Cyclooxygenase-2 Is an Obligatory Factor in Methamphetamine-Induced Neurotoxicity

David M. Thomas and Donald M. Kuhn

Departments of Psychiatry & Behavioral Neurosciences (D.M.T., D.M.K.) and Center for Molecular Medicine and Genetics (D.M.K.), Wayne State University School of Medicine, and John D. Dingell Veterans Affairs Medical Center (D.M.T., D.M.K.), Detroit, Michigan

Received November 5, 2004; accepted February 15, 2005

ABSTRACT

Methamphetamine causes persistent damage to dopamine nerve endings of the striatum. The mechanisms underlying its neurotoxicity are not fully understood, but considerable evidence points to oxidative stress as a probable mechanism. A recent microarray analysis of gene expression changes caused by methamphetamine revealed that cyclooxygenase-2 (COX-2) was induced along with its transcription factor CCAAT/enhancer-binding protein (Thomas DM, Francescutti-Verbeem DM, Liu X, and Kuhn DM, 2004). We report presently that methamphetamine increases striatal expression of COX-2 protein. Cyclooxygenase-1 (COX-1) expression was not changed. Mice bearing a null mutation of the gene for COX-2 were resistant to methamphetamine-induced neurotoxicity. COX-1 knockouts, like wild-type mice, showed extensive dopamine nerve terminal damage. Selective inhibitors of COX-1 [5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethyl pyrazole (SC-560)], COX-2 [N-[2-(cyclohexyloxy)-4-nitrophenyl] methanesulfonamide (NS-398), rofecoxib], or COX-3 (antiipyride) or a nonselective inhibitor of the COX-1/2 isoforms (ketoprofen) did not protect mice from neurotoxicity. Finally, methamphetamine did not change striatal prostaglandin E2 content. Taken together, these data suggest that COX-2 is an obligatory factor in methamphetamine-induced neurotoxicity. The functional aspect of COX-2 that contributes to drug-induced neurotoxicity does not appear to be its prostaglandin synthetic capacity. Instead, the peroxidase activity associated with COX-2, which can lead to the formation of reactive oxygen species and dopamine quinones, can account for its role.
that COX-2 plays an important role in METH-induced neurotoxicity. We report presently that METH increases the expression of COX-2 protein in striatum, whereas expression of COX-1 remains unchanged. Genetic inactivation of the COX-2 gene, but not the COX-1 gene, renders mice resistant to METH-induced neurotoxicity. These results establish COX-2 as an obligatory mediator of the DA nerve terminal damage caused by METH.

Materials and Methods

Materials. (+) Methamphetamine hydrochloride, NS-398, ketoprofen, antipyrine, SC-560, DA, methanol, EDTA, and all buffers and HPLC reagents were purchased from Sigma-Aldrich (St. Louis, MO). Rofecoxib was generously provided by Merck Research Laboratories (Rahway, NJ). PGE\(_2\) enzyme immunoassay kits and antibodies against COX-1 (monoclonal) and COX-2 (polyclonal) were obtained from Cayman Chemical (Ann Arbor, MI). Horseradish peroxidase-conjugated secondary antibodies were obtained from Amersham Biosciences (Piscataway, NJ). Bicinchoninic acid protein assay kits were purchased from Pierce Chemical (Rockford, IL) and Western Lightning Chemiluminescence Reagent Plus was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Biomax MR film was from Eastman Kodak (Rochester, NY).

Animals. Wild-type, female C57BL/6 mice were obtained from Harlan (Indianapolis, IN). Female mice with null mutations (homozygous) of the COX-1 (strain B6;129P2-Ptgse\(^{1\text{m}1\text{Lnc}}\) or COX-2 gene (strain B6;129P2-Ptgse\(^{2\text{m}1\text{Smc}}\)) and their wild-type littermates were purchased from Taconic Farms (Germantown, NY). The COX-1 and COX-2 knockout mice are bred on a mixed C57BL/6J background.

Pharmacological and Physiological Procedures. All animals were group-housed in acrylic cages with microisolator lids prior to and during experimentation. The ambient temperature within the treatment room was 23.0 ± 0.2°C and was monitored throughout all experiments. METH was injected i.p. at a dose of 5 mg/kg in four injections with a 2-h interval between each injection. Controls received physiological saline on the same schedule as METH. Injection volumes were 0.1 ml/10 g b.wt. Mice were also treated with various COX inhibitors to determine their effects on METH-induced neurotoxicity. These drugs and their injection schedules were administered as follows. Rofecoxib (COX-2 inhibitor) was administered by gavage in 0.5% methylcellulose in a dose of 25 mg/kg once per day for 5 days preceding METH treatment. Rofecoxib was also administered 1 h before the first METH injection, 1 h after the last METH injection, and once daily thereafter until the time of sacrifice. NS-398 (COX-2 inhibitor) was administered i.p. in a dose of 10 mg/kg in 40% dimethyl sulfoxide 1 h before the first METH injection, 1 h after the last METH injection, and twice daily thereafter until the time of sacrifice. SC-560 (COX-1 inhibitor) and ketoprofen (COX-2 inhibitor) were administered i.p. in doses of 10 mg/kg in 40% dimethyl sulfoxide 1 h before the first METH injection and 1 h after the last METH injection. Antipyrine (COX-3 inhibitor) was administered i.p. in a dose of 100 mg/kg in physiological saline 30 min prior to each METH injection. Body temperature was monitored throughout all treatments by telemetry using IPTT-200 implantable temperature transponders from BioMedic Data Systems, Inc. (Seaford, DE). Core body temperatures were recorded noninvasively using the DAS-5001 console system from BioMedic Data Systems, Inc. These mice were sacrificed 48 h after the above treatments for neurochemical analyses.

Immunohistochemistry. Striatal tissue was dissected from brain at the indicated times (below) after METH treatment and stored frozen at −80°C. Frozen tissue was disrupted by sonication in 1% SDS, 10 mM Tris pH 7.4, 1 mM sodium orthovanadate at 95°C, and insoluble material was sedimented by centrifugation. Protein was determined by the bichinionic acid method and equal amounts of protein (100 μg) were resolved by SDS-polyacrylamide gel electrophoresis and then electrophorased to nitrocellulose. Blots were blocked in Tris-buffered saline containing Tween 20 (0.1% v/v) and 5% nonfat dry milk for 1 h at room temperature. COX-1 and COX-2 proteins were detected on the same blots by incubating with anti-COX-1 (diluted 1:400) or anti-COX-2 (diluted 1:500), respectively, for 16 h at 4°C. After exposure to primary antibodies, blots were washed and incubated with the appropriate horseradish peroxidase-conjugated anti-IgG secondary antibody (1:3000, 1 h at room temperature). Immunoreactive bands were visualized by enhanced chemiluminescence.

Determination of Striatal DA and PGE\(_2\) Content. Depletion of striatal DA after METH treatment is widely used as an index of METH-induced toxicity to DA nerve endings. DA depletion from striatum faithfully reflects other measures of DA nerve ending damage caused by METH, such as reduced tyrosine hydroxylase immunoreactivity or reduced ligand binding to the DA transporter. Striata were dissected from brain 48 h after METH treatment and stored frozen at −80°C. Tissues were weighed and sonicated in 5 volumes of 0.1 M perchloric acid at 4°C. Insoluble protein was removed by centrifugation and catecholamines were adsorbed to alumina. After elution from alumina, the amount of DA in samples was determined by HPLC with electrochemical detection by comparison with a standard curve of authentic DA. Striatal PGE\(_2\) content was determined by enzyme immunoassay using a commercially available kit as previously described (Ayoub et al., 2004).

Data Analysis. The effects of METH (± COX inhibitors) on striatal DA and PGE\(_2\) content were tested for significance by analysis of variance. Individual treatment groups were compared with appropriate controls using Dunnett’s multiple comparison test in Graph-Pad Prism 4. Differences were considered significant if \(p < 0.05\). Areas under the curve (AUC) for core body temperature responses over the 9-h treatment periods were calculated by comparison against a common baseline for all groups using GraphPad Prism 4 and were expressed in arbitrary units.

Results

A neurotoxic regimen of METH increased expression of COX-2 protein in the striatum of wild-type C57BL/6 mice. The immunoblot in Fig. 1A shows that COX-2 was increased by 50% as early as 3 h after the final METH injection. COX-2 expression continued to increase up to 48 h after treatment, reaching a peak of 390% of control at 24 h. The levels of COX-1 protein were not changed by METH over the same time course. These results are in good agreement with our previous finding that COX-2 gene expression is increased by METH, whereas COX-1 gene expression remains unchanged (Thomas et al., 2004a). Using the time point of peak expression (24 h), we examined COX-2 protein levels in the wild-type littermates of COX-1 and COX-2 knockout mice (Fig. 1B). As with the wild-type C57BL/6 mice, these animals also exhibit robust increases in COX-2 expression of up to 320% of controls at this time point.

To test active involvement of COX-2 in METH-induced neurotoxicity, mice with targeted deletion of the genes for either COX-1 or COX-2, as well as their wild-type littermates, were tested for their responses to drug. Figure 2 shows that striatal DA content was the same among the five
groups of mice under control conditions. METH lowered striatal DA by about 70% in all wild-type mice. COX-1 knockout mice responded to METH in the same fashion as wild-type mice, showing reductions in striatal DA to 35% of control. On the other hand, COX-2 knockout mice were resistant to METH-induced neurotoxicity. DA levels were reduced to 81% of control in COX-2 knockout mice. The effect of METH on striatal DA content was significant in all five groups (*, p < 0.01, Dunnett's multiple comparison test) and METH-treated COX-2 KO mice were significantly different from all other METH-treated mice (**, p < 0.01, Dunnett's multiple comparison test).

In view of results showing that C57BL/6 wild-type mice responded to METH in an identical manner as the wild-type littermates of the COX-1 and COX-2 knockout mice and considering that drug effects in the COX-2 knockouts were so different from the responses in COX-1 knockouts, strain effects on METH actions can be ruled out, and C57BL/6 mice are used hereafter as wild-type controls.

One aspect of the COX-2 knockout phenotype is a hampered thermoregulatory response to pyrogens (Li et al., 2001). This is potentially significant because lowered core temperature is neuroprotective against METH (Bowyer et al., 1992; Miller and O'Callaghan, 1994). Figure 3 shows the core body temperature responses of mice to METH. The core temperatures of all groups of mice under control conditions did not differ significantly, so the results are included in a single control plot to make inspection of the data easier. It can be seen that METH increased body temperature by about 1°C in wild-type mice, starting 1 h after the first METH injection and lasting for approximately 6 h. The initial response to METH of both COX-1 and COX-2 knockout mice was a fall in core temperature of 1–2°C below control which lasted for about 2 h. Thereafter, core temperatures of both groups increased gradually during the METH treatment regimen, and by 4 h after the start of drug treatment, the COX-1 and COX-2 knockout groups had core temperatures that were the same as METH-treated wild-type mice. Core temperatures of all groups returned to control levels by 1 to 2 h after the last METH injection as shown in Fig. 3. AUCs were also calculated for each group to provide a measure of the overall response to METH throughout the entire treatment period (60 to 480 min). These measures confirm the data in Fig. 3 and indicate that wild-type mice treated with METH (AUC = 2514) showed the largest total response by comparison with controls (AUC = 2160). The lesser responses of the COX-1 (AUC = 2232) and COX-2 (AUC = 2142) knockout
mice to METH can be accounted for by their initial 120-min period of hypothermia after drug treatment.

In view of results showing that genetic inactivation of COX-2 was protective against METH-induced neurotoxicity, we tested various pharmacological inhibitors of the COX enzymes for neuroprotection in wild-type mice. All doses and treatment schedules used for the COX inhibitors were based on published, confirmed accounts of COX inhibition (see Materials and Methods). Figure 4 shows that the selective COX-2 inhibitors NS-398 and rofecoxib did not prevent drug-induced reductions in DA. SC-560, a selective COX-1 inhibitor, also failed to protect mice from METH-induced neurotoxicity. The nonselective COX-1/2 inhibitor ketoprofen and the COX-3 inhibitor antipyrine were also ineffective in preventing the effects of METH on DA nerve terminals as shown in Fig. 4. All treatment groups were significantly different from controls (p < 0.01, Dunnett’s multiple comparison test), and none of the groups treated with a COX inhibitor + METH was different from METH alone. None of the COX inhibitors altered striatal DA content when administered without METH (data not shown).

The effects of the COX inhibitors on METH-induced hyperthermia are shown in Fig. 5. When given alone, NS-398 caused a drop in body temperature of 1–2°C that started almost immediately after injection and lasted for 2 to 3 h (data not shown). Figure 5A presents results in mice treated with METH plus COX-2 inhibitors. The hyperthermic response to METH was very similar to what was observed in Fig. 3. However, core body temperature responses to METH were quite different in the NS-398 and rofecoxib groups. Mice treated with NS-398 responded with a further drop in body temperature of about 2°C that developed rapidly after initiation of METH treatment and remained below control for 3 h. On the other hand, mice treated with rofecoxib developed a slightly greater hyperthermia than mice treated with METH alone. This effect of rofecoxib persisted through three METH injections and returned to control levels thereafter. The overall effects of the COX-2 inhibitors were reflected in AUCs and confirmed that METH (AUC = 2562)- and METH + rofecoxib (AUC = 2628)-treated mice were hyperthermic by comparison with controls (AUC = 2208), whereas the METH + NS-398 group was hypothermic (AUC = 2133).

The effects of SC-560, ketoprofen, and antipyrine in METH-treated mice are presented in Fig. 5B. By themselves, each COX inhibitor lowered body temperature by 1–2°C for about 2 to 3 h (data not shown). As seen with the COX-2 inhibitors (Fig. 5A), the responses to these COX inhibitors were quite varied. SC-560, a COX-1 inhibitor, caused a profound hypothermia immediately after the start of METH treatment, with core temperatures lowered by almost 2°C for 1 h. The SC-560 + METH-treated group remained at control
core temperature levels for the next 2 h and then converted to a hyperthermia of 2–3°C above controls. This group remained above control temperatures for the remainder of the experiment. Ketoprofen, a nonselective COX-1/2 inhibitor, caused effects on core temperatures that were very similar to SC-560, producing a drop of about 2°C below controls that lasted for 2 to 3 h. Thereafter, the ketoprofen + METH group remained near the temperatures of the METH group. Antipyrine initially enhanced METH-induced hyperthermia by about 0.5°C, after which body temperatures returned to levels between controls and METH-treated mice for the remainder of the experiment. Calculation of AUCs for each group confirmed the METH-induced hyperthermia (AUC = 2766) by comparison with controls (AUC = 2202). AUCs for groups treated with SC-560 (AUC = 2514), ketoprofen (AUC = 2385), and antipyrene (AUC = 2526) revealed an overall hyperthermia throughout the treatment period, as all groups were between controls and METH-treated mice.

In view of results showing that METH increased expression of COX-2 protein (Fig. 1 above), the effects of drug treatment on striatal PGE$_2$ content was determined. Figure 6 shows that the levels of PGE$_2$ remained relatively unchanged for up to 48 h after METH treatment. There was a slight tendency of a reduction in PGE$_2$ levels 12 to 24 h after treatment, but this effect was not statistically significant.

**Discussion**

Evidence linking the COX enzymes and prostaglandins to METH-induced neurotoxicity is very limited in scope. Kita and colleagues showed that METH increased COX-2 expression (Kita et al., 2000), and ketoprofen has been shown to provide partial protection against METH-induced neurotoxicity (Asanuma et al., 2003). This situation is somewhat surprising in view of the larger role of the prostanooids in mediating other forms of neuronal injury, inflammation, and degeneration (Hurley et al., 2002). A comprehensive analysis of gene expression changes provoked by a neurotoxic regimen of METH substantiated these earlier findings by showing a drug-induced increase in expression of the genes for COX-2 and C/EBP (Thomas et al., 2004a). The importance of COX-2 in METH neurotoxicity was further substantiated by the finding that genetic inactivation of the COX-2 gene rendered mice almost totally resistant to drug-induced nerve terminal damage. COX-1 knockout mice were no different from wild-type mice in their response to METH, manifested as significant reductions (about 70%) in striatal DA content. COX-2 expression was increased by METH in the COX-1 knockout mice to the same extent observed in wild-type mice treated with METH (data not shown).

One element of the COX-2 knockout phenotype with possible relevance to METH neurotoxicity is an altered thermoregulatory response. COX-2 knockout mice have a diminished febrile response to pyrogens (Li et al., 2001). Treatments that prevent METH-induced hyperthermia, such as MK-801 or lowered ambient temperature (Bowyer et al., 1994; Miller and O’Callaghan, 1994), also prevent its associated neurotoxicity. Therefore, we studied the core temperature response of COX-2 knockout mice to METH. The initial response of COX-2 knockouts to METH treatment was a rapid fall in body temperature of about 2°C. The response of COX-1 knockouts was the same as COX-2 knockouts, whereas wild-type mice showed the expected hyperthermia. This drug-induced hypothermia in the COX knockout mice lasted for 1 to 2 h and returned gradually to levels shown by METH-treated wild-type mice. It is significant that both COX-1 and COX-2 knockout mice showed the same core temperature response to METH, whereas only COX-2 knockouts were resistant to its neurotoxicity. It is also interesting that COX-1 knockout mice demonstrated the same extent of DA depletion as wild-type mice, yet COX-1 knockouts responded to METH with hypothermia whereas wild-type mice developed hyperthermia. Taken together, these data establish that the body temperature responses of COX knockout mice to METH are not predictive of neurotoxicity and suggest that an altered thermoregulatory mechanism in COX-2 knockout mice is not mediating their resistance to METH-induced neurotoxicity.

The effects of pharmacological inactivation of COX-2 in wild-type mice apparently did not agree with the results seen in mice lacking COX-2. NS-398 and rofecoxib, two relatively selective COX-2 inhibitors, did not provide any measure of protection against the neurotoxic effects of METH. To test the possibility that COX-1 was playing a subtle role that was not immediately apparent, even in the COX-2 knockout mice, the COX-1 selective inhibitor SC-560 was given to mice before METH. It too failed to prevent METH toxicity. Ketoprofen, a nonselective inhibitor of both COX-1/2 isoforms, did not protect mice from METH-induced neurotoxicity. A new splice variant of COX-1 referred to as COX-3 (Chandrasekharan et al., 2002) participates in the regulation of body temperature in mice via its constitutive production of prostaglandins (Ayoub et al., 2004), so we tested an inhibitor of this enzyme as well to rule out a possible role for it in METH toxicity. Antipyrine, like other COX inhibitors used presently, did not prevent METH-induced neurotoxicity. The doses and treatment regimens for each COX inhibitor used were based on published, confirmed accounts of COX inhibition in mice, so tests of COX inhibition were not further confirmed presently. Therefore, it is possible that COX-2 was not inhibited by either NS-398 or rofecoxib. This does not seem likely in view

**Fig. 6.** Effects of METH on striatal PGE$_2$ levels. Mice were treated with a neurotoxic regimen of METH and sacrificed at the indicated times. Striatal tissue was analyzed for PGE$_2$ content by enzyme immunoassay. Data are presented as picograms of PGE$_2$ per milligram of tissue (wet weight) and represent mean ± S.E.M. of groups containing four to five mice per treatment time.
of the body temperature responses of mice treated with METH + COX-2 inhibitors (Fig. 5A), suggesting that NS-398 and rofecoxib were pharmacologically active. The inhibitors of COX-1, COX-3, and COX-1/2 produced widely divergent effects on METH-induced hyperthermia (Fig. 5B), suggesting that these drugs were also given in pharmacologically active doses. Finally, the COX inhibitors caused mild hypothermia by comparison with controls when given without METH, suggesting effective COX inhibition (Ayoub et al., 2004). It is not immediately apparent why ketoprofen was ineffective against METH in our studies, although it partially reduced METH toxicity when tested by Asanuma and colleagues (Asanuma et al., 2003). These investigators used lower doses of METH (4 mg/kg versus 5 mg/kg) and a slightly different ketoprofen treatment regimen (2 or 4 mg/kg before each of four METH injections) than used presently, but the effect of ketoprofen on body temperature seen by Asanuma et al. (2003) after METH administration was identical to our findings of an initial hypothermia that transitioned to a hyperthermia. COX inhibitors are not uniformly neuroprotective, and their effects on MPTP-induced damage to the DA neuronal system is unpredictable. Some COX inhibitors protect against MPTP (Teismann and Ferger, 2001; Maharaj et al., 2004), whereas others do not (Sairam et al., 2003). The reasons for widely varying effects of COX inhibitors on drug-induced neural damage are not clear.

The apparent differences in outcome between genetic inactivation of COX-2 (i.e., protective) versus pharmacological inhibition of the enzyme (i.e., COX-2 inhibitors not protective) with regard to METH-induced neurotoxicity could be explained if COX-2 was mediating a process that was independent of its prostaglandin biosynthetic capacity. One clue for the operation of such a mechanism emerged from studies showing that PGE2 levels were not increased in striatum after METH intoxication (Fig. 6), despite the fact that COX-2 protein levels were elevated. It is well known that the COX enzymes express peroxidase enzymatic activity in addition to the cyclooxygenase function (Smith et al., 2000). This is highly significant with respect to METH-induced neurotoxicity because the peroxidase activity associated with COX-2 converts DA to its quinone (Hastings, 1995). DA quinones are elevated in striatum by METH (LaVoie and Hastings, 1999), and they have also been implicated recently in MPTP-induced degeneration of DA neurons (Teismann et al., 2003). The quinones of DA can modify numerous proteins whose function is altered by the neurotoxic amphetamines including tryptophan hydroxylase (Kuhn and Arthur, 1998), tyrosine hydroxylase (Kuhn et al., 1999), and the DA transporter (Whitehead et al., 2001). The covalent modification of proteins by catechol quinones can generate additional reactive species and radicals through creation of redox-cycling centers/moieties (Paz et al., 1991).

One of the primary pharmacological effects exerted by the neurotoxic amphetamines, including METH, is a release of DA into the cytoplasm from synaptic vesicles and eventually into the synaptic space via reverse transport through the DA transporter (Sulzer et al., 1995). This liberation of DA from its presynaptic storage sites, along with increased production of COX-2 protein, would create conditions that favor the production of catechol quinones. In addition to their direct effects on proteins, membrane-bound DA quinones (Le et al., 2001) and free DA quinones (D. M. Thomas and D. M. Kuhn, manuscript in preparation) cause extensive microglial activation. Recently, emerging data has implicated microglial activation as an early event in the neurotoxic cascade that is initiated by the neurotoxic amphetamines (LaVoie et al., 2004; Oriol et al., 2004; Thomas et al., 2004b). Therefore, the requirement for COX-2 as an essential factor in at least METH-induced neurotoxicity offers a potential mechanism by which this drug of abuse stimulates the production of DA quinones and leads to microglial activation. This possibility is currently under investigation.

A major clinical danger associated with high-dose METH abuse in humans is the development of hyperthermia. The neurotoxic effects of METH can be blocked by drugs or treatments that prevent hyperthermia (Bowyer et al., 1994; Miller and O’Callaghan, 1994), making it difficult to determine whether hyperthermia is a direct participant in drug-induced neurotoxicity or a response that is coincident but does not actually contribute to nerve terminal damage. Several aspects of our data support the latter contention. First, COX-1 and COX-2 knockout mice respond in an identical manner to METH with a rapidly developing hyperthermia, yet COX-1 mice show the same toxicity as wild-type mice, and COX-2 knockouts show very little DA nerve terminal damage. Second, the COX-2 inhibitors did not prevent METH-induced neurotoxicity but when administered with METH, NS-398 caused a significant hypothermia and rofecoxib-enhanced hyperthermia. Third, inhibitors of COX-1, COX-3, or COX-1/2, when administered along with METH, led to varying body temperature responses, including hypothermia that transitioned over time into hyperthermia (see Fig. 5B). Taken together, these results with COX knockout mice and COX enzyme inhibitors indicate that core temperature responses to METH (i.e., hypothermia or hyperthermia) cannot predict the subsequent neurochemical response (i.e., toxicity or neuroprotection) to drug administration and lead us to agree with the conclusion that hyperthermia is neither necessary nor sufficient for amphetamine-induced neurotoxicity (Ali and Itzhak, 1998; Darvesh and Gudelsky, 2004).

Acknowledgments

We thank Drs. David DeVitt (Michigan State University, East Lansing, MI), Daniel Simmons (Brigham Young University, Provo, UT) and Robert Langenbach (National Institutes of Health, National Institute of Environmental Health Sciences, Research Triangle Park, NC) for helpful comments and expert advice on COX-2 involvement in METH actions. We also thank Merck Research Labs for the generous gift of rofecoxib.

References


Address correspondence to: Dr. Donald M. Kuhn, Department of Psychiatry and Behavioral Neurosciences, Wayne State University School of Medicine, 2125 Scott Hall, 540 E. Canfield, Detroit, MI 48201. E-mail: donald.kuhn@wayne.edu