., 2000; LoPachin et al., 2002, 2003). These parameters were determined twice per week. To evaluate gait and postural abnormalities, rats were placed in a clear plexiglass box and were observed for 3 min (LoPachin et al., 2002). Following observation, a gait score was assigned from 1 to 4, where 1 = a normal, unaffected gait; 2 = a slightly abnormal gait (tip-toe walking, hindlimb adduction); 3 = moderately abnormal gait (obvious movement abnormalities characterized by dropped hocks and tail dragging); 4 = severely abnormal gait (dragging hindlimbs and complete absence of rearing). Hindlimb grip strength was measured as the force (kg) required to pull a rat away from a strain gauge apparatus (LoPachin et al., 2002). Three successive grip strength measurements were averaged for each HD-intoxicated