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MORPHINE CAUSES PERSISTENT INDUCTION OF NITRATED NEUROFILAMENTS IN CORTEX AND SUBCORTEX EVEN DURING ABSTINENCE

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Abstract—Morphine has a profound role in neurofilament (NF) expression. However, there are very few studies on the fate of NFs during morphine abstinence coinciding with periods of relapse. Mice were treated chronically with morphine to render them tolerant to and dependent on morphine and sacrificed thereafter while another group, treated similarly, was left for 2 months without morphine. A long-lasting alteration in the stoichiometric ratio of the three NFs was observed under both conditions in both the cortex and subcortex. Morphine abstinence caused significant alterations in the phosphorylated and nitrated forms of the three NF subunits. Nitrated neurofilament light polypeptide chain (NFL) was significantly increased during chronic morphine treatment which persisted even after 2 months of morphine withdrawal. Mass spectrometric analysis following two-dimensional gel electrophoresis (2DE)-gel electrophoresis of cytoskeleton fractions of both cortex and subcortex regions identified enzymes associated with energy metabolism, cytoskeleton-associated proteins as well as NFs which showed sustained regulation even after abstinence of morphine for 2 months. It is suggestive that alteration in the levels of some of these proteins may be instrumental in the increased nitration of NFL during morphine exposure. Such gross alteration in NF dynamics is indicative of a concerted biological process of neuroadaptation during morphine abstinence.

Key words: cytoskeleton, neurofilaments, nitration, morphine, proteomics, withdrawal.

INTRODUCTION

Neurofilaments (NFs) are obligate heteropolymers consisting of four subunits, namely NF light (NFL; ~68 kDa), medium (NFM; ~150 kDa) and heavy (NFH; ~200 kDa) polypeptide chain as well as alpha-internexin (66 kDa), all of which assemble through a complex series of steps to form an antisymmetric tetramer filament of 10-nm diameter (Cohlberg et al., 1995; Goldman et al., 1999). While NFs regulate neuronal plasticity by affecting the dynamics and function of cytoskeletal elements such as microtubules and actin thereby regulating neurite outgrowth (Shea and Beermann, 1994), axonal caliber and transport (Hoffman and Lasek, 1980), the individual role of the subunits is not well understood.

There are numerous studies on the regulation of NFs during chronic exposure to drugs of abuse. NFL, NFM and NFH have been reported to be decreased in the Ventral tegmentum area (VTA) or the prefrontal cortex following repeated cocaine, morphine, or alcohol administration (Beitner-Johnson and Nestler, 1991; Beitner-Johnson et al., 1992; Garcia-Sevilla et al., 2004) as well as following chronic nicotine administration (Bunnemann et al., 2000). Decreased levels of immunoreactive NFL, NFM and NFH proteins in the frontal cortex have been reported in chronic opiate addicts (Garcia-Sevilla et al., 1997; Ferrer-Alcon et al., 2000). One striking limitation of these studies was in the experimental designs, where investigations were carried out while the drugs (cocaine, heroin or morphine) were still present within the system, and very few studies have extended the analysis into periods of abstinence. However, addiction is a long-lived adaptation process characterized by the phenomenon of relapse, which occurs much after the cessation of drug intake. This long-lived adaptation process entails stable changes in the structures of brain as observed during chronic exposure to drugs of abuse and during abstinence (Robinson and Kolb, 2004; Pal and Das, 2013). Thus, there is a need for studies which incorporate much longer periods of abstinence.

NFs also undergo various posttranslational modifications. The most abundant post translational modification of NF is phosphorylation, which plays an important role in the regulation of NF assembly and their accumulation (Nixon and Sihag, 1991), NF stability (Shea et al., 1993), cytoskeleton plasticity (Grant and Pant, 2000) and the association of NFs with molecular motors (Yabe et al., 2000; Motil et al., 2006). The

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Abbreviations: 2DE, two-dimensional gel electrophoresis; DTT, dithiothreitol; EGTA, ethylene glycol tetraacetic acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione; GSS, glutathione synthase; IEF, isoelectric focusing; MS, mass spectrometry; NF, neurofilament; NFH, neurofilament heavy polypeptide chain; NFL, neurofilament light polypeptide chain; NFM, neurofilament medium polypeptide chain; PAGE, polyacrylamide gel electrophoresis; pNF, phosphorylated neurofilament; PP5, protein phosphatase 5; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

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approach of the present study was also to investigate the phosphorylation and nitration of NFs during long-term morphine exposure and during morphine abstinence. In addition, high throughput proteomics approaches were carried out to identify other cytoskeleton protein molecules exhibiting sustained alterations in their expression levels, from immediately after chronic drug use till 2 months of drug withdrawal. Proteins identified under such paradigm were expected to provide greater correlation with the progression of addiction. We investigated the cytoskeleton proteomes of the cortex and subcortex, the two major areas of the reward system of the brain.

EXPERIMENTAL PROCEDURES

Animal treatment

Adult male *swiss* mice (20–25 g) were maintained under controlled conditions (22 °C, 55% humidity, 12-h-day/night rhythm) with free access to solid pellet diet and water *ad libitum* throughout the study. All efforts were followed in a way to minimize animal suffering during subcutaneous injection. Animals were rendered tolerant to morphine by chronic injection of increasing doses of morphine, three times a day, for 6 days, as described previously (Pal and Das, 2013) which has been shown to cause physical dependence in the animals. Briefly, animals were divided equally into three groups. Control animals received only saline. Chronic animals were injected with morphine, thrice a day, with an increasing dose (initially 10 mg/kg and finally 35 mg/kg with an increment of 5 mg/kg every day) for 6 days. Withdrawal animals were also treated with morphine as above and then kept for an additional 2 months without any drug. For each experiment with the control, chronic and withdrawal treated groups, tissues were pooled from eight mice per group. At least three such experiments were carried out for a given study.

Animals were handled following the guidelines of the Institutional Animal Care and Use Committee prepared according to that of the Indian National Science Academy. Animal experimentation was approved by the institutional animal ethics committee appointed by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) of the animal welfare division under the ministry of Environment and Forest, Government of India for the purpose of control and supervision of experimentation in animals.

Subcellular fractionation

The animals were sacrificed and the brains were carefully removed from the culvarium. Frontally, olfactory lobes were cut off and caudally, part of the midbrain along the cerebellum, pons and medulla were removed with a vertical incision at the junction of the cerebral cortex and pituitary, slicing between the superior and inferior colliculi. The adhering meninges and blood clots were removed by washing, and the cortices were separated at the median by cutting the corpus callosum and peeling off the cortices along with the white matter, keeping the hippocampi intact. Part of the pituitary that sticks at the ventral part is also removed. The cortices were pooled and used as cortex. The remaining preparation containing the hippocampi, thalamus, part of midbrain, hypothalamus and septal region were taken as subcortex. Cytoskeletal fractions were prepared following the modified method of Cox and Emili (2006). Briefly, tissue was minced and homogenized in five volumes of homogenizing buffer (0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 15 mM Tris–Cl pH 7.5, 0.5 mM dithiothreitol (DTT), protease inhibitor cocktail) with a glass–glass homogenizer using 20–25 strokes. It was filtered through a nylon mesh and the resultant filtrate was mixed with one-ninth volume of hypotonic buffer (50 mM Tris–Cl pH 7.5, 2 mM $MgCl_2$, 0.5% Triton X-100), vortexed and kept on ice for half an hour. This mixture was centrifuged at 16,000 × *g* for 20 min to get cytoskeletal pellet which was suspended in a buffer containing Tris 20 mM, 2% sodium dodecyl sulfate (SDS), 1% Tween-20, warmed briefly to solubilize completely and stored at −20 °C.

Western blotting

Fifty micrograms of protein was resolved in 7.5% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membrane (Millipore, 45 μm) by electroblotting. Membranes were probed with selective antibodies: anti-NFM (sc-20013, 1:500), anti-NFH (sc-32729, 1:250), anti-pNFL (sc-12965, 1:100), anti-GSS (sc-166882, 1:500) from Santacruz Biotechnology (Dallas, Texas, USA); anti-NFL (mab-1615, 1:1000), anti-pNFM (12-144, 1:5000), anti-pNFH (NE1022, 1:1000), anti-PP5 (07-1225, 1:1000) from Millipore and alpha-internexin (ab-5334, 1:5000) from Abcam (Chembridge, USA). Blots were reprobed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:500) from Millipore (Billerica, Massachusetts, USA) to ensure equal loading and transfer. Blocking and incubations were performed as per the manufacturers' protocol. Following incubation with a peroxidase-conjugated secondary antibody, blots were revealed by enhanced chemiluminescence using ECL (Sigma). Band density was quantified by measuring the intensity of band against protein-specific antibodies compared to that of band intensity of GAPDH in the same blot by using Image J software (version 1.45).

Calculation of molar ratios

For calculation of the molar ratio of the three NF subunits, the relative intensities of individual NF subunit in controls, chronic morphine treatment and 2 months of morphine withdrawal were calculated in blots probed with specific antibodies against subunits. We have considered the molar stoichiometric ratios of neurofilament heavy polypeptide chain (NFH):neurofilament medium polypeptide chain (NFM):neurofilament light polypeptide chain (NFL) subunits in the controls as 1:2:6 as reported by others (Shechet and Lasek, 1980; Chiu and Norton, 1982). Considering the band intensity of NFH in the control as 1 unit, the intensities of NFH during chronic morphine and morphine abstinence were assigned values calculated