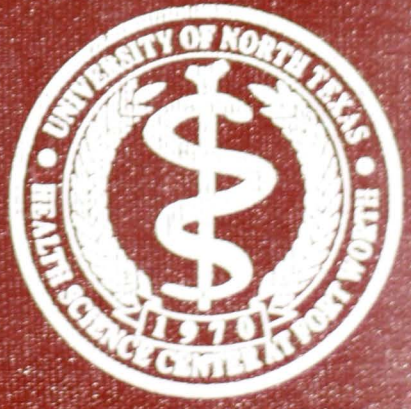
UNIVERSITY OF NORTH TEXAS
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Oxidative stress has been implicated in aging and neurodegenerative disorders. Pain sensitivity and responses to opioids change with aging. The effect of aging and oxidative stress on opioid receptor system is not yet well understood. To study the effects of aging on pain sensitivity and opioid-induced antinociception, and to determine the possible association of oxidative stress with these pain parameters, *in vivo* studies were conducted. To further elucidate the effects of oxidative stress on opioid receptor proteins and their function, *in vitro* studies were carried out.

The effects of aging on pain sensitivity and opioid-induced antinociception were studied in male Fischer 344 rats. Oxidative stress markers in cerebral cortex, hippocampus, striatum and midbrain of these rats were estimated. It was concluded that sensitivity to nociceptive stimulus increases and responses to opioids decrease with aging and age-related oxidative damage is negatively correlated with opioid-induced antinociception.

To characterize the effects of oxidative stress on function of opioid receptors, changes in intracellular cyclic adenosine monophosphate (cAMP) were measured in human SK-N-SH neuronal cells under oxidative stress conditions. It was found that oxidative stress decreased the function of *mu* opioid receptor (MOR) but not that of *delta*

or *kappa* opioid receptors (DOR and KOR respectively). Antioxidant intervention preserved the function of MOR.

Western immunoassays revealed that MOR but not DOR and KOR proteins were decreased under oxidative stress conditions. Thus, these findings show a selective impairment of the MOR function and reduction in MOR protein under conditions of oxidative stress.

The results from the *in vivo* and *in vitro* projects demonstrate the involvement of aging and oxidative stress in modulation of pain sensitivity, opioid-induced antinociception and opioid receptor function and expression.

OPIOID RECEPTORS IN AGING AND OXIDATIVE STRESS

Atul M. Raut, M.B.B.S., M.D.

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OPIOID RECEPTORS IN AGING AND OXIDATIVE STRESS

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Atul M. Raut, M.B.B.S., M.D.

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I dedicate this work to my parents **Mathuradas** and **Maya Raut** and my sister, **Jyoti**, for supporting, encouraging and most of all, loving me throughout my life. Without them, I could not have gotten this far.

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CHAPTER IV

LIST OF ABBREVIATIONS

3-NPA	3-Nitroproprionic acid
cAMP	Cyclic adenosine monophosphate
CTOP	d-Phe-Cys-Tyr-d-Trp-Orn-Thr-Pen-Thr-NH_2
DAMGO	D-Ala^2, $NMePhe^4$, Gly-ol enkephalin
DCF-DA	2', 7'-dichlorofluorescin diacetate
DOR	*delta* opioid receptor
DPDPE	D-Pen^2, D-Pen^5 enkephalin
IBMX	Isobutylmethylxanthine
KOR	*kappa* opioid receptor
MOR	*mu* opioid receptor
PGE_1	Prostaglandin E_1
ROS	Reactive oxygen species

CHAPTER - I

INTRODUCTION

Pain

Pain is a complex physiological, behavioral and subjective response to nociceptive inputs. The term "nociception" is defined as detection of noxious stimuli or stimuli that can damage the tissue (Basbaum and Jessell, 2000). Pain has two components which involve dedicated projection system from spinal cord to higher centers in the brain (Basbaum and Jessell, 2000). One component is a modality of somatic sensory perception. It permits the localization of pain and enables discrimination among different pain stimuli. The second component is affective. It activates circuits in the brain that results in negative emotions. The affective component contributes to perception of pain and interferes with behavioral aspects. This component also activates brain stem systems that increase arousal; and regions of hypothalamus and amygdala induce responses to stress (Craig, 2003). Pain serves a survival function by drawing attention to site of injury and leading to behavioral reflexive protection of injured tissue that permits healing (Handwerker and Kobal, 1993). Hence, regular physiological functioning of pain processing pathways is crucial for maintenance of normal body structures and functions.

Primary Nociceptors

Nociceptors are free nerve endings which serve as cutaneous receptors involved in nociception (Lembeck and Gamse, 1982). The cell bodies of peripheral nociceptors are located in dorsal root ganglia (Fig. 1). Nociceptors transduce mechanical, thermal and chemical nociceptive stimuli through action potentials that are transmitted to the spinal cord. Nociceptive neurons synapse in the dorsal horn of the spinal cord on local interneurons and on projection neurons which carry nociceptive information to higher brain centers.

Ascending Pain Pathways

Axons of projection neurons from dorsal horn cross the midline and ascend to higher centers in the form of spinoreticular and spinothalamic tracts (Basbaum and Fields, 1984) (Fig. 2). Neurons of spinoreticular tract terminate in the reticular formation of brain stem. The reticular neurons send projections to multiple brain regions, predominantly to thalamus through reticulothalamic tract. Spinothalamic tract ascend to higher centers in two divisions. The lateral division (neospinothalamic tract) relays information to venteroposterolateral (VPL) and venteroposteromedial (VPM) nuclei of the thalamus. VPL and VPM neurons send their projection to somatosensory cortex. The medial division of spinothalamic tract (paleospinothalamic tract) terminates in the intralaminar nuclei of the thalamus. Intralaminar thalamic neurons send widespread cortical projections which include major projection to association cortex such as prefrontal cortex. It is unknown how nociceptive information is perceived as pain but it

has been reported that cerebral association cortices, ventral striatum and amygdala receive inputs from the paleospinothalamic tract and subcortical circuits and process emotion (Basbaum and Jessell, 2000).

Descending Analgesic Pathways

After the nociceptive stimulus is perceived as pain, cortical neurons and amygdala send descending projections to periaqueductal gray (PAG) area in midbrain (Basbaum and Fields, 1984) (Fig. 2). Projections from PAG terminate on the rostral ventral medulla, which in turn projects to the dorsal horn through dosrolateral funiculus of the spinal cord. These neurons activate enkephalinergic interneurons that are located in the dorsal horn. Within the dorsal horn, enkephaline-containing interneurons also make direct post-synaptic contact with neurons of spinothalamic tract and inhibit them. Thus, the descending analgesic pathway activation results in inhibition of nociceptive somatic and affective components of pain.

Opioid Receptors

The function and responsiveness of the opioid system is based on opioid receptors. Opioid receptors belong to the G-protein-coupled receptor superfamily (Ueda et al., 1988). In the nervous system, opioid agonists stimulate heterotrimeric proteins of the Gi/o family and control neuronal function through several receptor-effector mechanisms, including activation of receptor-operated potassium channels, inhibition of voltage-gated calcium channels and inhibition of adenylyl cyclase (Ueda et al., 1988,

Preis, 1998, Gilman, 1986) (Fig. 3). Pharmacological binding studies using highly selective ligands have classified opioid receptors into three subtypes: *mu* (MOR), *delta* (DOR) and *kappa* (KOR) (Loh and Smith, 1990). Molecular cloning studies have confirmed the pharmacological classification of the opioid receptors (Evans et al., 1992, Meng et al., 1993, Wang et al., 1994). All of the cloned opioid receptors possess the same general structure of an extracellular N-terminal region, seven transmembrane domains and intracellular C-terminal tail structure (Gilman, 1986) (Fig. 4).

The *mu* opioid receptor (MOR) is the principal target of the clinically efficacious opioid analgesics such as morphine. There are three subtypes of MOR: mu_1, mu_2, and mu_3. In the CNS, MORs are predominantly expressed in the cerebral cortex, hippocampus, thalamus, caudate putamen, nucleus accumbens and septal nuclei (Zastawny et al., 1994) but the highest levels of MORs have been shown to be present in midbrain, hypothalamus and cerebral cortex (Delfs et al., 1994). Mu_1 *and* mu_2 receptors are mainly important for analgesia. The mu_3 receptor represents another splice variant of MOR. This receptor has very low affinity for endogenous opioid peptides and is coupled to nitric oxide release (Stefano et al., 1996). Activation of mu_1 opioid receptors contributes to supraspinal analgesia (Pasernak, 1993) while activation of mu_2 contributes to spinal analgesia, respiratory depression and decreased gastrointestinal transit (Pasternak, 1993, Matthes et al., 1996, Ward and Takermori, 1983). Acivation of MORs may also result in miosis, nausea, vomiting and euphoria (Pasternak, 1993).

There are two subtypes of delta receptor: $delta_1$ and $delta_2$. Both of these are involved in supraspinal analgesia while only $delta_2$ receptor is also involved in spinal

analgesia. *Delta* receptors are predominantly expressed in cerebral cortex, striatum, basal ganglia and dorsal horn of the spinal cord (Mansour et al., 1988). Activation of *delta* receptor contributes primarily to analgesia.

Kappa receptors are divided into 3 subclasses based on the pharmacological studies. *Kappa$_1$* receptors produce analgesia at spinal level while *kappa$_3$* receptors act through supraspinal mechanism. Mechanism of action of *kappa$_2$* receptor is not clear. Supraspinal *kappa* receptors are mostly localized in cortex, hypothalamus and striatum (Pasternak, 1993).

Endogenous opioids have different affinities for different opioid receptor subtypes. Enkephalins and β-endorphins have high affinity for *mu* and *delta* receptors while dynorphins have high affinity for *kappa* receptors (Reisine and Pasternak, 1996). Specific cDNA encoding an "orphan" receptor has been identified which has a high degree of homology to the "classical" opioid receptors. Structurally, this receptor is an opioid receptor and has been named as opioid receptor-like (ORL1) (Rosenberger et al., 2001). A peptide, nociceptin, also known as orphanin FQ is an endogenous ligand for ORL1 (Meunier et al., 1995, Reinscheid et al., 1995). This peptide is distributed widely in the CNS and contributes to pain modulation at supraspinal, spinal and peripheral levels (Darland et al., 1998).

Role of Opioids in Analgesia

In spite of advances in understanding of pain processing pathways and opioid receptors, morphine still remains a mainstay of treatment of moderate to severe pain for

over the centuries. By the beginning of the 20th century, morphine became available for the use in treatment of many types of pain, insomnia, aortic regurgitation, angina, cardiac dyspnea and diarrhea (Reisine and Pasternak, 1996).

Today, morphine and its congeners are still considered the "gold standard" for treatment of moderate to severe pain according to The World Health Organization (1990). This has led substantial increase in the global use of morphine. International Narcotics Control Board (1995) has reported that world consumption of morphine for the relief of pain increased six-fold from 1983 to 1993. A primary reason for this may be a shifting paradigm in the treatment of chronic pain (Melzack, 1990). As opposed to previous "as needed" regimen, around the clock scheduled doses of opioids prevent recurrence of pain more effectively (Bushnell and Justins, 1993, Foley, 1985, Melzack, 1990). Morphine is most effective in the treatment of nociceptive pain (McQuay, 1988) but also may be effective of neuropathic pain in some patients (Portenoy et al., 1992). Morphine also has been found to be useful in treatment of patients on mechanical ventilation (Marx et al., 1993) or those with dyspnea associated with left ventricular failure (Timmis et al., 1980). Pain in cancer patients is effectively relieved by various morphine regimens (Foley, 1985).

Pain and Opioid Analgesia in Aging

In the US, the percentage of persons aged 65 and over will increase from 12.6% of the population in 2000 to 14.9% in 2015 and 20.3% in 2030 (Kinsella and Victoria, 2001). Elderly suffer from pain more often than younger people. In old age, the

prevalence of pain may be as high as 70% (Gloth, 1996). It is estimated that 25-50% of aged population residing independently experience significant persistent pain as do 45-80% of those in nursing homes (Am Ger Soc, 2002). Morbidity rates of chronic pain were 400 per 1000 in a population of 81 year old individuals as compared to 75 per 1000 in 18-30 year old population (Davis and Srivastava, 2003). Opioid analgesics remain the mainstay of clinical management of moderate to severe pain. Pain management with opioids often shows large variability in the effects of these drugs in populations that differ in age. A few studies have shown that, in comparison to younger individuals, older patients require less morphine to achieve equivalent levels of analgesia (Macintyre and Jarvis, 1996, Vigano et al., 1998). Pain sensitivity has been found to increase in elderly individuals (Belville et al., 1971, Moore et al., 1990). Elderly people have been shown to experience lower peak effect and longer duration of action of pain relief after morphine administration (Moore et al., 1990). However, the underlying mechanism of changes in sensitivity to pain and increased efficacy of opioids in old age is not clear. Hence, it is important to identify the effect of aging and age-related neurodegeneration on pain and opioid system.

Age-related physiological changes alter the pharmacokinetics and pharmacodynamics of analgesics, narrowing their therapeutic index and increasing the risk of toxicity and drug-drug interactions. For example, with morphine, the risk of respiratory depression increases substantially after 60 years of age (Cepeda et al., 2003). Thus, aging adds to difficulty in management of pain. Poorly controlled persistent pain in the elderly leads to depression, mood disturbances, and cognitive failure and reduces

activities of daily living thus affecting the quality of life in elder patients. Due to limited knowledge and understanding about changes in opioid system with increasing age, management of pain in elderly patients often appears to be irrational and arbitrary. Age is a factor in opioid analgesia and should be used as a guide in determination of dosing regimens (Kaiko, 1980).

A few studies have focused on the effects of aging on basal pain sensitivity and opioid antinociception in animals. Hoskins et al., (1986) reported that as compared to adult mice, aged male ICR mice displayed less antinociceptive response to morphine. In the same study, it was also found that the regional distribution of ^{3}H- morphine in cortex, midbrain, medulla, striatum and periaqueductal region was similar in both age groups. The authors suggest that the age-related differences might not be due to differences in the levels of morphine attained in the brain since such levels were similar in both age groups. A decrease in opioid-induced antinociception in aged animals has also been reported by other investigators (Chan and Lai, 1982, Kavaliers et al., 1983, Wallace et al, 1980) while Saunders et al., (1974) have reported an increase in morphine-induced antinociception in aging. Spratto and Dorio (1978) found that opioid-induced antinociception decreased (5 mg/kg of morphine), increased (7.5 mg/kg of morphine) and remained unchanged (3 mg/kg and 10 mg/kg of morphine) with aging.

The differences in observed basal pain sensitivity and opioid analgesia in aging could be due to possible differences in affinity and/or number and/or responsiveness of opioid receptors in different age groups. Aging may result in decrease in number of neurons and dendritic spines leading to decline in the number of membrane receptors for

neurotransmitters and neuromodulators (Messing et al., 1980). It has been reported that number of catecholamine receptors decreased with aging in rats and humans (Govoni et al., 1978, Greenberg and Weiss, 1978, Maggi et al., 1979). Thus, it is possible that number of opioid receptors decrease with aging, leading to alterations in basal pain sensitivity and changes in opioid analgesia.

Oxidative Stress and Aging

A free radical theory of aging has received a widespread attention. This theory proposes that deleterious actions of free radicals are responsible for deterioration in neuronal function associated with aging (Kasapoglu and Ozben, 2001). Oxidative stress is a ubiquitous phenomenon in all cell types and it is primarily generated in mitochondria. Oxygen is the terminal electron acceptor in the respiratory chain of mitochondria during production of ATP. During the process of respiration, reactive oxygen species (ROS) like superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hydroxyl radical ($^{\cdot}OH$) and peroxynitrite radical ($ONOO^-$) are produced (Boveris, 1984). These active oxidants cause lipid peroxidation of the neuronal cell membranes leading to neuronal damage (Tatsumi and Fliss, 1994). Number of studies have reported increases in lipid peroxidation and protein oxidation in various regions of aged mammalian brains (Leutner et al., 2001, Liu et al., 2002, Smith et al., 1991), thus supporting the role of oxidative stress in aging process.

Eukaryotic cells have specific defenses against these ROS including superoxide dismutase, glutathione peroxidase and catalase whose function is to reduce ROS in

intracellular space (Halliwell and Gittridge, 1999). The excessive generation of ROS beyond capacity of these defensive mechanisms leads to oxidative stress. This oxidative stress has been shown to be associated with neurodegeneration (Floyd, 1999). Severe oxidative stress induces cellular injury leading to apoptosis and/or necrosis, disrupts cellular functions like signaling cascades and transcriptional/posttranscriptional control of gene expression and thus contributes to nerve cell damage (Nose, 2000). Oxidative stress plays a substantial role in neurodegenerative diseases like Alzheimer's disease and Parkinson's disease (Honda et al., 2004). Age-related neurodegeneration has been shown to be associated with oxidative stress (Floyd, 1999)

It has been shown that there is a significant increase in oxidative stress marker of protein oxidation i.e. protein carbonyls in aged mice brain regions like cortex, hippocampus, striatum and midbrain (Forster et al., 2000, Dubey et al., 1996). Since these brain regions predominantly express opioid receptors and play a vital role in pain processing (Delfs et al., 1994, Mansour et al., 1988, Pasternak, 1993, Basbaum and Fields, 1984), the above findings imply that oxidative stress may affect basal pain sensitivity, opioid analgesia and opioid receptors.

Role of Mitochondrial Dysfunction in Oxidative Stress

Brain represents only 2% of the body weight but accounts for 20% of total body oxygen consumption (Simpkins et al., 2005). Neurons of central nervous system have a very high oxygen demand. Hence, mitochondria, the energy production machinery of the cell, play the most important role in survival and function of neurons. Dysregulation of

mitochondrial function results in excessive production of reactive oxygen species overcoming antioxidant capacity of the cell; leading to disruption of normal pro-oxidant: antioxidant status of the cell (Dykens 1995). The mitochondrial insult results in opening of cation channels in outer membrane of mitochondria (Green and Kroemer 2004). Collectively, oxidative stress and excessive Ca^{2+} loading (due to channel opening) cause disruption of inner mitochondrial membrane which decreases mitochondrial membrane potential. This process is called permeability transition (Green and Kroamer 2004). Brain mitochondria from aged mice have lower threshold for calcium-induced, cyclosporin-sensitive calcium release that also contributes to activation of permeability transition pore (Mather and Rottenberg, 2000). Permeability transition results in reduced ATP production, cytochrome C release and caspase activation, eventually leading to apoptosis. Simultaneously, there may also be matrix swelling, membrane rupture and necrosis of the cells (Simpkins et al., 2005).

Effects of Oxidative Stress on Receptor Systems

Because central nervous system function is critical in homeostasis, the attrition of a sufficient number of neurons can lead to age associated disability and loss of various physiological functions such as receptor mediated signal transduction. Oxidative damage leads to increased membrane rigidity which can lead to a decline in receptor signaling. It has been reported that, in aging, there is a significant loss of sensitivity in central neuronal receptors to agonist stimulation (Joseph and Roth, 1992). This loss of sensitivity has been predominantly observed in hippocampal and striatal muscarinic cholinergic

systems and in striatal doparminergic systems (Joseph and Roth, 1992). In younger animals, it has been shown that kainic acid and ionizing radiation-induced oxidative damage causes age-related loss of different adrenergic and cholinergic receptors and disrupt their signal transduction (Joseph and Roth, 1992). In one study, it has been reported that binding density of *mu* opioid receptors in striatum in male Charles River rats was significantly reduced by 3-NPA, a mitochondrial toxin which exerts oxidative stress (Page et al., 2000). Decrease in number of receptors and less efficient signal transduction may be responsible for a significant decline in sensory and motor functions in aging.

SK-N-SH Cell Model

SK-N-SH cells are human neuroblastoma cells (Kohl et al., 1980, Kuramoto et al., 1981). These cells differentiate to a neuronal phenotype and have all the morphological and biochemical characteristics of neurons (Preis, 1998). These cells are widely used to investigate opioid receptors (Bennett and Ratka, 2003, Baker et al., 2000, Hurle et al., 1999). Differentiation of SK-N-SH cells with retinoic acid is associated with considerable reduction in the proliferation rate and induction of neuritic processes (Preis, 1998). As compared to undifferentiated cells, differentiated SK-N-SH cells show increased expression of *mu* and *delta* opioid receptors (Yu et al., 1988). As compared to *mu* and *delta* receptors, *kappa* receptors in these cells have not been well characterized. It has been shown that *kappa* receptor agonists U-50488 H and U-69593 inhibited the amplitude of K^+-induced $[Ca^{2+}]_i$ increase in SK-N-SH cells but this effect was not

blocked by *kappa* receptor antagonist nor-binaltorphimine (Hurle et al., 1999). Opioid receptor-mediated signal transduction pathways such as cyclic adenosine monophosphate (cAMP) pathway are reported to be induced during retinoic acid differentiation. Exposure of SK-N-SH cells to prostaglandin E_1 (PGE_1) results in significant rise in the intracellular cAMP level which can be attenuated by morphine via activation of opioid receptors (Preis, 1998). SK-N-SH cells, therefore, can serve as a useful model for investigating changes in regulation and function of *mu* and *delta* opioid receptors and need to be explored for characterization of *kappa* receptor function.

3-Nitropropionic Acid

3-Nitropropionic acid (3-NPA) is a mitochondrial toxin. It is a succinate dehydrogenase inhibitor that causes uncoupling of mitochondrial oxidative phosphorylation leading to interruption of energy metabolism (Ludolph et al., 1991). 3-NPA has been used in *in vivo* conditions to model acute insults like ischemia (Geddes et al., 2000) as well as neurodegenerative disorders like Alzheimer's disease (Beal, 1995) and Huntington's disease (Beal, 1994). 3-NPA has also been used to induce oxidative stress in *in vitro* conditions (Wang et al., 2001). 3-NPA provokes generation of ROS, decline in mitochondrial membrane potential and depletion of intracellular ATP (Wang et al., 2001). 3-NPA-induced rise in ROS can result in loss of glycolytic enzyme glyceradehyde-3-phasphate dehydrogenase activity due to reduction in glutathione levels resulting in oxidative damage (Binienda et al., 1998). ROS induce oxidative damage to proteins, sugars, lipids and nucleic acids. In the neuronal cell bodies, RNA is the primary

target of oxidative stress (Honda et al., 2004). Thus, oxidative stress damages the neuronal membranes, intracellular RNA and proteins leading to functional deficits in the neurons.

Rationale for Dissertation Research

The long-term objective of the proposed studies is to determine the effect of aging on sensitivity to pain, opioid analgesia and opioid receptors and to determine if oxidative stress contributes to these effects. Pain is an important health problem in old age. Due to limited knowledge and understanding about effects of aging on opioid system, management of pain in these individuals remains unsatisfactory. Literature reports show that geriatric pain is difficult to manage and its management is inadequate (Bernabei et al., 1999, Donovan et al., 1987, Foley, 1995). Hence, probing the issue of pain and opioid analgesia in this population is important.

Although a few studies have shown that aging affects responses to opioid analgesic therapy, effects of aging on opioid receptors expression and function are still unknown. From controversial clinical reports, it is difficult to speculate on changes at the receptor level. Also, effects of oxidative stress on opioid receptors have not been determined yet. In the light of free radical theory of aging, it is important to study if pain and opioid analgesia alter with aging and if these changes are associated with oxidative damage to neurons. The research is also aimed to determine the effects of oxidative stress conditions on opioid receptor expression and function *in vitro*.

The proposed research study is directed at finding a rationale for improving opioid therapy in elderly patients. The new findings about effect of oxidative damage and aging on opioid system will lead to better understanding of complex processes which regulate responses to opioid analgesics in older individuals. Moreover, the proposed study will form a basis for future research on effect of aging and neurodegenerative disorders on opioid system-mediated responses to pain and analgesics.

Over the long term, as a result of an improved knowledge of the impact of aging on opioid system, more rational management of pain with opiates in older patients will be possible. Better understanding of the changes in opioid receptors during aging will be applied in designing most effective and safe dose regimens for opioid analgesics. Fundamental advances within our understanding of the effects of aging and neuronal damage on opioid system will emphasize age as an important factor to consider in management of pain. In view of a significant rise in the size of aged population in the near future, this research project is important and timely.

Hypothesis and Specific Aims

The present research project is designed to investigate effect(s) of oxidative stress and aging on *mu, delta* and *kappa* opioid receptors under *in vivo* and *in vitro* conditions. It was hypothesized that:

(1) There is a decrease in pain threshold and opioid-induced antinociception with advancing age in male Fischer 344 rats and this decrease is caused by oxidative damage to various brain regions.

(2) Oxidative stress decreases the function of *mu*, *delta* and *kappa* opioid receptors by reducing the responsiveness of intracellular cAMP to the receptor activation and reducing the expression of these receptors in SK-N-SH neuronal cells.

Research efforts were focused on two specific aims.

Aim 1: To determine the effects of aging on basal nociceptive sensitivity and opioid analgesia and to study the possible correlation between oxidative damage in various brain regions and these effects in male Fischer 344 rats. The hot plate test was performed to measure changes in response to thermal nociceptive stimulus. Pain measures were performed in three age groups of rats: 3-6 months, 9-12 months and 21-24 months. Hot plate responses to opioid agonists, morphine and fentanyl (*mu* opioid receptor agonist) were measured. Specific brain regions were dissected. Markers of oxidative stress were studied by lipid peroxidation and protein oxidation assays. Pilot studies exploring effect of aging on *mu* opioid receptor (MOR) protein levels in cerebral cortex were carried out with Western blot technique. The effects of aging on pain sensitivity and opioid analgesia were characterized and their correlation with oxidative damage in various brain regions was determined.

Aim 2: To characterize effect(s) of oxidative stress on opioid receptor-mediated reduction in intracellular cAMP and expression of opioid receptors in SK-N-SH neuronal cells. Oxidative stress was induced by exposing differentiated, opioid-responsive SK-N-SH neuronal cells to 3-Nitropropioinic acid (3-NPA), a succinate dehydrogenase inhibitor. *Mu*, *delta* and *kappa* opioid receptors in these cells were investigated. The

changes in intracellular cyclic AMP were used as a marker of opioid receptor function. Effect(s) of oxidative stress on expression of opioid receptor proteins was studied by Western blot technique. Generation of reactive oxygen species was studied with 2', 7'-dichlorofluorescin diacetate assay. Assessment of expression and function of opioid receptors under 3-NPA treatment was used to determine how *mu*, *delta* and *kappa* opioid receptors are affected by the oxidative stress.

Together, these findings contribute to better understanding of the effects of aging and oxidative stress on opioid receptors proteins and their function. Results from this study form a basis for future studies to explore more specific changes in opioid receptor signaling pathways in aging. Ultimately, better understanding of changes in opioid receptor system, evoked by oxidative stress and aging, may provide a rationale for more effective treatment strategies for pain in elderly patients.

Figure 1. Primary afferent nociceptors, nociceptive neurons synapses in the dorsal horn of the spinal cord and ascending projections to higher brain centers (Lembeck and Gamse, 1982).

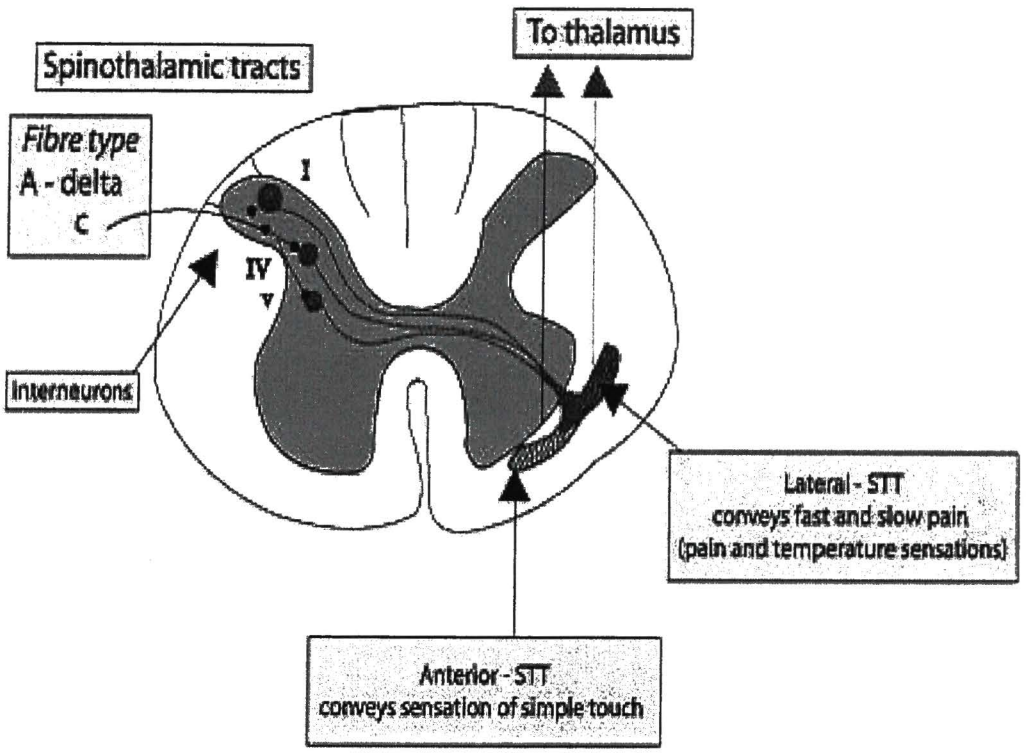

To thalamus
Spinothalamic tracts
Fibre type
A - delta
C
I
IV
V
Interneurons
Lateral - STT
conveys fast and slow pain
(pain and temperature sensations)
Anterior - STT
conveys sensation of simple touch

Figure 2. Ascending and descending pain pathways (adapted from Nestler et al. 2001).

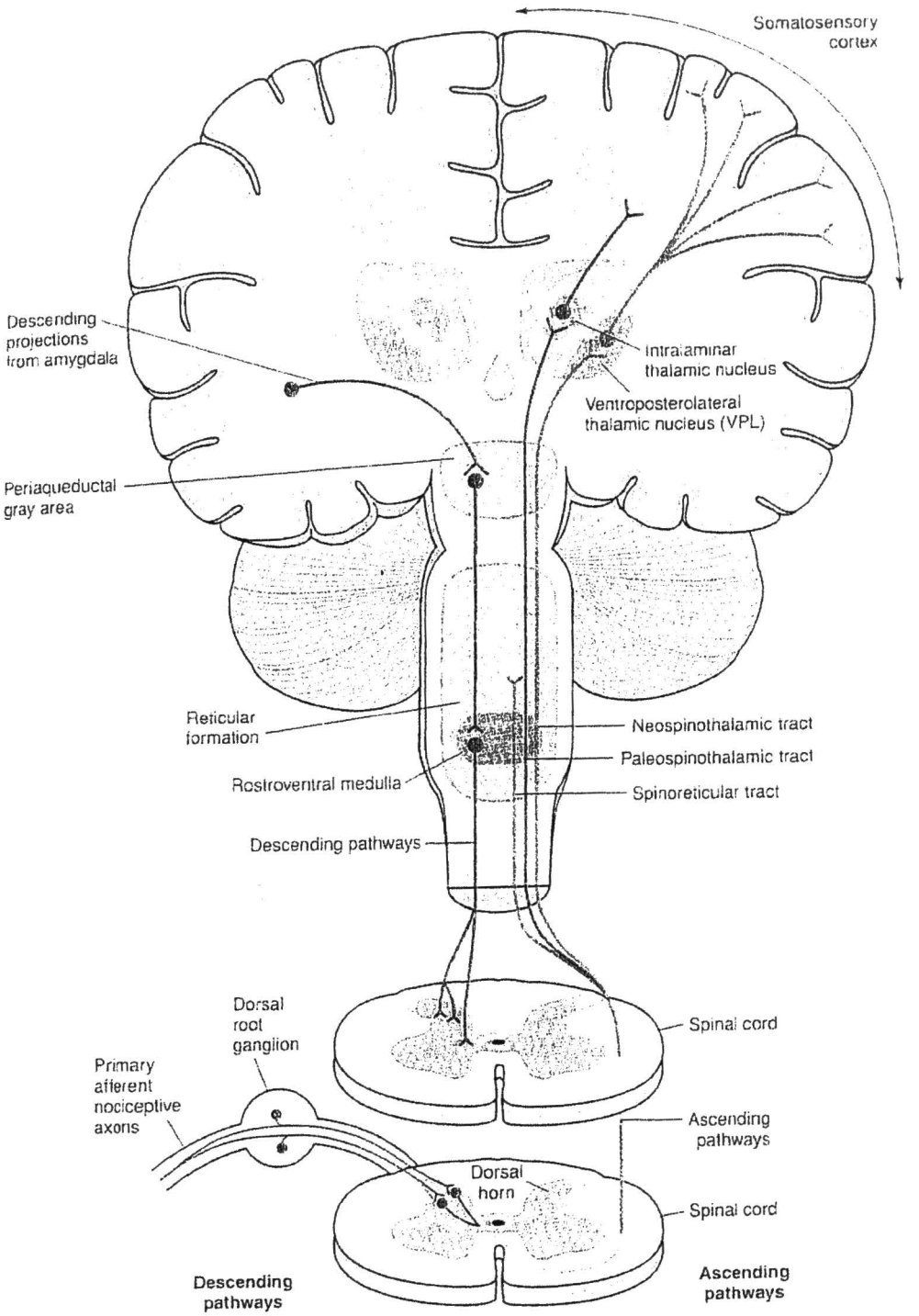

Somatosensory cortex
Descending projections from amygdala
Intralaminar thalamic nucleus
Ventroposterolateral thalamic nucleus (VPL)
Periaqueductal gray area
Reticular formation
Neospinothalamic tract
Paleospinothalamic tract
Rostroventral medulla
Spinoreticular tract
Descending pathways
Dorsal root ganglion
Spinal cord
Primary afferent nociceptive axons
Ascending pathways
Dorsal horn
Spinal cord
Descending pathways
Ascending pathways

Figure 3. Opioid receptor, GTP-binding protein and second messengers (Nestler, 1996)

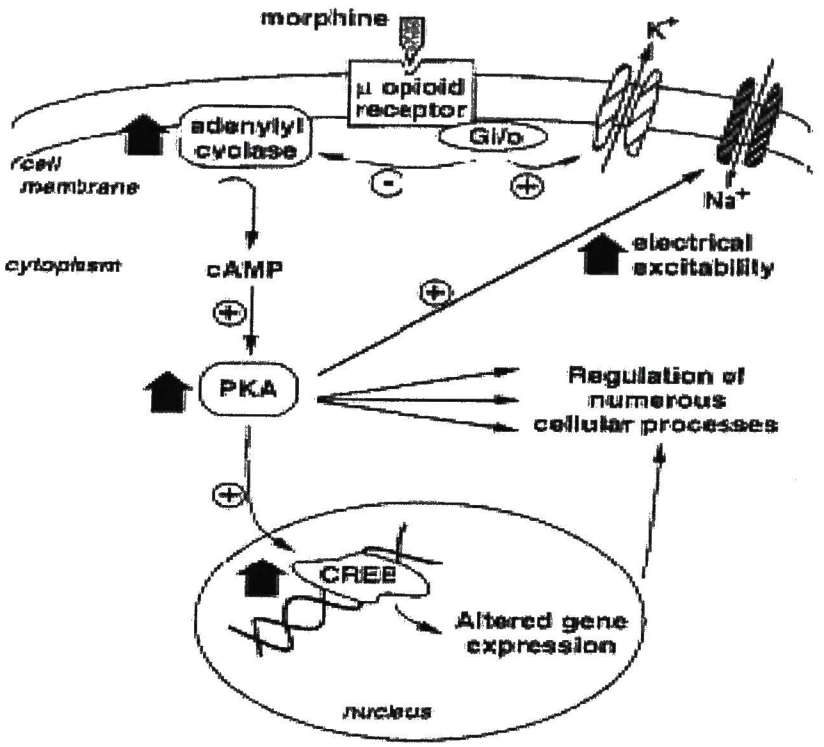
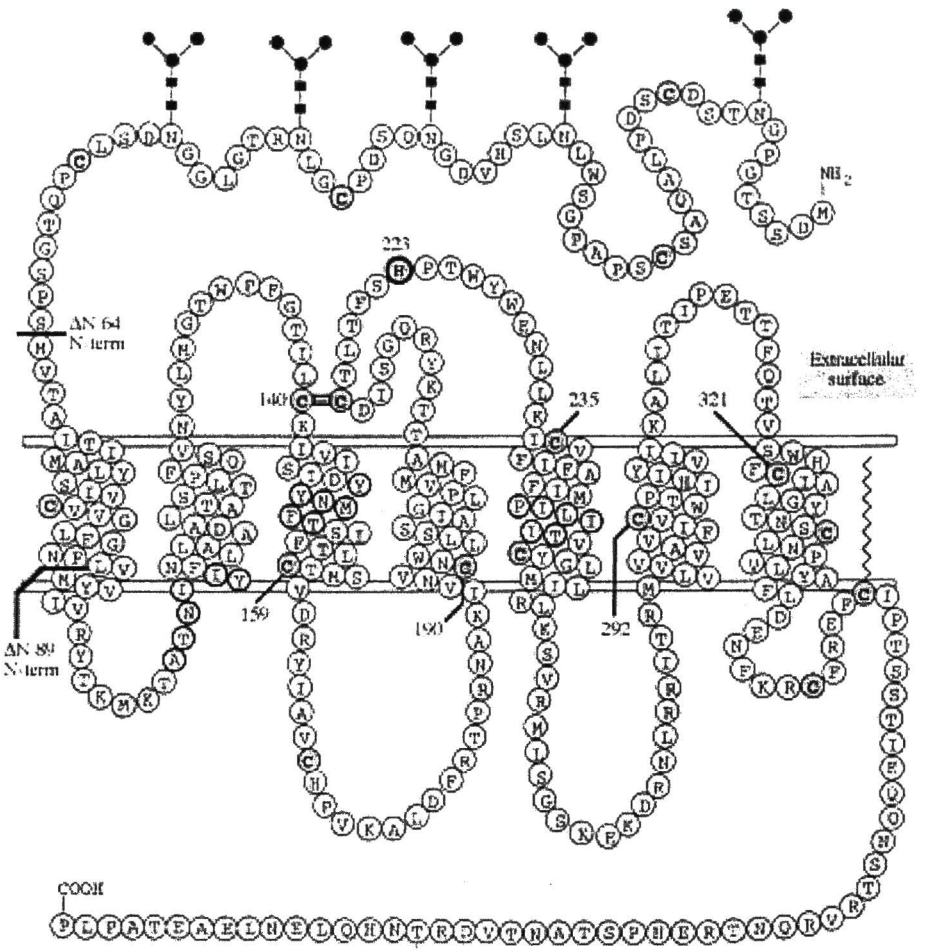

morphine
μ opioid receptor
K+
adenylyl cyclase
Gi/o
cell membrane
Na+
cytoplasm
cAMP
electrical excitability
PKA
Regulation of numerous cellular processes
CREB
Altered gene expression
nucleus

Figure 4. General structure of opioid receptors. All three opioid receptors (MOR, DOR and KOR) possess the same general structure of an extracellular N-terminal region, seven transmembrane domains and intracellular C-terminal tail structure. The particular amino acid sequence and proposed transmembrane topology shown in the figure is of the rat MOR (Reisine and Pasternak, 1996).


ΔN 64
N-term
ΔN 89
N-term
223
140
159
190
235
292
321
Extracellular surface
NH2
COOH

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CHAPTER II

OXIDATIVE DAMAGE AND SENSITIVITY TO PAIN AND OPIOIDS IN AGING RATS

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Abstract

Oxidative stress plays an important role in aging and development of neurodegeneration. Aberrant pain associated with oxidative stress-induced neurodegenerative disorders is inadequately managed because the neurodegeneration-related changes in sensitivity to pain and opioids are poorly understood. The knowledge about effects of oxidative stress on the opioid system is very limited. We have demonstrated significant decrease in opioid receptor function in neuronal cells exposed to oxidative stress. The goal of this project was to study the relationship between oxidative damage and sensitivity to pain and opioids in aging rats. Three age groups (3-6, 9-12, and 21-24 months) of male Fischer 344 rats were tested for pain threshold and responses to morphine and fentanyl using the hot plate method. Oxidative stress markers in various brain regions were measured. The baseline sensitivity to thermal nociceptive stimulus increased significantly with age. There was a significant age-dependent and dose-dependent decrease in the antinociceptive effects of morphine and fentanyl. Levels of oxidative stress markers were the highest in brain regions from the oldest group of rats. There was a significant negative correlation between morphine antinociception and protein oxidation in cortex, striatum, and midbrain (r^2 = 0.73, 0.87, and 0.77, respectively), and lipid peroxidation in cerebral cortex, hippocampus and striatum (r^2 = 0.73, 0.61 and 0.71, respectively). Significant negative correlation was observed between fentanyl antinociception and protein oxidation in cortex, striatum and midbrain (r^2 = 0.39, 0.55, and 0.3 respectively), and lipid peroxidation in cerebral cortex, hippocampus and striatum (r^2 = 0.27, 0.39 and 0.26, respectively). A pilot study suggested a decrease in

MOR protein level in cerebral cortex in the oldest rats. The results from this research show that (1) at the advanced age, the sensitivity to pain is significantly increased and the antonociceptive effect of opioids significantly decreased, (2) oxidative stress is negatively correlated with the opioid-induced antinociception, and (3) age-related changes in pain and analgesia may be due to oxidative stress-induced reduction in opioid receptors protein level.

Keywords: Age, analgesia, antinociception, oxidative stress, pain.

Introduction

Elderly suffer from pain more often than younger people. In old age, the prevalence of pain may be as high as 70% (Gloth, 1996). It is estimated that 25-50% of aged population residing independently experience significant persistent pain as do 45-80% of those in nursing homes (Am Ger Soc, 2002). Morbidity rates of chronic pain were 400 per 1000 in a population of 81 year old individuals as compared to 75 per 1000 in 18-30 year old population (Davis and Srivastava, 2003). Opioid analgesics remain the mainstay of clinical management of moderate to severe pain. Pain management with opioids often shows large variability in the effects of these drugs in populations that differ in age. A few studies have shown that, in comparison to younger individuals, older patients require less morphine to achieve equivalent levels of analgesia (Macintyre and Jarvis, 1996, Vigano et al., 1998). Pain sensitivity has been found to increase in elderly individuals (Belville et al., 1971, Moore et al., 1990). Elderly people have been shown to experience lower peak effect and longer duration of action of pain relief after morphine administration (Moore et al., 1990). Due to limited knowledge and understanding about changes in opioid system with increasing age, management of pain in elderly patients often appears to be irrational and arbitrary. Age is a factor in opioid analgesia and should be used as a guide in determination of dosing regimens (Kaiko, 1980). Literature reports show that geriatric pain is difficult to manage and its management is inadequate (Bernabei et al., 1999, Donovan et al., 1987, Foley, 1985).

A few animal studies have reported the differences in the basal pain sensitivity and antinociceptive response to opioids in different age groups. Decline in morphine-

induced antinociception has been reported in aged male ICR mice (Hoskins et al., 1986). In the same study, it was found that the regional distribution of ^{3}H- morphine in cortex, midbrain, medulla, striatum and periaqueductal region was similar in young and old age groups. Thus, the age-related differences might not be due to differences in the levels of morphine attained in the brain since such levels were same in both age groups. Similar findings were reported by other investigators (Chan and Lai, 1982, Kavaliers et al., 1983, Wallace et al, 1980) while other studies reported reductions and/or increases in morphine antinociception with aging (Saunders et al., 1974, Spratto and Dorio, 1978). The underlying mechanism of changes in the pain sensitivity and altered efficacy of opioids in old age still remains unclear.

During past few decades, much attention has been focused on free radical theory of aging. According to free radical theory, aging is a result of cellular damage caused by destructive reactive oxygen species (ROS) generated during the normal process of aerobic respiration (Harman, 1956). Oxidative stress has been implicated in aging and neurodegenerative diseases (Kasapoglu and Ozben, 2001). Age-related neurodegeneration has been shown to be associated with oxidative stress (Floyd, 1999). It has been shown that there is a significant increase in oxidative stress marker of protein oxidation i.e. protein carbonyls in aged mice brain regions such as cortex, hippocampus, striatum and midbrain (Forster et al., 2000, Dubey et al., 1996).

Since these brain regions predominantly express opioid receptors (Zastawny et al., 1994, Pasternak, 1993, Mansour et al, 1988, Delfs, 1994) and play a vital role in pain

processing (Basbaum and Fields, 1984), the above findings imply that oxidative stress may affect basal pain sensitivity, opioid analgesia and opioid receptors.

However, it has not yet been explored if oxidative damage in aging is associated with alterations in basal pain sensitivity and responses to opioid analgesia. We have recently reported that 3-nitroprorpionic acid induced oxidative stress reduced *mu* opioid receptor (MOR) function in opioid-responsive SK-N-SH neuronal cells (Raut et al., 2006). The present research project was designed to determine the effects of aging on basal nociceptive sensitivity and opioid-induced antinociception and to study the possible correlation between oxidative damage and these effects. Pilot studies exploring effect of aging on *mu* opioid receptor (MOR) protein in cerebral cortex in rats were also carried out.

Materials and Methods

Animals

The protocol was approved by Institutional Animal Care and Use Committee (IACUC) of the University of North Texas Health Science Center. Male Fisher 344 rats of three different age groups i.e. 3-6 months, 9-12 months and 21-24 months were studied. The rats were housed under standard conditions within AAALAC accredited conditions (temperature 22 $\pm$ 2^{0}C and light illuminated daily 0700-2000 hrs). Rats were provided tap water and laboratory chow *ad libitum*. The animals were allowed 10 days acclimate to animal facilities. During acclimatization, rats were handled daily by the experimenter.

Hot Plate Testing

Three different age groups consisting of 40 rats per group were studied. Each group was divided into 4 subgroups of rats. Each subgroup received either vehicle (control) or one of three different doses of morphine. The rats in the control group remained in the control group and the rats in the experimental groups remained in the respective experimental groups throughout all the experimental sessions. After the wash out period of one week, the experiments were repeated with fentanyl. Hot plate testing was done at baseline and 30, 45 and 60 minutes before and after morphine administration and 15, 30 and 45 minutes before and after fentanyl administration. Experimental sessions were carried out in animal housing room. Before each experimental session, the rats were weighed and at least 30 minutes later, the experimental testing started.

The rat's responses to a thermal nociceptive stimulus at 56 $\pm$ 0.1^{0}C were determined using the hot plate method. The hot plate apparatus provides a constant temperature which is low enough to prevent harm to the animal yet high enough to be perceived as thermal pain. Each rat was placed on the heated plate and the time lapse (in seconds) until front or hind paw lick was recorded as hot plate latency (HPL). A cut off time of 30 seconds was be used to avoid thermal trauma to the paws.

Antinociceptive effect was calculated as % Maximum Possible Effect (%MPE) using the following formula:

% MPE = [(Observed HPL) – (Baseline HPL) / (30-Baseline HPL)] x 100

Drug Administration

Morphine and fentanyl were obtained from Sigma Chemicals (St. Louis, MO). The drugs were dissolved in saline. Morphine was administered at doses 5-15 mg/kg subcutaneously (Kramer and Bodnar, 1986, Hoskins et al., 1986, Craft et al., 1995). Fentanyl was injected subcutaneously at the doses of 25- 50 μg/kg (Redwine and Trujillo, 2003, Rivat et al., 2002). To avoid diurnal variations in animal responses to a painful stimulus, all hot plate tests were performed between 0800 and 1100 hours.

Dissection of Brain Regions

The rats were sacrificed by decapitation. The whole brain was quickly excised on an ice cold Petri dish and the following brain regions were dissected: midbrain, striatum, hippocampus and cerebral cortex. The brain tissue was stored in antioxidant buffer (50 mM phosphate buffer (pH 7.4), 1 mM BHT, 100 μM DTPA) at -80^{0}C until further analysis for oxidative stress marker assays and Western blot immunoassay.

Lipid Peroxidation Assay

Lipid peroxidation products were measured as thiobarbituric acid-reactive substances (TBARS) (Ohkawa et al., 1979). Thiobarbituric acid was added to the samples and the products were measured by fluorometry at excitation 525 nm and emission 550 nm. Concentrations of the samples were calculated by using a standard curve calibrated with standard solutions of thiobarbituric acid.

Protein Oxidation Assay

Protein carbonyl content, as a marker of protein oxidation, was measured as described previously (Levine et al., 1990). Briefly, the supernatants of brain tissue were used for reaction with 2, 4-dinitrophenylhydrazine (DNPH). For each sample, the supernatants were divided into two equal volumes. Four volumes of 10 mM DNPH in 2 M HCl were added to one of the sample pair, and four volumes of 2 M HCl alone was added to the other one (for reagent blank assay). Samples were then incubated for 1 hr at room temperature in the dark and were precipitated with an equal volume of 20% trichloroacetic acid (TCA). After 10 min on ice, samples were centrifuged at 3,000 x *g* for 5 min and supernatants were discarded. Protein pellets were washed in 10% TCA once and in ethanol/ethyl acetate (1:1) three times to remove free DNPH and additional lipid contaminants. Final protein precipitates were dissolved in denaturing buffer solution. The differences (Δ) in absorbance between the DNPH-treated and the HCl-treated samples were determined by spectrophotometry at 375 nm and the amount of carbonyl contents (C) was calculated by using a molar extinction coefficient (ϵ) of $22{,}000^{-1}$ cm^{-1} [C in nmol/ml = A_{375} (Δ) x $10^6/\epsilon$]. Data are expressed as nmoles of carbonyls per mg of soluble extracted protein.

Statistical Analysis

Data are expressed as mean ± S.E.M. The statistical evaluation of data was performed by analysis of variance (ANOVA) followed by the Tukey post hoc test using GraphPad Prism Version 4.0 statistical software package. Correlation was studied with

linear regression analysis and Pearson's coefficient (r) was calculated. p values <0.05 were considered as statistically significant.

Test-retest reliability of hot plate measurements was assessed by estimating Pearson's coefficient for datasets collected from a randomly selected 10 rats from young, adult and old age groups (n=30). Correlation between basal hot plate latencies at initial session and basal hot plate latencies after 7 days' session was studied. The value of Pearson's coefficient served as an index for the consistency of the hot plate latencies across different experimental sessions.

Results

Effect of aging on sensitivity to thermal nociceptive stimulus in male Fischer 344 rats

Figure 1 presents the results on the baseline sensitivity to thermal nociceptive stimulus in young (3-6 months), adult (9-12 months) and old (21-24 months) rats. The baseline hot plate latency in old rats was 2.4 $\pm$ 0.1 seconds, in adult rats it was 4.9 $\pm$ .1 seconds and in young rats it was 5.2 $\pm$ 0.1 seconds. The hot plate latency values in adult and old rats were significantly lower as compared to the latencies measured in the young rats (young vs. adult p^* <0.05, young vs. old p^* < 0.001).

Basal hot plate latency and body weight

Basal nociceptive threshold (as indicated by baseline hot plate latency) was not significantly correlated with body weight in young, adult and old age groups (r^2 = 0.00, 0.00 and 0.19 respectively, Fig.2).

Opioid-induced antinociception and body weight

There was no significant correlation between body weight and maximum analgesia with morphine (15 mg/kg at 60 minutes) in homogenous population of all the three age groups (r^2 = 0.09, Fig. 3).

Test-retest reliability

Correlation studies between basal hot plate measurements during consecutive experimental sessions showed a significant correlation (r^2 = 0.23, $p < 0.05$).

Effect of aging on morphine-induced antinociception

There was a gradual increase in morphine-induced antinociception from 30 to 60 minutes and the maximum effect was observed at 60 minutes (Fig. 4). At 30 minutes after 5 mg/kg of morphine administration, old rats had 6.8 $\pm$ 0.7 % MPE which was significantly lower than 10.4 $\pm$ 0.5 % in the young rats (Fig. 4 A). Similarly, old rats showed significantly lower antinociceptive effects than young rats at 45 and 60 minutes after morphine administration at 5 mg/kg of dose.

At the dose of 10 mg/kg, as compared to 36.5 ± 2 % MPE in young rats, old rats showed 29.1 ± 2.3 % MPE which was statistically significant (Fig. 4B). Similar significant differences in morphine-induced antinociception were observed at 45 and 60 minutes. No significant group differences in morphine-induced antinociception were observed between young and adult rats at doses of 5 and 10 mg/kg.

At 30 minutes, 15 mg/kg of morphine caused significantly lower antinociception in adult and old rats (46 ± 2.7 and 36.4 ± 1.8 respectively) as compared to the young rats (60.4 ± 2.4%, Fig. 4C). At 45 and 60 minutes, similar significant differences in morphine-induced antinociception were observed in the rats of three age groups of rats.

Effect of aging on fentanyl-induced antinociception

Maximum antinociceptive effects of fentanyl were observed at 30 minutes after the drug administration. At 15 minutes after 25 μg/kg of fentanyl administration, old rats had 6.9 ± 0.7 % MPE which was significantly lower than 9.6 ± 0.7 % in the young rats (Fig. 5 A). Similarly, old rats showed significantly lower antinociceptive effects than young rats at 30 and 45 minutes after 25 μg/kg of fentanyl administration.

At the dose of 37.5 μg/kg, old rats had significantly lower antinociception (17.4 ± 1.2 % MPE) as compared to 24.5 ± 1.2 % MPE in young rats (Fig. 5B). Similar significant differences in fentanyl-induced antinociception were observed at 30 and 45 minutes. No significant group differences in fentanyl-induced antinociception were observed between young and adult rats at doses of 25 and 37.5 μg/kg.

At 15 minutes, 50 μg/kg of fentanyl had significantly lower antinociception in adult and old rats (35.3 ± 1.7 % and 29.8 ± 1.7 % respectively) as compared to the young rats (44.3 ± 2.7 %) (Fig. 5C). At 30 minutes, similar significant differences in fentanyl-induced antinociception were observed in the rats of three age groups. The difference between fentanyl-induced antinociception in adult and young rats became statistically insignificant at 45 minutes.

Oxidative Stress Markers in Various Brain Regions

Protein Oxidation

The levels of protein oxidation marker, i.e. protein carbonyls showed a gradual increase with aging in cerebral cortex, hippocampus, striatum and midbrain (Fig 6). In cerebral cortex, old rats showed significantly higher carbonyl content (2.2 ± 0.1 nmoles/mg protein) as compared to young rats (1.5 ± 0.2 nmoles/mg protein) (Fig 6 A). Similarly, protein carbonyl content in hippocampus in old rats was significantly higher than the young rats (3.9 ± 0.2 and 2.2 ± 0.3 nmoles/mg protein respectively) (Fig. 6B). Striatum showed the highest (approximately 3 fold) difference in protein carbonyl levels between young and old age groups (Fig. 6C). Striatum showed significantly higher levels of protein content in adult and old rats (2 ± 0.1 and 3 ± 0.2 nmoles/mg protein respectively) as compared to young rats (1.3 ± 0.1 nmoles/mg protein) (Fig. 6C). Similarly, midbrain showed significantly higher levels of protein carbonyls in adult and old rats (2.1 ± 0.1 and 2.9 ± 0.3 nmoles/mg protein respectively) as compared to young rats (1.7 ± 0.1 nmoles/mg protein) (Fig. 6D).

Lipid Peroxidation

TBARS content, a marker of lipid peroxidation increased progressively with aging in cerebral cortex, hippocampus, striatum and midbrain (Fig 7). In cerebral cortex, old rats showed significantly higher levels of TBARS (0.5 $\pm$ 0.1 nmoles/mg protein) as compared to young rats (0.2 $\pm$ 0.02 nmoles/mg protein) (Fig 6 A). Similarly, TBARS content in hippocampus in old rats was significantly higher than young rats (0.3 $\pm$ 0.1 and 0.2 $\pm$ 0.02 nmoles/mg protein respectively) (Fig. 6B). Striatum showed significantly higher levels of protein content in adult and old rats (0.4 $\pm$ 0.02 and 0.5 $\pm$ 0.04 nmoles/mg protein, respectively) as compared to young rats (0.2 $\pm$ 0.01 nmoles/mg protein) (Fig. 6C). No significant group differences were observed in TBARS content in midbrain of the three age groups of the rats (Fig. 7D).

Oxidative Damage and Opioid Antinociception

Protein oxidation and morphine antinociception

There was a significant negative correlation between maximum antinociception with morphine (15 mg/kg at 60 minutes) and protein oxidation in cortex, striatum, and midbrain (r^2 = 0.73, 0.87, and 0.77, respectively) (Fig. 8).

Protein oxidation and fentanyl antinociception

A significant negative correlation was observed between fentanyl-induced antinociception (50 μg/kg at 30 minutes) and protein oxidation in cortex, striatum and midbrain (r^2 = 0.39, 0.55 and 0.30, respectively) but not in hippocampus (Fig. 9).

Lipid peroxidation and morphine antinociception

Morphine-induced antinociception (15 mg/kg at 60 minutes) had a significant negative correlation with lipid peroxidation in cerebral cortex, hippocampus and striatum (r^2 = 0.73, 0.61 and 0.71, respectively) (Fig. 10). Lipid peroxidation in midbrain was also negatively correlated with morphine antinociception but the results were statistically insignificant.

Lipid peroxidation and fentanyl antinociception

There was a significant negative correlation between fentanyl-induced antinociception (50 μg/kg at 30 minutes) and lipid peroxidation in cerebral cortex, hippocampus and striatum (r^2 = 0.27, 0.39 and 0.26, respectively) (Fig. 11). No significant correlation was observed between lipid peroxidation in midbrain and fentanyl antinociception.

Discussion

Effect(s) of aging on opioid system are poorly understood. The factors responsible for alterations in pain sensitivity and opioid analgesia during aging have not yet been clearly identified. It is not known if age-related oxidative damage plays any role in modulation of opioid system function. The present project studied the effect(s) of aging on pain sensitivity and opioid-induced antinociception in young, adult and old rats and the possible association of these effects with oxidative damage to various brain regions. The results demonstrate that aging increases pain sensitivity and decreases opioid

antinociception and moreover, responses to opioids are negatively correlated with oxidative damage to cerebral cortex, hippocampus, striatum and midbrain.

Since effects of opioids have been well characterized in Fischer 344 rats (Gosnell & Krahn 1993, Woolfolk & Holtzman 1995), this rat strain was chosen as an animal model. Opioid antinociception was tested with morphine and fentanyl (*mu* opioid receptor selective agonist) (Reisine and Pasternak, 1996). In this study, we have used at least 7 days interval between drug exposures in the rats to avoid development of tolerance. Previous studies have reported that repeated testing of opioids does not lead to tolerance if the exposure is separated by 5-7 days interval (Cook et al., 2000, Walker et al., 1999, Smith and Gray 2001). We used the dose ranges: for morphine, 5-15 mg/kg and for fentanyl, 25-50 μg/kg. These doses were based on reports from previous studies as mentioned in methods section and our pilot studies. The doses below 5 mg/kg (morphine) and 25 μg/kg (fentanyl) did not elicit any antinociceptive effects as measured by the hot plate method, and the doses above 15 mg/kg (morphine) and 50 μg/kg (fentanyl) resulted in catatonia in the rats that interfered with measurement of the hot plate latency.

In the present study, a progressive increase in nociceptive sensitivity and a decrease in antinociceptive responses to opioids (morphine and fentanyl) were observed in aging rats. These data are consistent with earlier reports. Progressive increase in pain sensitivity and reductions in morphine analgesia on thermal nociceptive tests have been reported in 3-6 and 24-27 months old male ICR mice (Hoskins et al., 1986), Charles River rats at 2, 5 and 10 months of age (Chan and Lai, 1982) and in 2-24 months old C57BL/6J mice (Webster, 1976).

However, Smith and French (2002) have reported that there was no significant difference in baseline nociceptive sensitivity in young (3 months) and old (21 months) Fischer male rats on warm water tail-withdrawal test. Tail-withdrawal test reflects spinal responses (Dennis et al., 1980) while the hot plate method, which has been used in the present study, determines supraspinal responses to painful stimulus (Dennis et al., 1980, Ramabardran and Banisnath, 1986). Thus, different method predominantly reflects nociception at different levels of the central nervous system. Therefore, dissimilarities between results due to different pain testing methods may occur. Aging may cause a more profound decline in function of opioid system at supraspinal level than at spinal level; accounting for the observed differences in pain parameters measured by hot plate and tail emersion tests. Morphine analgesia as measured by the vocalization test was higher in older (9-10 months) as compared to younger (2-3 months) Sprague-Dawley rats (Saunders et al., 1974). The discrepancies in the results might be attributed to the differences in definition of aged animals; the age of the oldest group used by Saunders et al. was 9-10 months as compared to 21-24 months in our study. Animal strain differences and difference in the algesia measurement methods i.e. hot plate (thermal) *versus* vocalization (electrical) test could also account for variability in the results.

Observed age-related increase in sensitivity to nociceptive stimulus and reduction in responses to opioids in the present study may be due to multiple factors. Since aging rats gain body weight, we determined the relationship between body weight and sensitivity to pain stimulus and opioid-induced antinociception. Increase in body weight could lead to increased pressure between paw surface and the hot plate that might result

in faster paw withdrawal from the thermal stimulus. This might have resulted in increased sensitivity to pain (i.e. shorter hot plate latency) in aged rats. Correlation analysis between body weight and sensitivity to thermal stimulus showed that there was no correlation between these two factors in any of the age groups. Similarly, no significant correlation was found between opioid-induced antinociception and body weight in these three age groups of rats. These findings rule out the possible contribution of age-related increase in body weight to increase in sensitivity to painful stimulus in old rats.

The differences in observed basal pain sensitivity and opioid analgesia in aging could be due to the possible differences in affinity and/or number and/or responsiveness of opioid receptors in different age groups. A significant reduction in density of binding sites for ^{3}H-dihydromorphine was observed in thalamus and midbrain in aged (26 months old) female Fischer 344 rats (Messing et al., 1980). It can be postulated that this reduction may be due to either loss of binding sites or impaired receptor plasticity leading to decline in receptor function in old age.

There could be other possible explanations for the observed effects of aging on opioid antinociception. With increasing age, there are alterations in central neurotransmitter systems like dopaminergic, adrenergic and cholinergic system (Kanowski, 1977, Pradhan, 1980). Morphine analgesia and central dopaminergic and cholinergic systems have synergistic effects (Chan, 1979, Chan and Yip, 1979). Thus, the progressive decrease in morphine antinociception with aging may reflect a decline in dopaminergic, adrenergic and cholinergic systems in senescent brain. Binding studies have shown that opioid receptor concentration was reduced in aged rats, especially in

brain regions associated with dopaminergic systems (Jensen et al., 1980). Although morphine antinociception in aged (24-27 months) male ICR mice was significantly lower than that of in young (3-6 months) mice, regional distribution of morphine in cerebral cortex, midbrain, striatum and periaqueductal gray area was not different in these two age groups (Hoskins, 1986). Thus, the age-related differences in opioid antinociception might be due to changes in opioid system rather than the differences in levels of morphine attained in the brain.

In the light of free radical theory of aging (Harman, 1956) and our previous findings that oxidative stress decreases MOR function (Raut et al., 2006), the present study focused also on possible association between oxidative damage to cerebral cortex, hippocampus, striatum and midbrain, and opioid antinociception in three age groups of rats. Brain regions such as cerebral cortex, striatum and midbrain play an important role in pain processing (Basbaum and Fields, 1984). After the nociceptive stimulus is perceived as pain, descending projections to periaqueductal gray (PAG) area in midbrain originate from cortical neurons and striatum (Basbaum and Fields, 1984). These brain regions express opioid receptors predominantly (Zastawny et al., 1994, Pasternak, 1993, Mansour et al, 1988, Delfs, 1994) and many studies have shown that markers of oxidative damage to proteins and lipids in these regions increase with aging (Forster et al., 2000, Dubey et al., 1996, Leutner et al., 2001, Liu et al., 2002).

In our study, two markers of oxidative damage were measured; protein oxidation by the protein carbonyl assay and lipid peroxidation by the TBARS assay. With aging, protein carbonyl content progressively increased in cerebral cortex, hippocampus,

striatum and midbrain. These findings are in accord with many of the previous reports. Protein carbonyl content in these brain regions was significantly higher in old (27 months) C57BL/6 mice as compared to young (8 months) mice (Dubey et al., 1996). Whole brain homogenates of old (22 months) C57BL/6 mice showed significantly higher protein carbonyl content than that of young (4 months) mice (Forster et al., 2000). Farr et al., (2003) found that brain homogenates of 12-month-old SAMP8 mice, in comparison with 4-month-old mice, had increased levels of protein carbonyls.

We have also found that TBARS content (marker of lipid peroxidation) was higher in adult and old aged rats than young rats. These results are consistent with the previous findings. It has been reported that adult (13 months) and old (18 months) CD1 Swiss mice had higher levels of TBARS content in brain homogenates as compared to young (7 months) mice (Navarro et al., 2002). TBARS content in mitochondria isolated from brain homogenates in adult (13 months) and old (18 months) Wistar rats was found to be higher than that of in the young (7 months) rats (Navarro and Boveris, 2004). Brain homogenates of 12-month-old SAMP8 mice, in comparison with 4-month-old mice, showed increased levels of TBARS (Farr et al., 2003). Thus, it was observed that there was a significant increase in oxidative damage in aging.

To explore the possibility that oxidative damage may play a role in modulation of opioid responses in aging, the correlation between opioid antinociception and oxidative stress markers was studied. Protein oxidation in cerebral cortex, striatum and midbrain and opioid antinociception showed a significant negative correlation. Oxidation of opioid receptor proteins in these regions may lead to decreased opioid receptor function leading

to reduced responses to opioids. There was no correlation between protein oxidation in hippocampus and opioid antinociception. This might be due to different opioid receptors densities in other brain regions and hippocampus. In contrast to other brain regions, we could not achieve quantifiable bands for opioid receptors in hippocampus in the pilot Western immunoassay (data not shown); that might suggest very low expression of opioid receptors in hippocampus. Lower levels of opioid receptors in hippocampus, even if oxidized in aging, may not be enough to influence opioid analgesia. Hence, although protein oxidation in hippocampus was significantly higher in old rats, it was not associated with decline in opioid-induced antinociception in old rats.

There was a significant negative correlation between lipid peroxidation in cerebral cortex, striatum and hippocampus and opioid antinociception. Lipid peroxidation of the neuronal cell membranes leads to neuronal damage (Tatsumi and Fliss, 1994). Neuronal damage in these brain regions might have been responsible for impairment of opioid system function or pain processing. Lipid peroxidation in midbrain in aged group was not significantly different from the younger groups. This may account for observed insignificant correlation between lipid peroxidation in midbrain and opioid antinociception.

Pearson's coefficient values indicated that, as compared to morphine-induced antinociception, fentanyl-induced antinociception showed relatively weaker correlation with oxidative damage. The weaker correlation might be due to selective action of fentanyl on MOR. Morphine acts on MOR and other opioid receptor subtypes (Reisine and Pasternak, 1996) that might be affected by oxidative damage. The oxidative damage

to multiple opioid receptors subtypes might result in further decline in morphine antinociception, thus showing stronger negative correlation with oxidative damage.

The negative correlation between oxidative stress markers and opioid-induced antinociception suggests that oxidative damage in various brain regions possibly affects number and/or function of opioid receptors. Aging may result in a decrease in number of neurons and dendritic spines leading to declines in the number of membrane receptors for neurotransmitters and neuromodulators (Messing et al., 1980). Reduction in number of catecholamine receptors has been observed in aged rats and humans (Govoni et al., 1978, Greenberg and Weiss, 1978, Maggi et al., 1979). Knowledge about the effects of oxidative stress on opioid receptor system is very limited. In one study, it has been reported that binding density of *mu* opioid receptors in striatum in male Charles River rats was significantly reduced by 3-NPA, a mitochondrial toxin which exerts oxidative stress (Page et al., 2000). In our pilot study, a decrease in MOR protein levels was observed in cerebral cortex in older male Fischer 344 rats (data not shown). Thus, age-related oxidative damage may cause a decrease in number of opioid receptors leading to reduction in responses to opioids.

Taken together, our findings suggest that the increase in oxidative damage in different brain regions and a decrease in opioid receptors proteins may contribute to an increase in pain sensitivity and a decrease in opioid antinociception in aging. Although the results in this study do not offer a definite explanation for the role of oxidative stress in modulation of opioid analgesia in aging, the data have shown that, in aging, there is a

decrease in pain threshold and responses to opioids and a negative correlation exists between oxidative damage and opioid-induced antinociception.

Conclusions

In conclusion, the results from our study show that in aged male Fischer 344 rats, there is an increase in nociceptive sensitivity and a decrease in opioid antinociception that are associated with increased oxidative damage in cerebral cortex, striatum, hippocampus and midbrain.

To more fully understand the mechanism(s) underlying the effects of aging on sensitivity to pain and opioids, further studies need to explore proteins and mRNA expression of opioid receptor subtypes in brain.

Figure 1: Effect of aging on sensitivity to painful stimulus in male Fischer 344 rats. The baseline sensitivity to thermal nociceptor were measured in young (3-6 months), adult (9-12 months) and old (21-24 months) rats using hot plate technique at 56 ± 0.1^{0} C. Hot plate latencies were measured in seconds. $N = 40$ in each group. * $p < 0.05$ as compared to young age group.

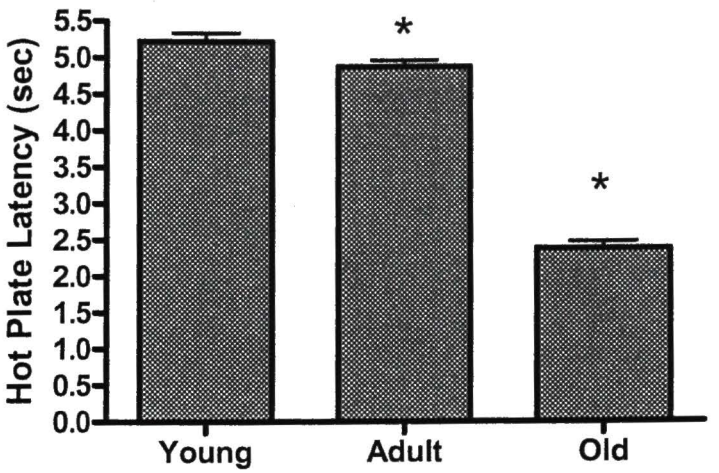
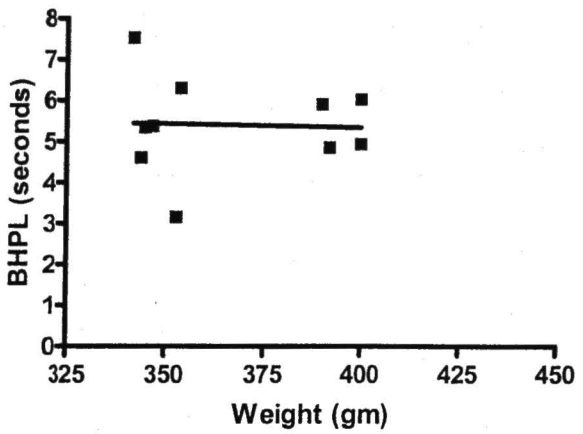

Hot Plate Latency (sec)
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Young
Adult
Old

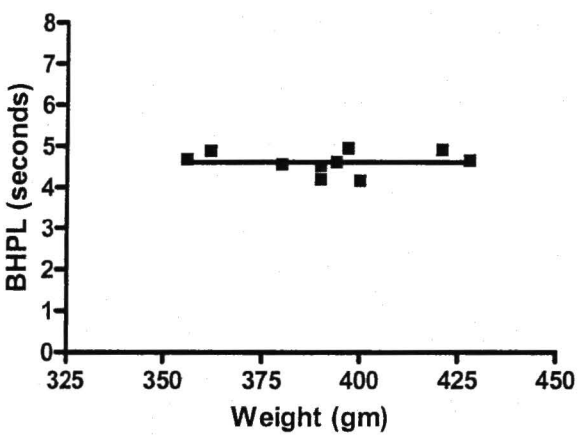
Figure 2. Correlation between body weight and basal hot plate latencies (BHPL) in 3 age groups of male Fischer 344 rats. Linear regression relationship between body weight and sensitivity to pain was analyzed using Pearson's coefficient in (A) Young (B) Adult and (C) Old rats. Rats were randomly selected from each age group. $N = 10$ in each age group.

A



$r^2 = 0.00$
$p = 0.91$

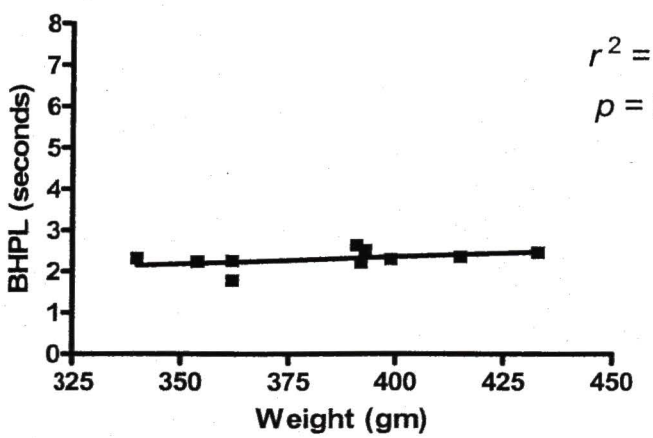
B



$r^2 = 0.00$

$p = 0.96$

C



$r^2 = 0.19$
$p = 0.19$

Figure 3. Correlation between body weight and morphine antinociception (expressed as % MPE) in homogeneous population of 3 age groups. Linear regression relationship between body weight and morphine antinociception (15 mg/kg at 60 minutes) was studied and Pearson's coefficient was calculated. $N = 30$.

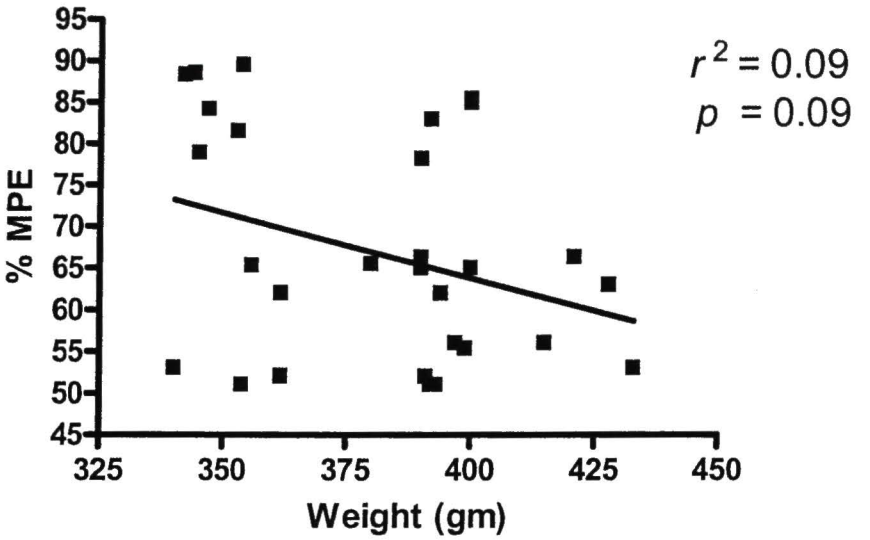
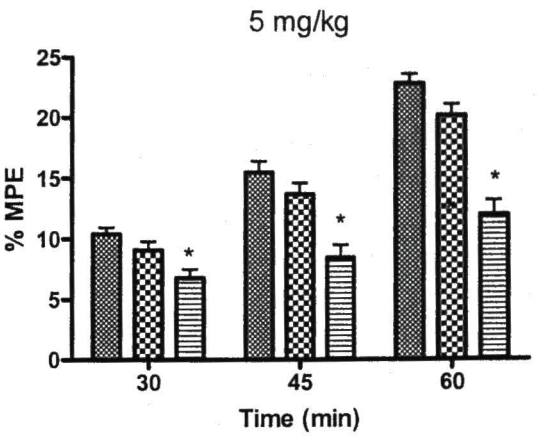
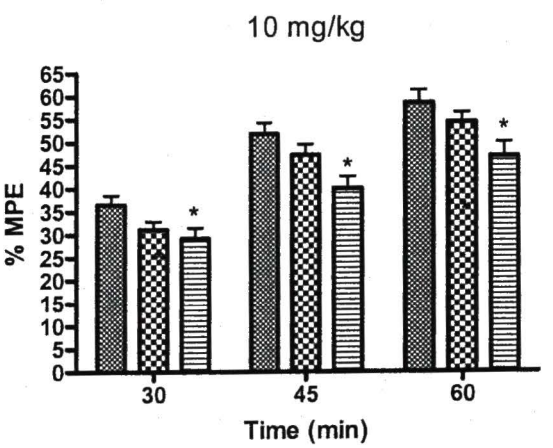

95
90
85
80
75
70
65
60
55
50
45
% MPE
325
350
375
400
425
450
Weight (gm)
$r^2 = 0.09$
$p = 0.09$

Figure 4. Effect of aging on morphine-induced antinociception. The rats were administered (A) 5 mg/kg, (B) 10 mg/kg and (C) 15 mg/kg of morphine and responses to thermal nociceptive stimulus were measured 30, 45 and 60 minutes after morphine administration. $N = 10$ in each group. * $p < 0.05$ compared with young age group.

A


5 mg/kg
% MPE
25
20
15
10
5
0
30
45
60
Time (min)

B


10 mg/kg
% MPE
65
60
55
50
45
40
35
30
25
20
15
10
5
0
30
45
60
Time (min)


Young
Adult
Old

C

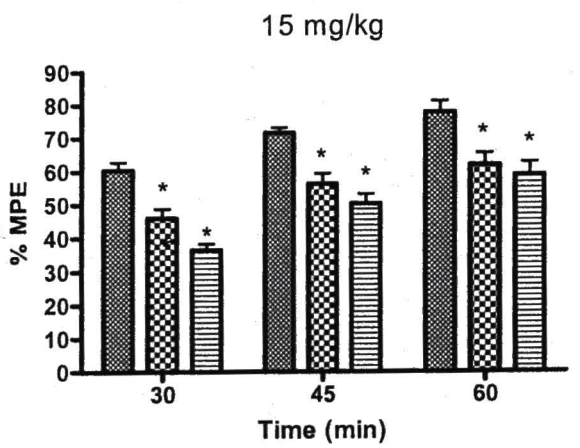
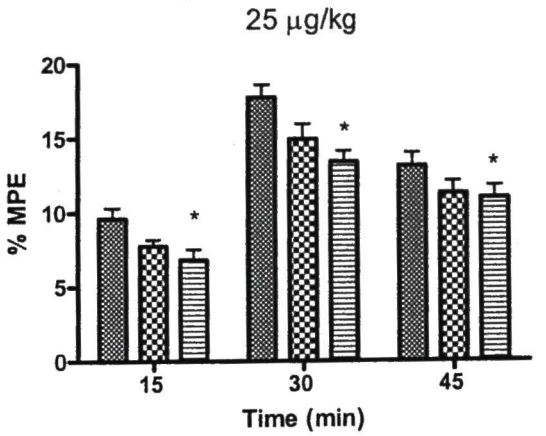
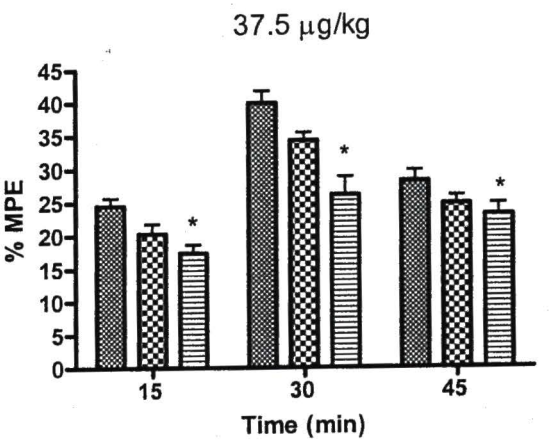

15 mg/kg
% MPE
90
80
70
60
50
40
30
20
10
0
30
45
60
Time (min)

Figure 5. Effect of aging on fentanyl-induced antinociception. Three different age groups of rats were administered (A) 25 μg/kg , (B) 37.5 μg/kg and (C) 50 μg/kg of fentanyl and antinociception was measured 15, 30 and 45 minutes after fentanyl administration. N = 10 in each age group. * $p< 0.05$ compared with young age group.

A


25 µg/kg
% MPE
20
15
10
5
0
15
30
45
Time (min)
*
*
*

B


37.5 µg/kg
% MPE
45
40
35
30
25
20
15
10
5
0
15
30
45
Time (min)
*
*
*


Young
Adult
Old

C

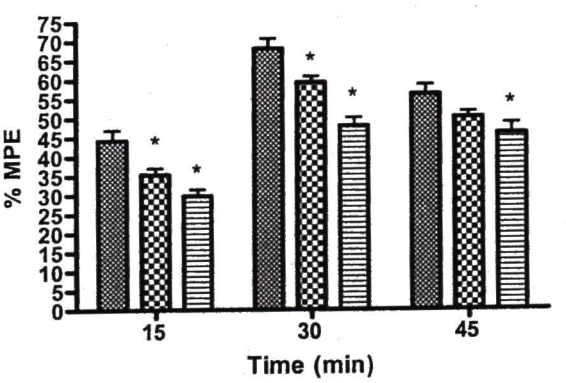
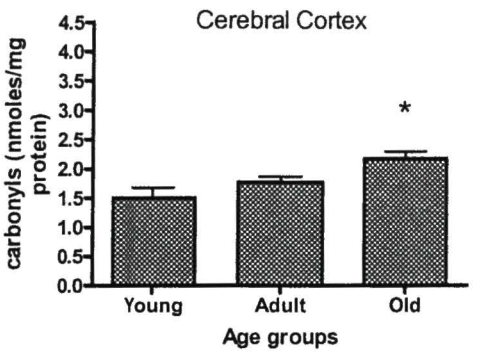
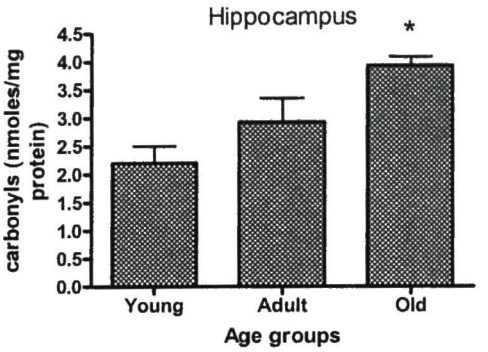

50 µg/kg
% MPE
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
15
30
45
Time (min)
*
*
*
*
*

Figure 6. Protein carbonyl contents (marker of protein oxidation) in (A) cerebral cortex, (B) hippocampus, (C) striatum, and (D) midbrain in male Fischer 344 rats of different ages. The data are from 6 determinations on tissue homogenates from 6 individual rats. * $p < 0.05$ vs. young age group.

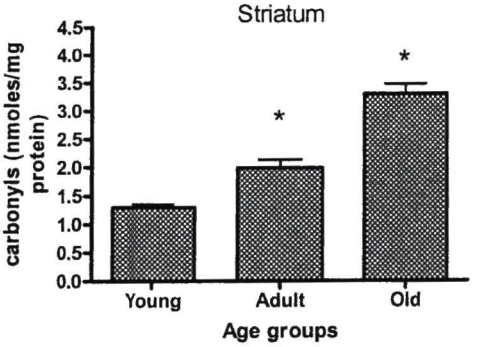
A


Cerebral Cortex
carbonyls (nmoles/mg protein)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Young
Adult
Old
Age groups

B


Hippocampus
carbonyls (nmoles/mg protein)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Young
Adult
Old
Age groups

C


Striatum
carbonyls (nmoles/mg protein)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Young
Adult
Old
Age groups

D

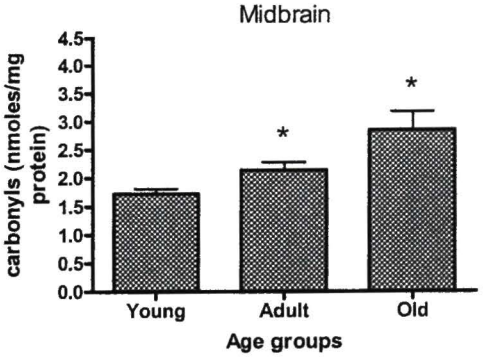

Midbrain
carbonyls (nmoles/mg protein)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Young
Adult
Old
Age groups

Figure 7. TBARS contents (marker of lipid peroxidation) in (A) cerebral cortex, (B) hippocampus, (C) striatum, and (D) midbrain in male Fischer 344 rats of different ages. The data are from 6 determinations on tissue homogenates from 6 individual rats. * p <0.05 vs. young age group.

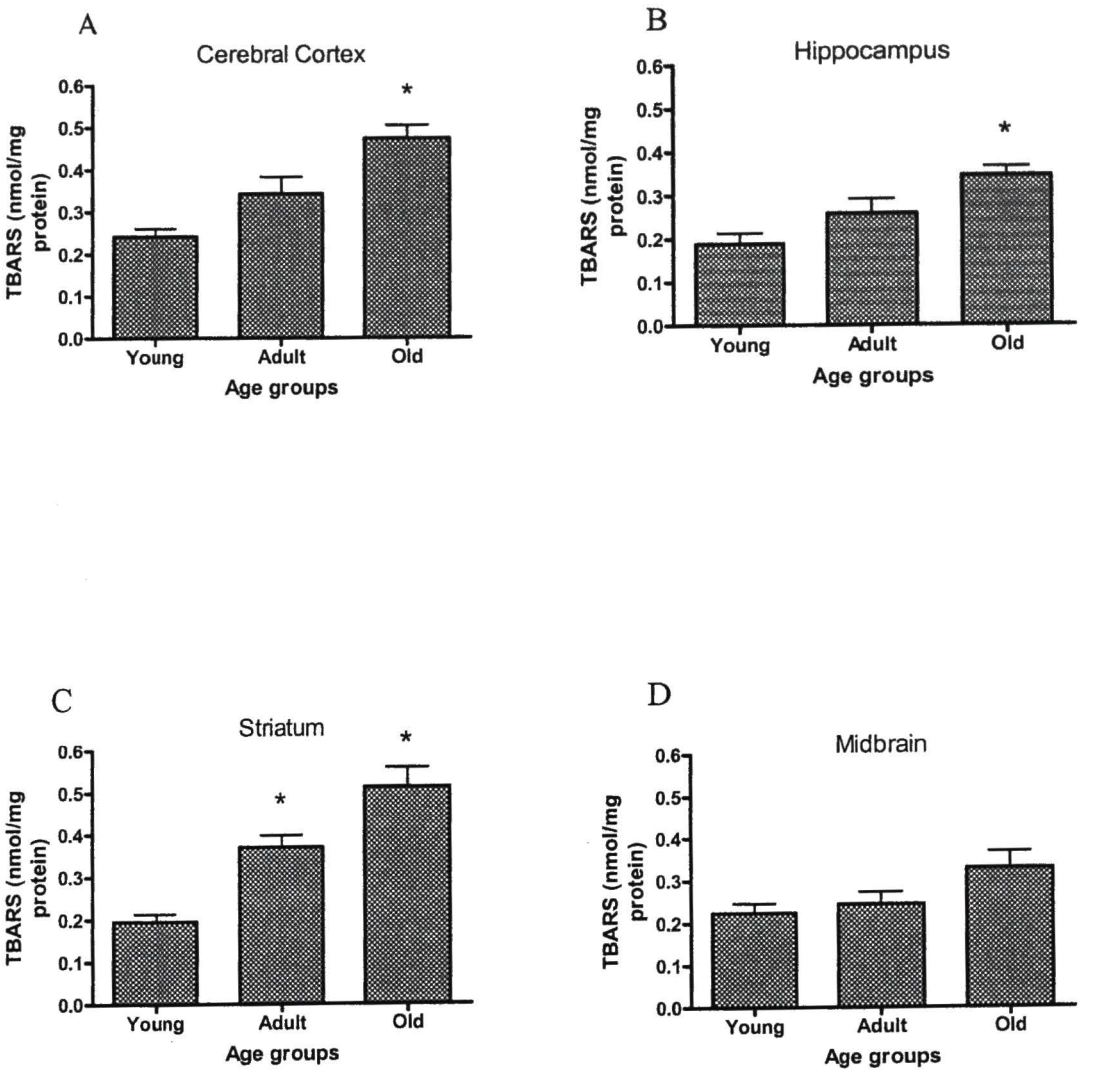

A
Cerebral Cortex
TBARS (nmol/mg protein)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
Young
Adult
Old
Age groups
B
Hippocampus
TBARS (nmol/mg protein)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
Young
Adult
Old
Age groups
C
Striatum
TBARS (nmol/mg protein)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
Young
Adult
Old
Age groups
D
Midbrain
TBARS (nmol/mg protein)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Young
Adult
Old
Age groups

Figure 8. Correlation between protein carbonyls in (A) cerebral cortex, (B) hippocampus, (C) striatum, and (D) midbrain and morphine-induced antinociception. Linear regression relationship between protein carbonyls and morphine antinociception (15 mg/kg at 60 minutes) was analyzed and expressed as Pearson's coefficient. $N = 6$ in each age group.

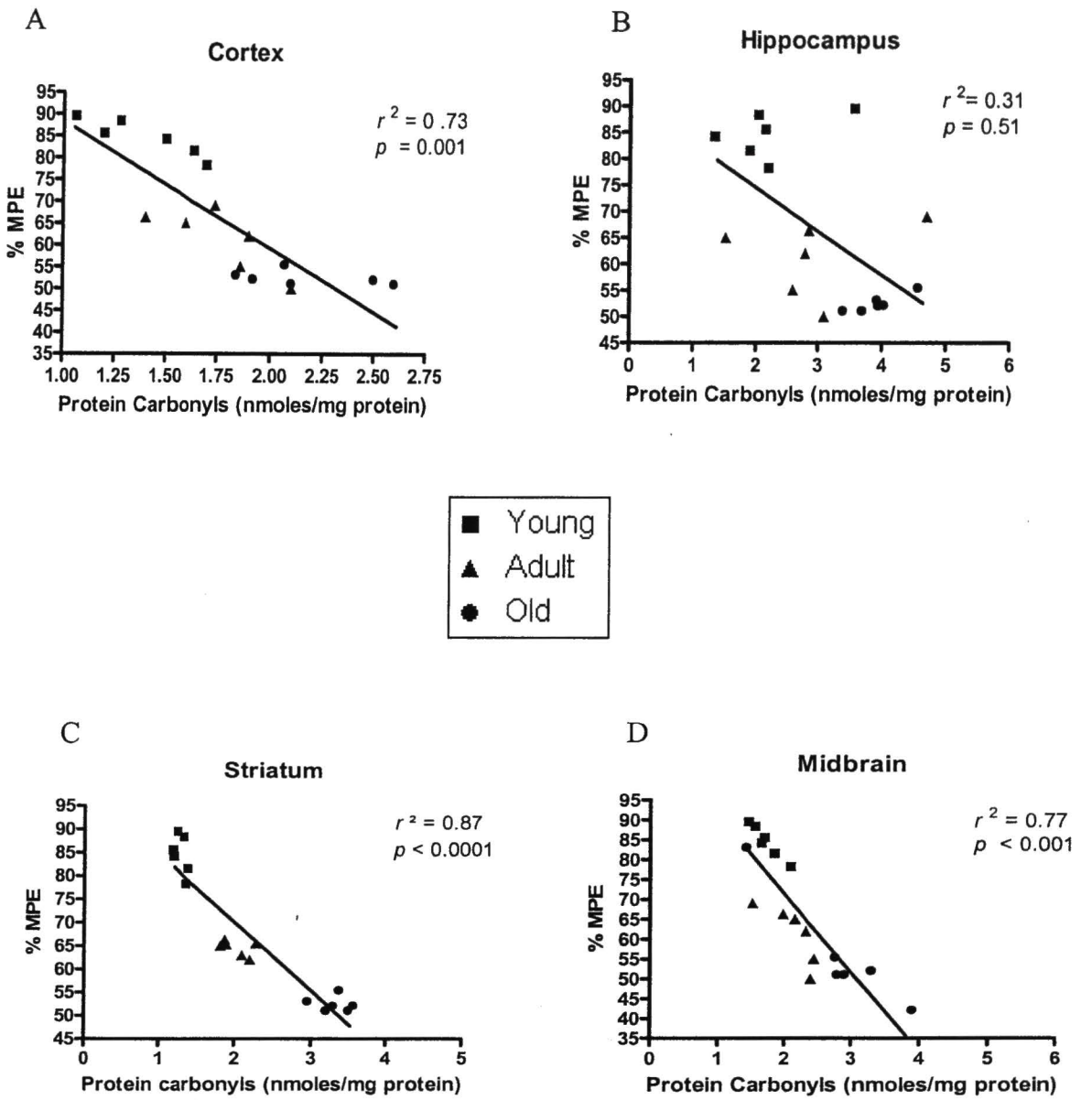

A
Cortex
r^2 = 0 .73
p = 0.001
% MPE
Protein Carbonyls (nmoles/mg protein)
B
Hippocampus
r^2= 0.31
p = 0.51
% MPE
Protein Carbonyls (nmoles/mg protein)
Young
Adult
Old
C
Striatum
r^2 = 0.87
p < 0.0001
% MPE
Protein carbonyls (nmoles/mg protein)
D
Midbrain
r^2 = 0.77
p < 0.001
% MPE
Protein Carbonyls (nmoles/mg protein)

Figure 9. Correlation between protein carbonyls in (A) cerebral cortex, (B) hippocampus, (C) striatum, and (D) midbrain and fentanyl-induced antinociception. Linear regression relationship between protein carbonyls and fentanyl antinociception (50 μg/kg at 30 minutes) was analyzed and expressed as Pearson's coefficient. $N = 6$ in each age group.

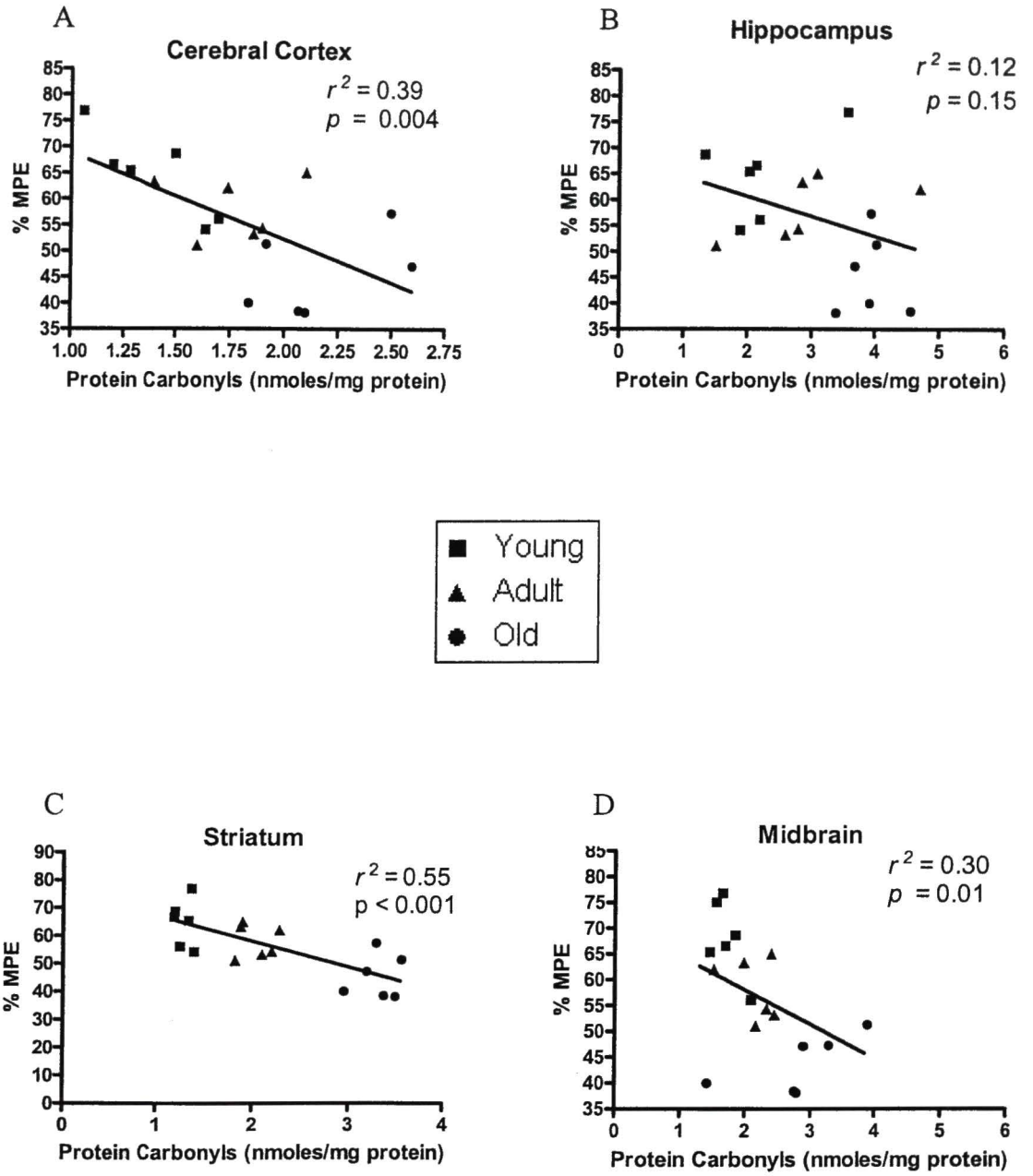

A
Cerebral Cortex
$r^2 = 0.39$
$p = 0.004$
% MPE
Protein Carbonyls (nmoles/mg protein)
B
Hippocampus
$r^2 = 0.12$
$p = 0.15$
% MPE
Protein Carbonyls (nmoles/mg protein)
Young
Adult
Old
C
Striatum
$r^2 = 0.55$
$p < 0.001$
% MPE
Protein Carbonyls (nmoles/mg protein)
D
Midbrain
$r^2 = 0.30$
$p = 0.01$
% MPE
Protein Carbonyls (nmoles/mg protein)

Figure 10. Correlation between TBARS content in (A) cerebral cortex, (B) hippocampus, (C) striatum, and (D) midbrain and morphine-induced antinociception (expressed as % MPE). Linear regression relationship between TBARS content and morphine antinociception (15 mg/kg at 60 minutes) was analyzed and expressed as Pearson's coefficient. $N = 6$ in each age group.

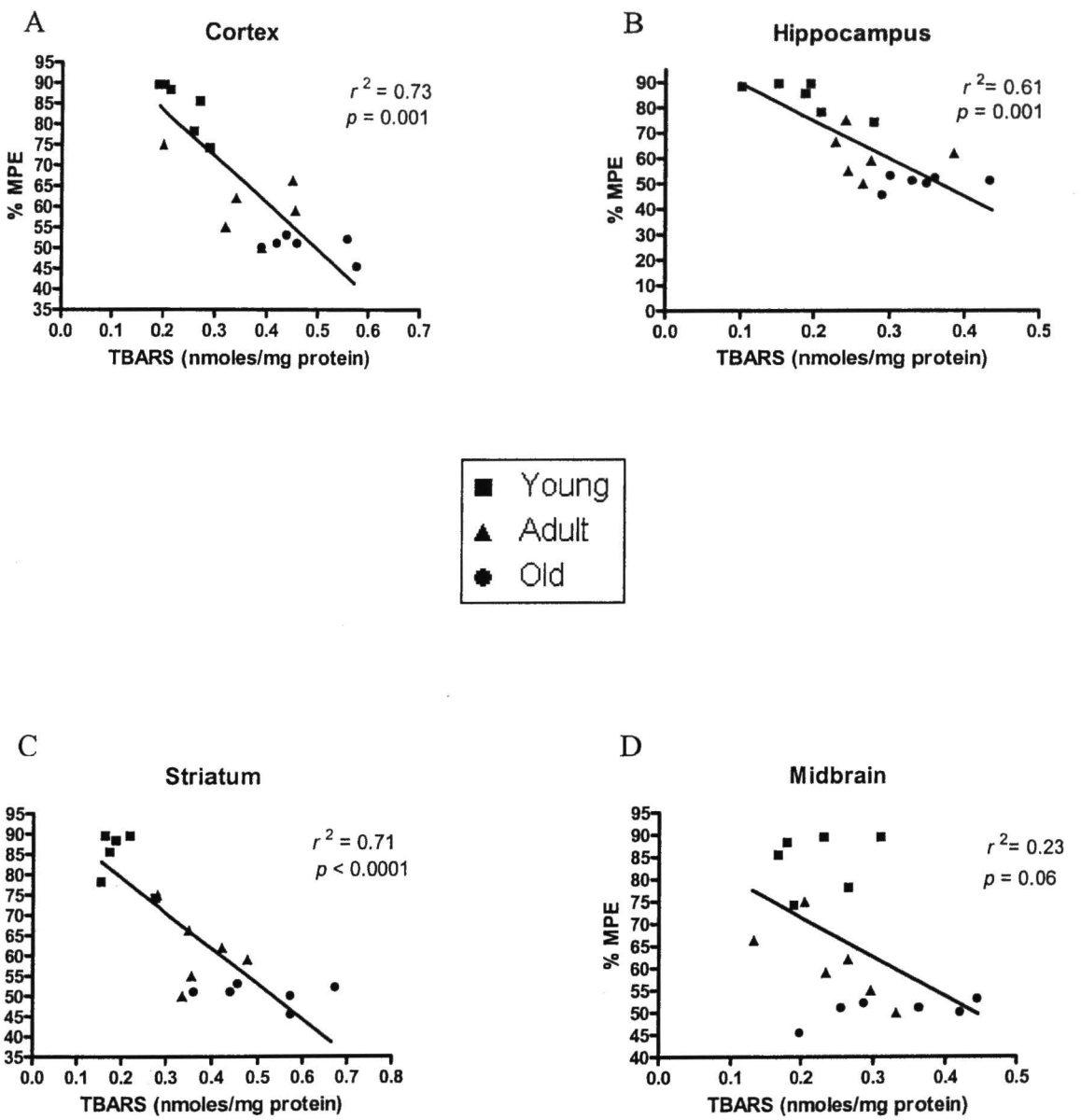
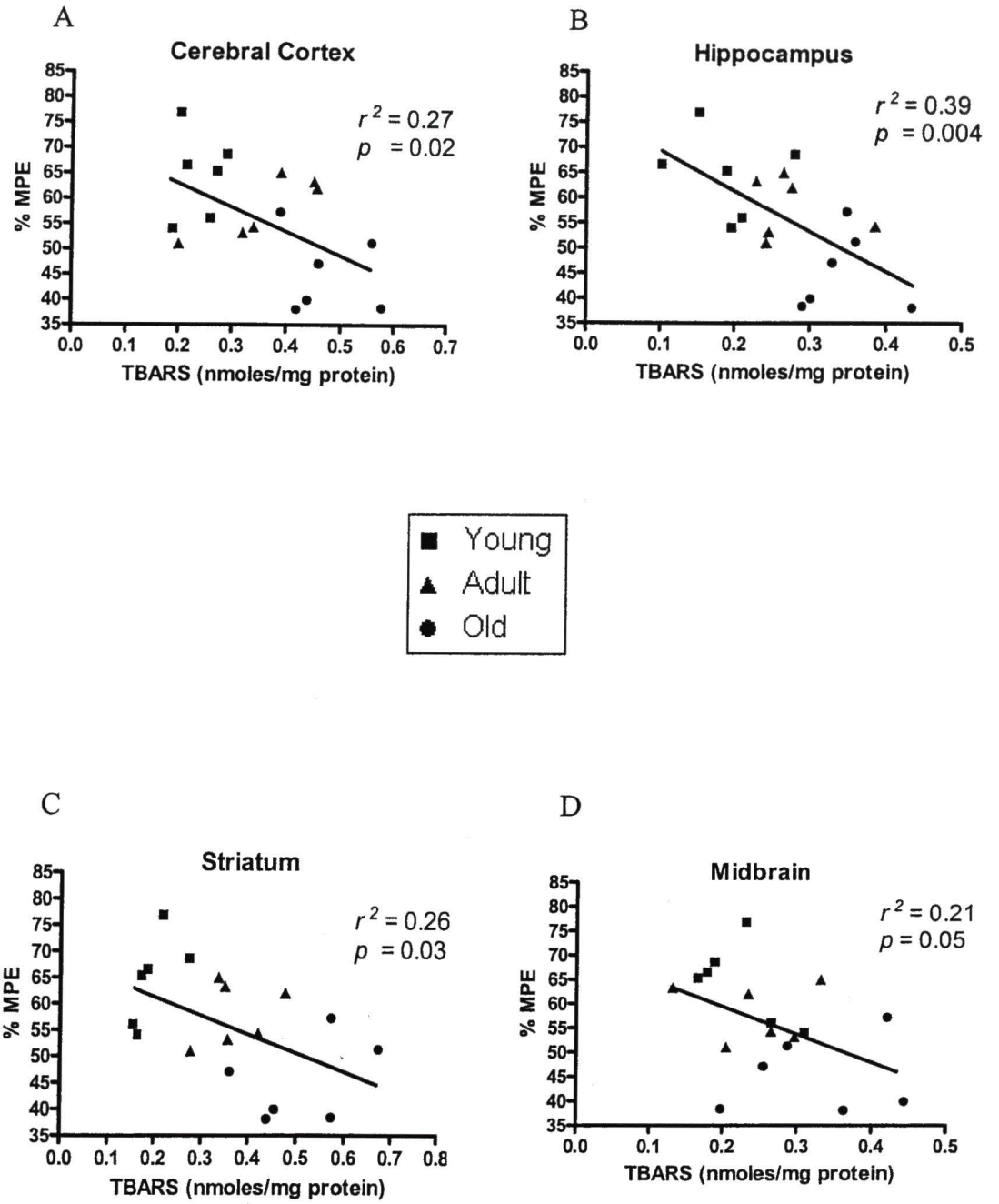

A
Cortex
r^2 = 0.73
p = 0.001
% MPE
TBARS (nmoles/mg protein)
B
Hippocampus
r^2= 0.61
p = 0.001
% MPE
TBARS (nmoles/mg protein)
Young
Adult
Old
C
Striatum
r^2 = 0.71
p < 0.0001
% MPE
TBARS (nmoles/mg protein)
D
Midbrain
r^2= 0.23
p = 0.06
% MPE
TBARS (nmoles/mg protein)

Figure 11. Correlation between TBARS content in (A) cerebral cortex, (B) hippocampus, (C) striatum, and (D) midbrain and fentanyl-induced antinociception (expressed as % MPE). Linear regression relationship between TBARS content and fentanyl antinociception (50 μg/kg at 30 minutes) was analyzed and expressed as Pearson's coefficient. $N = 6$ in each age group.


A
Cerebral Cortex
$r^2 = 0.27$
$p = 0.02$
% MPE
TBARS (nmoles/mg protein)
B
Hippocampus
$r^2 = 0.39$
$p = 0.004$
% MPE
TBARS (nmoles/mg protein)
Young
Adult
Old
C
Striatum
$r^2 = 0.26$
$p = 0.03$
% MPE
TBARS (nmoles/mg protein)
D
Midbrain
$r^2 = 0.21$
$p = 0.05$
% MPE
TBARS (nmoles/mg protein)

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CHAPTER III

DIFFERENTIAL EFFECTS OF IMPAIRED MITOCHONDRIAL ENEREGY PRODUCTION ON THE FUNCTION OF *MU* AND *DELTA* OPIOID RECEPTORS IN NEURONAL SK-N-SH CELLS

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Abstract

Oxidative stress contributes to changes in neurosensory processing, including pain, that occur during aging and neurodegeneration. The effects of neuronal oxidation on the opioid system are poorly understood. In this *in vitro* study, oxidative stress was induced by 3-nitroproprionic acid (3-NPA) in opioid-responsive differentiated SK-N-SH cells. Changes in the inhibitory effects of opioid receptor agonists on intracellular cAMP were used as a marker of the function of *mu* and *delta* opioid receptors (MOR and DOR, respectively). Cells were treated with morphine and selective MOR and DOR agonists and antagonists to characterize the function of each receptor subtype. Cyclic AMP (cAMP) was measured by enzyme immunoassay. Levels of reactive oxygen species (ROS) were assessed using the 2, 7-dichlorofluorescin diacetate assay. Exposure of cells to 3-NPA resulted in an increase in ROS. After 3-NPA exposure, there was a significant attenuation of the inhibitory effect of morphine and DAMGO but not of DPDPE on cAMP. In cells pretreated with CTOP, 3-NPA did not change the inhibitory effect on cAMP. These findings demonstrate for the first time that under conditions of mitochondrial damage, the function of MOR is significantly decreased, while the function of DOR does not change, suggesting that the effect of 3-NPA on opioid receptors is subtype-specific.

Keywords: Opioid receptors; MOR; DOR; ROS; 3-NPA; cAMP

Introduction

Aging and neurodegenerative diseases are associated with oxidative stress. The free radical theory of aging proposes that deleterious effects of reactive oxygen species (ROS) are responsible for the deterioration of neuronal function in degenerative pathologies and aging (Floyd, 1999, Kasapoglu and Ozben, 2001). A number of studies have reported increased lipid peroxidation and protein oxidation in aged mammalian brain (Leutener et al., 2001, Smith et al., 1991). The effects of oxidative damage on other receptor systems, such as adrenergic and cholinergic, have been studied (Joseph and Roth, 1992, Joseph et al., 1996, Kramer et al., 1987), but the effect of oxidative stress on opioid receptors is unknown. Opioid receptors play a critical role in pain and analgesia. Since free-radical-induced oxidative stress leads to an impairment of neuronal function, it may have a detrimental effect on opioid receptors. Pain is an important health problem in old age, with the prevalence of pain reaching as high as 70% (Gloth, 1996). Elderly individuals show increased sensitivity to pain and altered efficacy of opioids (Belwille et al., 1971, Macintyre and Jarvis, 1996, Moore et al, 1990). Due to limited understanding of the effects of oxidative stress on the opioid system, management of pain in elderly individuals and patients with neurodegenerative disorders is highly inadequate (Donovan et al., 1987, Foley, 1995). It is unclear how oxidative stress contributes to changes in sensitivity to pain and opioid therapy in neurodegenerative pathologies because we do not understand oxidative-stress-induced changes in the opioid system and its receptors and signal transduction pathways.

In this study, we investigated the effects of mitochondrial damage on the function of MOR and DOR in opioid-responsive SK-N-SH cells. The SK-N-SH cells differentiate to a neuronal phenotype (Preis et al., 1988) with an increased expression of MOR and DOR (Yu et al., 1988). Activation of opioid receptors with morphine significantly attenuated the prostaglandin E_1 (PGE_1)-induced increase in cAMP levels (Loh and Smith, 1990, Preis et al., 1988). A similar experimental approach was used in our previous studies (Baker et al., 2000, Ratka et al., 1991, Ratka and Simpkins, 1997) i.e., changes in the inhibitory effect of morphine on the PGE_1-induced accumulation of cAMP were measured to assess the function of opioid receptors. 3-Nitroproprionic acid (3-NPA) is known to be a mitochondrial toxin (Ludolph et al., 1991). Exposure to 3-NPA resulted in an increase of ROS, the induction of oxidative stress, a decline in mitochondrial membrane potential, and the depletion of intracellular ATP (Wang et al., 2001), leading to impaired cellular energy production. 3-NPA has been used to induce oxidative stress in both *in vitro* and *in vivo* conditions to model neurodegenerative disorders (Geddes et al., 2000). Trolox is an antioxidant, a water soluble vitamin E analog which is a peroxyl radical scavenger that rapidly penetrates biological membranes (Castle and Perkins, 1986). It inhibits membrane damage and has been shown to protect cells *in vivo* (Mickel et al., 1989) and *in vitro* (Wu et al., 1990).

Materials and Methods

Supplies

SK-N-SH cells were purchased from American Type Tissue Collection (ATTC, Rockville, MD, USA). RPMI-1640 media from GIBCOBRL (Grand Island, NY, USA) were used. Morphine sulfate, fetal bovine serum (FBS), penicillin, streptomycin were obtained from Sigma (St. Louis, MO, USA). Enzyme immuno assay kits for cAMP assays were purchased from Cayman Chemicals (Ann Arbor, MI, USA). DAMGO, spiradoline and DPDPE were purchased from Sigma (St. Louis, MO, USA). Trolox was purchased from Calbiochem (San Diego, CA, USA)

SK-N-SH Cell Culture and Differentiation

The monolayer of SK-N-SH cells from passages 20-30 were maintained in RPMI 1640 media supplemented with 10% FBS and 100 μg streptomycin/ml and 100 IU penicillin/ml. In RPMI 1640 media, 14μM of phenol red served as a pH indicator. The cells were plated at an initial density of 2×10^5 cells/ ml in 24 -well plastic culture plates. Medium was changed at 2 day intervals. Newly seeded cells were allowed at least 24 hrs to adhere. The cells were treated with retinoic acid to induce neuronal differentiation; medium containing 10μM of retinoic acid was replaced every other day for 6 days (Yu and Sadee, 1988).

Treatments with 3-NPA

On the day of the experiment, appropriate 3-NPA concentrations were prepared in cell culture media. Before exposure to 3-NPA, medium was aspirated and the cell monolayer was rinsed with serum free medium. Subsequently, cells were exposed to medium containing 1- 20 mM 3-NPA for a period of 30 minutes - 24 hours at 37^0 C (Wang et al., 2001). After 3-NPA treatment, cell extraction was performed.

Treatments with Trolox

In the separate sets of experiments, appropriate Trolox concentration was prepared in cell culture media. Before exposure to Trolox, medium was aspirated and the cell monolayer was rinsed with serum free medium. Subsequently, cells were exposed to medium containing 10 mM Trolox for 8 hours (McClain et al., 1995, Salgo et al., 1995, Salgo and Pryor, 1996). After pretreatment with Trolox, cells were exposed to 3-NPA (10 mM, 2 hrs) and then, cell extraction was performed as mentioned below.

DCF-DA Assay

ROS generated by 3-NPA were measured with the 2', 7'-dichlorofluorescin diacetate (DCF-DA) assay as described previously (McPherson and Yao, 2001, Wang et al., 2001). Free radical generation in cells was assessed using the probe 2', 7'-dichlorofluorescin (DCFH). The cells were plated at a density of 10,000 cells/well in 96-well plates and differentiated with RA. The membrane-permeable diacetate form of DCFH, DCFH-DA, was added to the perfusate at a final concentration of 5 μM. Once in

the cell, esterases cleave the acetate groups on DCFH-DA, thus trapping DCFH intracellularly. Free radicals in the cells oxidize the DCFH, yielding the fluorescent product DCF. Fluorescence was measured with an excitation wavelength of 480 nm and emission wavelength of 535 nm. Intensity values were obtained as the percentage of initial values after subtraction of the background value.

Cell Extraction

For cAMP assay, cell extraction was performed according to method described previously (Yu et al., 1988). Briefly, medium was aspirated, cell monolayer rinsed with 1 ml of serum free RPMI-1640 medium at 37° C. Subsequently, cells were incubated for 10 minutes with 300 μl of 0.5 mM isobutylmethylxanthine (IBMX). After that time, 100 μl of 1 μM PGE_1 and 40 μl of 10 μM morphine sulfate were added and cells were incubated for 10 minutes at 37° C. The incubation was terminated by adding 1 ml of 0.1 M hydrochloric acid (HCl) in each well. After 10 minutes of exposure to HCl, cellular debris was scraped and transferred to glass tubes and centrifuged for 10 minutes at 1000 g. The supernatant was decanted into clean test tubes and assayed for the content of cAMP. To identify effect of 3-NPA on each receptor subtype, instead of morphine, either *mu* receptor agonist DAMGO (10 μM) or *delta* receptor agonist DPDPE (10 μM) or *kappa* receptor agonist spiradoline (10 μM) was added to the cells. In separate experiments, before exposure to 3-NPA, the cells were preincubated for 2 hrs with 1 μM of either *mu* receptor selective antagonist CTOP (d-Phe-Cys-Tyr-d-Trp-Orn-Thr-Pen-Thr-NH2) or *delta* receptor antagonist naltrindol.

Determination of cAMP

The amount of cAMP was measured by enzyme immuno assay (EIA) using a cAMP EIA kit provided by Cayman Chemicals (Ann Arbor, MI, USA). This assay is based on the competition between free cAMP and a cAMP-acetylcholinesterase (AChE) conjugate (cAMP tracer) for a limited number of cAMP specific rabbit monoclonal antibody binding sites. The product of this enzyme reaction was measured at 412-630 nm. Each experiment was performed at least three times with an independent pool of cells. Experimental points were obtained from 3-4 parallel wells and the sample from each well was assayed in triplicate by EIA. The cAMP data were normalized to protein content and levels of cAMP were presented as percent of control (vehicle-treated cells).

Protein Assay

Protein concentration of the cell extracts was determined by the Bradford Protein Assay (Bradford, 1976).

Data Analysis

Each set of experiments was repeated at least three times. Data are expressed as mean ± S.E.M. (n = 6 wells). The statistical evaluation of data was performed by analysis of variance (ANOVA) followed by the Tukey post hoc test using GraphPad Prism Version 4.0 statistical software package. p value <0.05 was considered as statistically significant.

Results

Effects of 3-NPA on the morphine-induced inhibition of cAMP in differentiated SK-N-SH cells

In the initial experiment, the effect of 2- and 24-h treatments with 10mM 3-NPA on the inhibitory effect of morphine on the PGE_1-stimulated accumulation of intracellular cAMP were characterized. In the absence of 3-NPA, PGE_1 stimulated cAMP to 258 ± 17% of control and morphine significantly inhibited cAMP to 133 ± 5% of control (Fig. 1A). In cells pretreated for 2 h with 3-NPA, PGE_1 increased cAMP to 317 ± 6% of control, and morphine decreased this level insignificantly to 265 ± 26%. After the 24-h pretreatment with 3-NPA, no significant inhibitory effect of morphine on cAMP was observed (Fig. 1A).

Since the levels of cAMP at baseline and after PGE_1 exposure were not affected significantly by treatment with 3-NPA, we decided to present only the changes in the inhibitory effect of opioid agonists on cAMP levels to emphasize the receptor function. The following formula was used to estimate the percent of inhibition caused by morphine (or a selective agonist):

[(cAMP after PGE_1 − cAMP after PGE_1 plus morphine (or a selective agonist)]/(cAMP in presence of PGE_1)×100.

In the presence of 10 mM 3-NPA, the inhibition of cAMP after morphine exposure was significantly reduced from 48 ± 5% to 16 ± 5% and to 11 ± 5% after 2 and 24 h, respectively (Fig. 1B). The results presented in Fig. 1 show that 3-NPA

significantly attenuated the inhibitory effect of morphine on intracellular cAMP in opioid-responsive SK-N-SH neuronal cells.

Dose-response and time-response effects of 3-NPA on the morphine-induced inhibition of cAMP in differentiated SK-N-SH cells

In a subsequent series of experiments, dose- and time- response studies with 3-NPA were performed. After exposure to 10 mM and 20 mM of 3-NPA for 2 h, the inhibition of cAMP was significantly reduced to 17 ± 4% and 15 ± 6%, respectively (Fig. 2A). Exposure to 10 mM 3-NPA for 2, 12 and 24 h resulted in a significant reduction of cAMP inhibition by morphine to 16 ± 5%, 8 ± 3% and 11 ± 5%, respectively (Fig. 2B).

Effects of 3-NPA (10 mM) on the levels of ROS in differentiated SK-N-SH cells

To determine the levels of ROS after 3-NPA exposure, cellular hydrogen peroxide and hydroxyl radicals were measured. As shown in Fig. 3, with an increase in the duration of treatment with 10 mM of 3-NPA, progressive rise in ROS was observed and became significant after 2, 12, and 24 h at 168 ± 5%, 243 ± 14%, and 309 ± 22%, respectively.

Effects of 3-NPA (10 mM, 2 hrs) on the inhibitory effects of morphine, DAMGO and DPDPE

Additional experiments were conducted to determine the effect of 3-NPA on individual opioid receptor subtypes. The cells were treated either with morphine (an

agonist to MOR, DOR and KOR), MOR selective agonist (DAMGO), or DOR selective agonist (DPDPE), and the effects of 3-NPA on cAMP inhibition by these treatments were compared. Exposure to 3-NPA significantly reduced the inhibitory effect of morphine on cAMP from 49 ± 5% to 15 ± 5% (Fig. 4).

Treatment with DAMGO resulted in a 37 ± 4% inhibition of cAMP, and exposure to 3-NPA significantly reduced this inhibition to 16 ± 3% (Fig. 4). DPDPE treatment caused 60 ± 5% reduction in cAMP and after treatment with 3-NPA, this inhibitory effect insignificantly decreased to 55 ± 5%. The results presented in Fig. 4 show that 3-NPA significantly reduced the function of MOR but did not change DOR function.

Effects of 3-NPA (10 mM, 2 hrs) on the function of MOR and DOR evidenced by prior treatment with selective antagonists

To substantiate the previous findings, experiments were performed using selective receptor antagonists. The cells were first treated for 2 h with either 1 μM naltrindol (DOR antagonist) or 1 μM CTOP (MOR antagonist), and after that, they were exposed to 3-NPA (10 mM, 2 h), and subsequently, to morphine. In the absence of 3-NPA, morphine showed a 31 ± 2% inhibition of cAMP after pretreatment with naltrindol, and 3-NPA reduced this inhibition to 17 ± 1% (Fig. 5A). In cells pretreated with CTOP, in the absence of 3-NPA, morphine showed a 26 ± 2% inhibition of cAMP, and this effect was insignificantly reduced to 24 ± 3% by 3-NPA (Fig. 5B).

The findings presented in Figs. 4 and 5 show that the function of MOR was selectively and significantly reduced by 3-NPA.

Effects of 3-NPA (10 mM, 2 hrs) on the inhibitory effects of morphine, DAMGO, DPDPE and spiradoline

After characterizing the MOR and DOR function in SK-N-SH neuronal cells, attempts were focused to determine if these cells respond to spiradoline (KOR selective agonist) suggesting possible expression and function of KOR in these cells. As opposed to inhibitory effects of morphine (49 ± 5%), DAMGO (37 ± 4%) and DPDPE (60 ± 5%) on cAMP inhibition, spiradoline exposure resulted in much lower inhibition of cAMP (11 ± 1 %) which was insignificantly reduced to 9 ± 1% by exposure to 3- NPA (Fig. 6).

Effects of Trolox on 3-NPA induced effects on MOR function

As depicted in Fig. 3, 3-NPA exposure resulted in significant rise in ROS in differentiated SK-N-SH cells. We assessed the effects of 3-NPA in the presence of an antioxidant, Trolox, which is a water soluble analog of vitamin E. Trolox alone did not change cAMP inhibition in the cells. Morphine induced inhibition of cAMP, which was reduced from 47 ± 5% to 16 ± 4 % by 3-NPA, was preserved by Trolox (39 ± 5%, Fig. 7). Similarly, DAMGO induced inhibition of cAMP, which was reduced from 35 ± 4% to 15 ± 2% by 3-NPA, was restored to 30 ± 3% by Trolox. These effects of Trolox were statistically significant.

Discussion

In this study, we demonstrate for the first time that under conditions of reduced cell energy metabolism, there is a significant reduction of the function of MOR but not

DOR in opioid-responsive neuronal cells. These findings imply that opioid receptor subtypes respond differently to the detrimental effect of cellular energy damage evoked by the interruption of mitochondrial function. To our knowledge, no published reports are available on the differential effects of reduced mitochondrial activity and its consequences on the function of opioid receptor subtypes, i.e., MOR and DOR.

Several possible explanations may be considered for the novel findings from our studies. Multiple opioid receptor subtypes and their effector systems can be targets for oxidative stress that lead to differential impairment of opioid receptors function. Different amino acid sequences of MOR and DOR proteins (Chen et al., 1993) could contribute to the differences in vulnerability of these receptor subtypes to oxidative stress. The extracellular loops of opioid receptors consist of amino acid residues, such as cysteine and methionine that are sensitive to oxidation (Law et al., 1999). Hence, changes in these amino acid residues may be critical in the mechanism underlying the selectivity of oxidative stress on opioid receptors.

The opioid-subtype-specific effects of oxidative stress may be related to differences in G proteins coupled to MOR and DOR. Studies with G_α-specific antibodies suggested that G_{i-2} mediates DOR's inhibition of adenylyl cyclase activity in NG 108-15 cells (McKenzie and Milligan, 1990), while G_o mediates MOR inhibition of adenylyl cyclase activity in SHSY- 5Y cells (Carter and Medzihradsky, 1993). It may be that G_o protein but not G_{i-2} protein is damaged by oxidative stress. The differences in the sensitivity of G protein isoforms to oxidative damage could account for the observed differences in the effect of oxidative stress on MOR and DOR function. Oxidative stress

may also influence the trafficking of MOR and DOR differently. Chu et al. (Chu et al., 1997) have reported that the internalization and trafficking of DOR and *kappa* opioid receptor (KOR) are differentially regulated, even when activated by the same agonist. DOR was found to be more rapidly internalized than KOR after stimulation by etorphine (Chu et al., 1997). It seems possible that oxidative stress may influence the internalization and trafficking of MOR differently than that of DOR.

MOR and DOR proteins may become oxidized under oxidative stress conditions. Studies have shown that free oxidative radicals cause oxidation of proteins in various regions of aged mammalian brain (Oliver et al., 1987, Smith et al., 1991). Our findings suggest that the MOR protein may be more vulnerable to oxidative damage than the DOR protein. In a pilot experiment, we observed that the level of the MOR protein was significantly reduced after treatment with 3-NPA.

The findings presented in this study also may be a result of functional interactions between MOR and DOR. The selective activation of DOR with DPDPE resulted in more cAMP inhibition compared to the inhibitory effects of DAMGO and morphine. Since morphine activates both opioid receptor subtypes, the effect of morphine on cAMP in differentiated SK-N-SH cells could be a result of the simultaneous activation of MOR and DOR. The stronger inhibitory effect of a selective DOR agonist may be due to functional interactions between MOR and DOR. It has been reported that MOR and DOR form heterodimers that act through adenylyl cyclase (Gomes et al., 2000). The cross-talk between MOR and DOR is well documented (Gomes et al., 2000, Rothman et al., 1993, Salahpour et al., 2000, Vaught et al., 1982) but it is not clear whether these interactions

are always synergistic or if they could lead to a decline in the function of the other receptor. It has been reported that sub-effective doses of DOR-selective agonists leu-enkephalin, d-Ala-2-d-Leu-5 enkephalin, and β h-endorphin potentiated MOR-mediated analgesia, whereas other DOR-selective agonists like metenkephalin and d-Ala-2-metenkephaline attenuated MOR-mediated analgesia (Vaught et al., 1982). Ligand binding studies have shown that MOR-selective ligands inhibit the binding of DOR-selective ligands in both a competitive and noncompetitive manner (Rothman et al., 1993). When DOR is selectively activated with an agonist in the absence of MOR activation, its activity may not be inhibited and is, therefore, higher. Thus, it is likely that the individual contributions of MOR and DOR to the inhibition of intracellular cAMP could be different from their interactive contributions. The differences in the contribution of opioid receptor subtypes, individually or as heterodimers, to cAMP inhibition may explain differences in the extent of cAMP inhibition after morphine (49 ± 5%), DAMGO (37 ± 4%) and DPDPE (60 ± 5%) observed in our study (Fig. 4).

In this research project, the function of *kappa* opioid receptor (KOR) was not well characterized. Like MOR and DOR, KOR is a G protein-coupled receptor that acts through the cAMP pathway (Law et al., 1982). KOR in SK-N-SH cells has not been extensively studied, and to our knowledge, it has not been reported whether this cell line expresses KOR or if this receptor subtype is functional in these cells. Only one study, by Hurle et al., (1999) reported that KOR agonists inhibited the amplitude of K^+-induced $[Ca^{2+}]_i$ increase in SK-N-SH cells, but this effect was not blocked by KOR antagonist nor-binaltorphimine. Therefore, even if SK-N-SH cells express KOR, these receptors

may not be functional, and hence, their contribution to cAMP inhibition might be insignificant.

Our finding that the antioxidant, Trolox, preserved MOR function, supports the hypothesis that oxidative stress leads to decline in opioid receptor function. 3-NPA has been shown to causes an increase in hydrogen peroxide and peroxinitrite (Wang et al., 2001) which are potent ROS. Trolox has been shown to protect the mammalian cells against peroxynitrite toxicity (Salgo et al., 1995). The protective effect of Trolox appears to be based on its capacity to inhibit membrane lipid peroxidation (Wu et al., 1990). Taken together, these findings substantiate the role of oxidative damage in decline in neuronal function. Apart from oxidative damage, other consequences of reduced mitochondrial succinate dehydrogenase activity induced by 3-NPA, e.g., reduced cellular ATP, could be involved in the changes in opioid receptors.

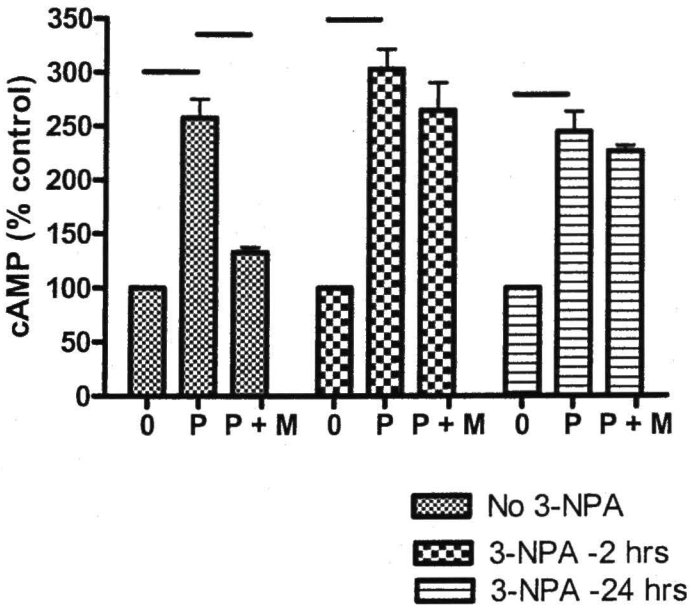
Conclusions

In summary, the results of our study show for the first time that an increase in ROS may be selectively detrimental to the function of MOR but not DOR, and the findings imply that opioid receptor subtypes differ significantly in their sensitivity to mitochondrial damage. Additional studies should be directed at elucidating the mechanism(s) underlying the differential responses of opioid receptor subtypes to mitochondrial energy impairment in neuronal cells. Future research findings may help to better understand the functioning of the opioid receptor system under conditions

associated with neuronal damage and, ultimately, may be applied to assessment and treatment of pain in pathologies such as aging, dementia, and Alzheimer's disease.

Figure 1. Effects of 3-NPA, PGE_1 and morphine on cAMP in differentiated SK-N-SH cells. (A) Levels of cAMP in control cells (vehicle = 0), after PGE_1 (P) and after PGE_1 plus morphine (P + M). Horizontal bars connect significantly different groups ($p < 0.05$). (B) Effect of 3-NPA on the inhibitory effect of morphine on PGE_1- stimulated cAMP. *$p < 0.05$ vs. no 3-NPA.

A


cAMP (% control)
350
300
250
200
150
100
50
0
0 P P+M 0 P P+M 0 P P+M
No 3-NPA
3-NPA -2 hrs
3-NPA -24 hrs

B

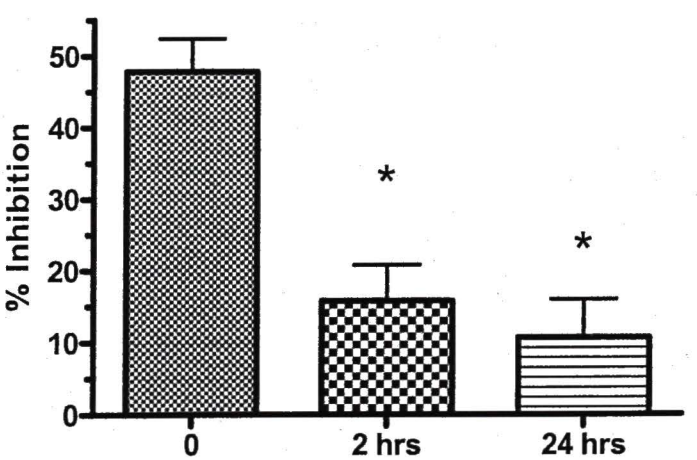
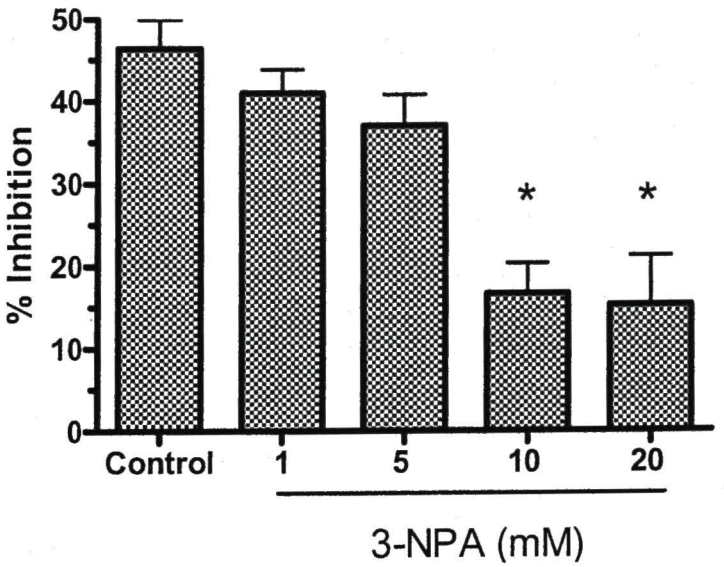

% Inhibition
50
40
30
20
10
0
*
*
0
2 hrs
24 hrs

Figure 2. Effects of 3-NPA on the morphine-induced inhibition of cAMP in differentiated SK-N-SH cells. (A) Dose-response effect of 3-NPA after a 2-hrs incubation. *$p < 0.05$ Vs. control. (B) Time-dependent effect of 3-NPA (10 mM). * $p < 0.05$ vs. control (0).

A


% Inhibition
50
40
30
20
10
0
*
*
Control
1
5
10
20
3-NPA (mM)

B

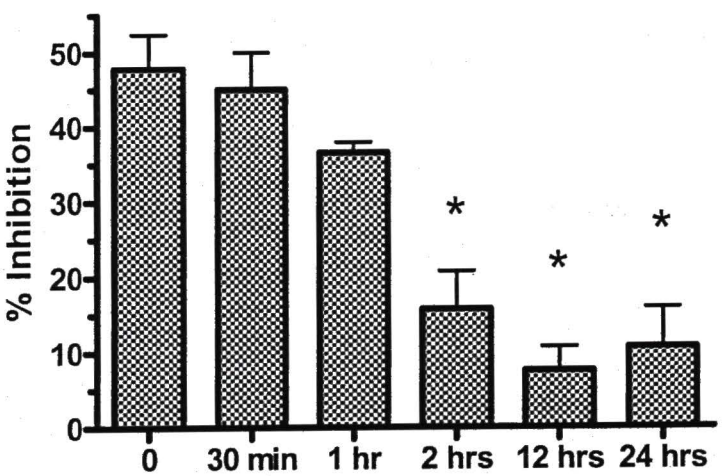

% Inhibition
50
40
30
20
10
0
*
*
*
0
30 min
1 hr
2 hrs
12 hrs
24 hrs

Figure 3. Effects of 3-NPA (10 mM) on the levels of reactive oxygen species (ROS) measured by DCF fluorescence in differentiated SK-N-SH cells. * $p < 0.05$ vs. control (0).

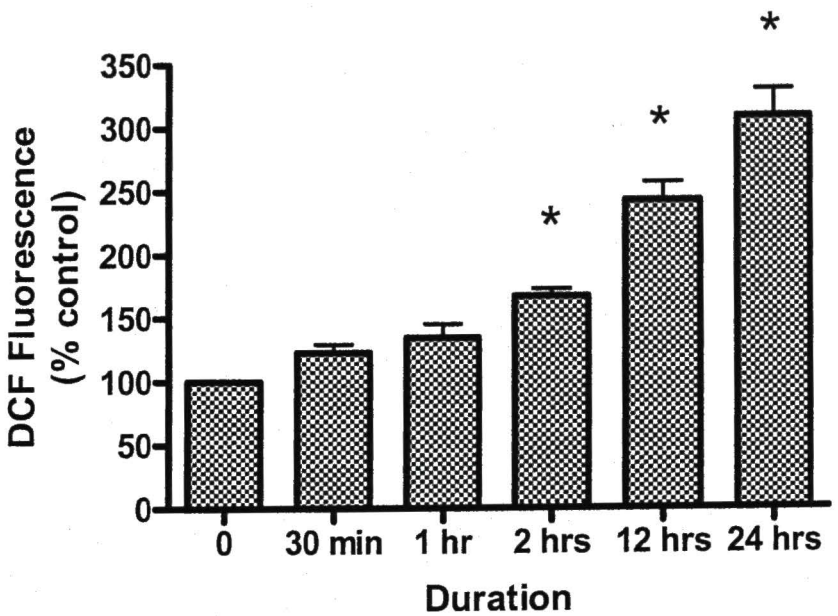

DCF Fluorescence
(% control)
350
300
250
200
150
100
50
0
*
*
*
0
30 min
1 hr
2 hrs
12 hrs
24 hrs
Duration

Figure 4. Effects of 3-NPA (10 mM, 2 hrs) on the inhibitory effects of morphine, DAMGO, and DPDPE on PGE1-stimulated cAMP in differentiated SK-N-SH cells. Horizontal bars connect significantly different groups ($p < 0.05$).

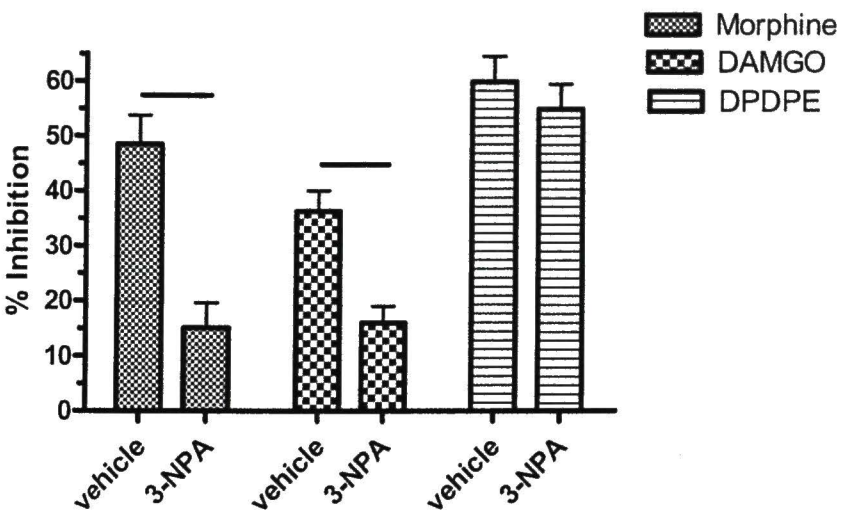
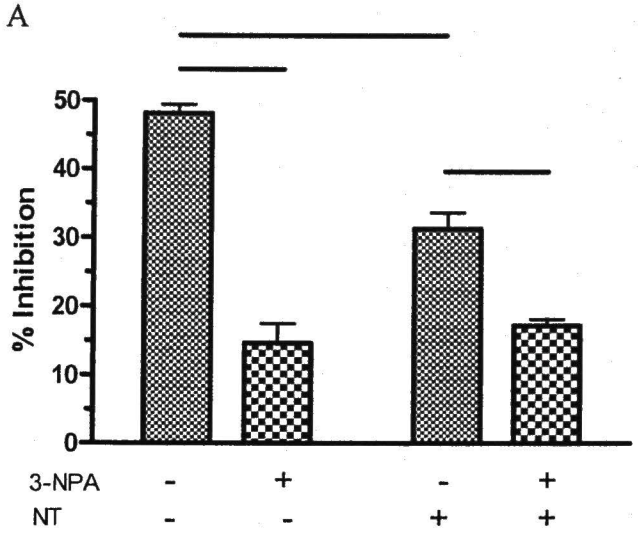

Morphine
DAMGO
DPDPE
% Inhibition
60
50
40
30
20
10
0
vehicle
3-NPA
vehicle
3-NPA
vehicle
3-NPA

Figure 5. Effects of 3-NPA (10 mM, 2 hrs) on the function of MOR and DOR. (A) Cells pretreated with NT (naltrindol, 1 μM, 2 hrs) and then exposed to morphine (10 μM). (B) Cells pretreated with CTOP (1 μM, 2 hrs) and then exposed to morphine (10 μM). Horizontal bars connect significantly different groups ($p < 0.05$).

A


% Inhibition
50
40
30
20
10
0
3-NPA - + - +
NT - - + +

B

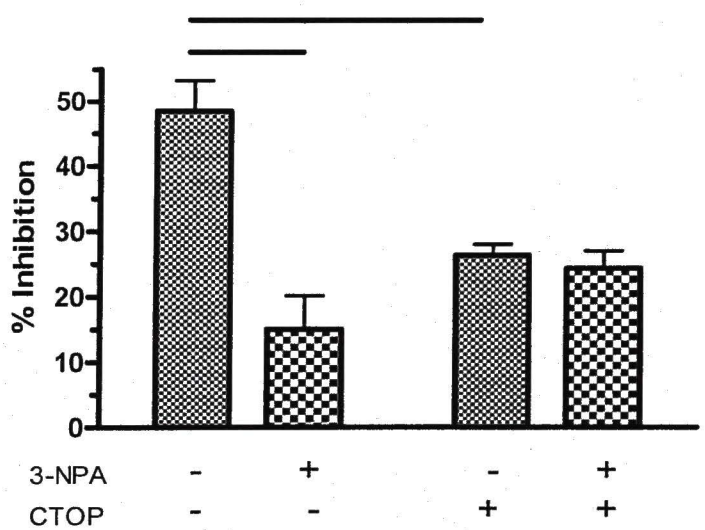

% Inhibition
50
40
30
20
10
0
3-NPA - + - +
CTOP - - + +

Figure 6. Effects of 3-NPA (10 mM, 2 hrs) on the inhibitory effects of morphine, DAMGO, DPDPE and spiradoline on PGE_1-stimulated cAMP in differentiated SK-N-SH cells. Horizontal bars connect significantly different groups ($p < 0.05$).

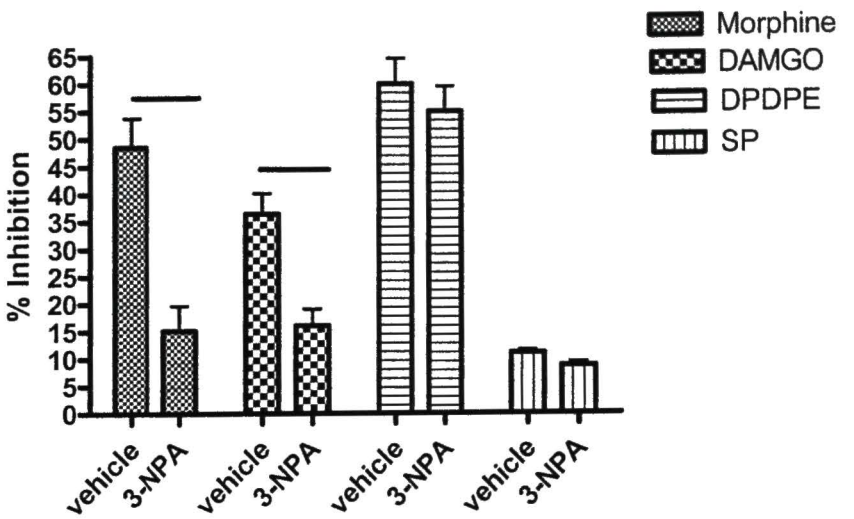
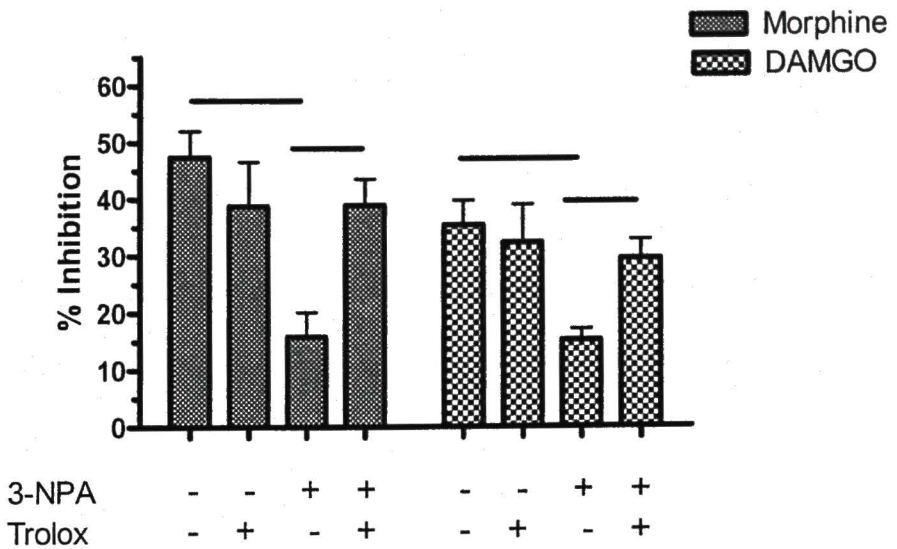

Morphine
DAMGO
DPDPE
SP
% Inhibition
65
60
55
50
45
40
35
30
25
20
15
10
5
0
vehicle
3-NPA
vehicle
3-NPA
vehicle
3-NPA
vehicle
3-NPA

Figure 7. Effects of Trolox (10 mM, 8 hrs) on the effects of 3-NPA on morphine and DAMGO induced inhibition of cAMP in differentiated SK-N-SH cells. Horizontal bars connect significantly different groups ($p < 0.05$).


Morphine
DAMGO
% Inhibition
60
50
40
30
20
10
0
3-NPA - - + + - - + +
Trolox - + - + - + - +

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CHAPTER IV

EFFECTS OF 3-NITROPROPRIONIC ACID INDUCED OXIDATIVE STRESS ON *MU, DELTA* AND *KAPPA* OPIOID RECEPTORS PROTEINS IN DIFFERENTIATED NEURONAL SK-N-SH CELLS

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Abstract

Oxidative stress has been implicated in aging and neurodegenerative diseases. Oxidative damage contributes to changes in neurosensory processing, including pain, that occur during aging and neurodegeneration. Although other receptor systems have been explored to study the effect of oxidative stress, the effects of oxidative stress on the opioid receptor system are poorly understood. Earlier, we have reported that 3-nitroproprionic acid (3-NPA) induced oxidative stress and impairment of mitochondrial energy metabolism reduced the function of *mu* but not *delta* opioid receptors. In the present study, the effects of 3-NPA-induced oxidative stress on the *mu, delta* and *kappa* opioid receptors (MOR, DOR and KOR respectively) proteins were studied by Western immunoassays in opioid-responsive differentiated SK-N-SH neuronal cells. 3-NPA had different effects on MOR, DOR and KOR proteins. There was a significant reduction in MOR proteins in SK-N-SH cells while the DOR and KOR proteins remained unaffected under oxidative stress conditions. These findings, for the first time, demonstrate selective impairment of the MOR proteins under conditions of oxidative stress.

Keywords: Opioid receptors; MOR; DOR; ROS; 3-NPA; Western blot, oxidative stress

Introduction

Aging and neurodegenerative diseases are associated with oxidative stress. The free radical theory of aging proposes that deleterious effects of reactive oxygen species (ROS) are responsible for the deterioration of neuronal function in degenerative pathologies and aging (Floyd, 1999, Kasapoglu and Ozben, 2001). Increased lipid peroxidation and protein oxidation in aged mammalian brain have been shown in multiple studies (Leutner et al., 2001, Smith et al., 1991). The effects of oxidative damage on other receptor systems, such as adrenergic and cholinergic, have been investigated (Joseph and Roth, 1992, Joseph et al., 1996, Kramer et al., 1987) but the effect of oxidative stress on opioid receptors is unknown. Recently, we have reported that oxidative stress induced by 3-nitroproprionic acid (3-NPA) affects *mu* and *delta* opioid receptors function differentially (Raut et al., 2006). The SK-N-SH cells differentiate to a neuronal phenotype (Preis et al., 1988) with an increased expression of MOR and DOR (Yu et al., 1988). Differentiated SK-N-SH cells have been widely used as an experimental model to study opioid receptors (Baker et al., 2000, Loh and Smith, 1990, Preis et al., 1988, Ratka et al., 1991, Raut et al., 2006, Ratka and Simpkins, 1997). 3-Nitroproprionic acid (3-NPA) is a mitochondrial toxin (Ludolph et al., 1991). Exposure to 3-NPA resulted in an increase of ROS, the induction of oxidative stress, a decline in mitochondrial membrane potential, and the depletion of intracellular ATP (Raut et al., 2006, Wang et al., 2001), leading to impaired cellular energy production. 3-NPA has been used to induce oxidative stress in both *in vitro* and *in vivo* conditions to model neurodegenerative disorders (Geddes et al., 2000).

Opioid receptors play a critical role in pain and analgesia. Since free-radical-induced oxidative stress leads to an impairment of neuronal function, it may have a detrimental effect on opioid receptors.

Indeed, previous study showed that pain is an important health problem in old age, with the prevalence of pain reaching as high as 70% (Gloth, 1996). Elderly individuals show increased sensitivity to pain and altered efficacy of opioids (Belville et al., 1971, Macintyre and Jarvis, 1996, Moore et al., 1990). Due to limited understanding of the effects of oxidative stress on the opioid system, management of pain in elderly individuals and patients with neurodegenerative disorders is highly inadequate (Donovan et al, 1987, Foley, 1995). It is unclear how oxidative stress contributes to changes in sensitivity to pain and opioids in neurodegenerative pathologies. Moreover, we do not understand oxidative-stress-induced changes in the opioid receptor system and signal transduction pathways.

The present study continues to further explore the findings reported previously (Raut et al., 2006) on the effects of 3-NPA on three classical subtypes of opioid receptors; MOR, DOR and KOR in opioid-responsive SK-N-SH neuronal cells.

Materials and Methods

Supplies

SK-N-SH cells were purchased from American Type Tissue Collection (ATTC, Rockville, MD, USA). RPMI-1640 media from GIBCOBRL (Grand Island, NY, USA) were used. Fetal bovine serum (FBS), penicillin, streptomycin and 3-nitroproprionic acid

were obtained from Sigma (St. Louis, MO, USA). The primary rabbit polyclonal anti-MOR and anti-KOR antibodies and secondary anti- rabbit horse radish peroxidase labled IgG antibodies were purchased from BioSource International, Inc. (Camarillo, CA). The primary rabbit polyclonal anti-DOR antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). For reprobing, anti- β1 subunit of NA^+-K^+-ATPase antibody was purchased from Novus Biologicals Inc. (Littleton, CO).

SK-N-SH Cell Culture and Differentiation

The monolayer of SK-N-SH cells from passages 20-30 were cultured and grown in RPMI 1640 media supplemented with 10% FBS and 100 μg streptomycin/ml and 100 IU penicillin/ml. In RPMI 1640 media, 14μM of phenol red served as a pH indicator. The cells were plated at an initial density of 2 x 10^5 cells/ml in 100 mm culture plates. Medium was changed at 2 day intervals. Newly seeded cells were allowed at least 24 hrs to adhere. The cells were treated with retinoic acid (RA) to induce neuronal differentiation; medium containing 10 μM of retinoic acid was replaced every other day for 6 days (Yu and Sadee, 1988).

Treatments with 3-NPA

On the day of the experiment, appropriate 3-NPA concentrations were prepared in cell culture media. Before exposure to 3-NPA, medium was aspirated and the cell monolayer was rinsed with serum free medium. Subsequently, cells were exposed to medium containing 3-NPA (1- 20 mM) for a period between 30 minutes and 24 hrs at

37^0 C (Wang et al, 2001). After 3-NPA treatment, plasma membrane preparation was performed for Western immunoassays.

Membrane Preparation

The isolation of plasma membrane was performed as previously described (Dibas et al., 1996). SK-N-SH cells were grown on 100 mm dishes and treated with RA for 6 days as described in previous section. The differentiated cells were treated with different concentrations of 3-NPA for different durations as described above. The cells were washed and scraped in PBS buffer (0.2 g/L KCl, 8 g/L NaCl, 2.16 g/L $Na_2HPO_4 \cdot 7H_2O$). After a brief centrifugation, the cells were resuspended in homogenization buffer (32 mM Tris–HCl, 16 mM sodium pyrophosphate, 1.6 mM EDTA, 0.8 mM EGTA, 0.64 mM NH_4 molybdate, 3.2 mM DTT) containing protease inhibitors (20 μg /ml leupeptin, 20 μg/ml aprotinin, 20 μg/ml soybean trypsin inhibitor and 40 μg/ml PMSF). The samples were quickly sonicated and the homogenate was centrifuged briefly to sediment unbroken cells and nuclei. The supernatant was centrifuged at 100 000×g for 35 min at 4°C. The resultant pellet was dissolved in homogenization buffer containing proteinase inhibitors, Triton-X 100 (0.1%) and NP-40 (0.1%) by gently aspirating several times and incubating on ice for 1 hr. Protein concentration of the membranes was determined using the BCA reagent and bovine serum albumin as the protein standard (Sigma Chemicals, St Louis, MO).

Western Immunoassay

Protein content of MOR, DOR and KOR in the plasma membrane preparation was measured by Western blot technique as previously described (Truong et al., 2003) with some modifications. Briefly, 10-40 μg of protein sample was mixed with SDS-sample buffer (62.5 mM Tris, pH 6.8, 0.1% (v/v) glycerol, 2% SDS, 0.05% 2-β mercaptoethanol and 0.005% (w/v) bromophenol blue), and incubated at 95^0C for 5 min. SDS-PAGE was performed on 10% gels according to the procedures described previously (Laemmli, 1970) using a Bio-Rad electrophoresis unit. Protein was then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) according to methods described previously (Towbin et al., 1979) in transfer buffer (192 mM Glysine, 20% methanol and 25 mM Tris pH 8.3) at room temperature overnight using Bio-Rad electrophoretic transfer unit. The nitrocellulose membrane was washed, blocked with 5% nonfat milk blocking solution prepared in TBS/T buffer (10 mM Tris HCL pH 8.0, 150 mM Nacl, 0.05% Tween-20), and incubated overnight on orbital shaker with primary antibody for MOR or DOR or KOR (1:1000). After incubation, the membrane was washed again and then incubated with the secondary antibody (1:10,000) for 30 minutes. After three washes with TBST, the blots were developed using an ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ). For stripping and reprobing, the membrane was incubated in stripping buffer (100 mM 20-mercaptoethanol, 2% sodium dodecyl sulphate, 62.5mM Tris-HCl, pH 6.7) at 45°C for 45 minutes, washed 4 times in TBS/T buffer at room temperature and blocked with 5% nonfat milk blocking solution. To ensure accurate protein transfer, the blots were stripped and reprobed with primary antibody for

β_1 subunit of Na^+-K^+ ATPase (1:2500) for 1 hr, washed and then incubated with secondary antibody as above. Densitometric analysis of the bands was done using the Scion image analysis software. The quantification of band intensity is represented as a percentage to the value of its corresponding control band in the same membrane.

Data Analysis

Each set of experiments was repeated at least three times. Western immunoblots were quantified using scanning densitometry. The values depict normalized densitometric values as % control. Data are expressed as mean ± S.E.M. (n = 4). The statistical evaluation of data was performed by analysis of variance (ANOVA) followed by the Tukey post hoc test using GraphPad Prism Version 4.0 statistical software package. p value <0.05 was considered as statistically significant.

Results

Western blot immunoassay of MOR, DOR and KOR proteins after 3 -NPA treatment

In the initial experiments, the effect of 2 hrs treatments with 10 mM 3-NPA on the MOR, DOR and KOR proteins in plasma membrane of differentiated SK-N-SH cells was characterized. Western immunoanalysis of MOR proteins revealed a band at approximately 55 Kd, DOR proteins at 58 Kd and KOR proteins at 45 Kd (Fig. 1A). As determined by comparison of optical density readings, 3-NPA treatment (10 mM for 2 hrs) significantly decreased the level of MOR protein to 49 ± 10% of control. 3-NPA

treatment reduced the DOR proteins by 4% to 96 ± 8% of control, the change being statistically insignificant as compared to control cells. No significant changes in the quantity of KOR proteins were detected after 3-NPA treatment (100 ± 10% of control).

Dose-response studies of 3-NPA on MOR, DOR and KOR proteins in differentiated SK-N-SH cells

In a subsequent series of experiments, dose-response studies with 3-NPA were performed to determine the effects of 0, 5, 10 and 20 mM of 3-NPA on MOR, DOR and KOR protein levels. After exposure to 10 mM and 20 mM of 3-NPA for 2 hrs, the quantities of MOR protein significantly decreased to 43 ± 7% and 24 ± 3% of control respectively (Fig. 2). After exposure to 5 mM, 10 mM and 20 mM 3-NPA for 2 hrs, the DOR protein levels were 101 ± 7%, 105 ± 4% and 93 ± 8% of control, respectively. Similarly, after treatment with 5 mM, 10 mM and 20 mM 3-NPA for 2 hrs, the KOR protein levels were 95 ± 8%, 98 ± 7% and 91 ± 5% of control, respectively. The levels of DOR and KOR proteins after 3-NPA treatments were not statistically different from the vehicle-treated cells.

Time-response studies of 3-NPA on MOR, DOR and KOR proteins in differentiated SK-N-SH cells

To determine the time-response effect of 10 mM of 3-NPA on opioid receptor proteins, the cells were treated with 3-NPA for 1 hr-24 hrs and Western blot immunoassays were carried out. Exposure to 10 mM 3-NPA for 2, 12 and 24 hrs resulted

in a significant reduction of MOR protein levels to 45 ± 6%, 58 ± 4% and 36 ± 3% of control, respectively (Fig. 3). After exposure to 10 mM 3-NPA for 1 hr, 2 hrs, 12 hrs and 24 hrs, the DOR protein levels were 99 ± 6%, 91 ± 4%, 91 ± 4% and 93 ± 7% of control respectively. After treatment with 10 mM 3-NPA for 1 hr and 24 hrs, the KOR protein levels were increased to 120 ± 16% and 118 ± 10% of control, respectively, but these changes were statistically insignificant. Treatment with 10 mM 3-NPA for 2 hrs (98 ± 11% of control) and 12 hrs (103 ± 8% of control) did not cause any significant changes in the KOR protein levels.

Discussion

In this study, we demonstrate for the first time that, under conditions of reduced cell energy metabolism induced by 3-NPA, there is a significant reduction in the level of MOR protein but not DOR and KOR proteins in opioid-responsive SK-N-SH neuronal cells. These findings imply that opioid receptor subtypes respond differently to the detrimental effect of cellular energy damage evoked by the interruption of mitochondrial function. In earlier studies conducted on the same cell model, we have shown that the function of MOR but not DOR was reduced under *in vitro* oxidative stress conditions induced by 3-NPA (Raut et al., 2006). To our knowledge, no literature reports demonstrated changes in opioid receptor protein levels under conditions of reduced mitochondrial energy under *in vitro* conditions. The novel findings from our current and previous studies clearly show that opioid receptor subtypes differ in their vulnerability to oxidative stress.

Different amino acid sequences of MOR and DOR proteins (Chen et al., 1993) could contribute to the differences in vulnerability of these receptor subtypes to oxidative stress. The extracellular loops of opioid receptors consist of amino acid residues, such as cysteine and methionine that are sensitive to oxidation (Law et al., 1999). Hence, oxidative damage to these amino acid residues may be critical in the mechanism underlying the selectivity of oxidative stress on opioid receptors.

Opioid receptors are G protein coupled receptors (Ueda et al., 1988, Gilman, 1986). The opioid-subtype-specific effects of oxidative stress may be related to differences in G proteins coupled to MOR and DOR. Studies with G_α-specific antibodies suggested that G_{i-2} proteins are coupled with DOR in NG 108-15 cells (McKenzie and Milligan, 1990), while G_o proteins are coupled with MOR in SHSY-5Y cells (Carter and Medzihradsky, 1993). G_o and G_{i-2} proteins may differ in their vulnerability to oxidative stress leading to damage to G_o but not G_{i-2}. Oxidation of G protein can lead to loss of anchoring effect of G protein to opioid receptor in the plasma membrane that can lead to migration of opioid receptor to cytosolic compartment. The differences in the sensitivity of G protein isoforms to oxidative damage could account for the observed differences in the effect of oxidative stress on MOR, DOR and KOR proteins.

Oxidative stress may also influence the trafficking of MOR, DOR and KOR differently. It has been reported that the internalization and trafficking of DOR and KOR are differentially regulated, even when activated by the same agonist (Chu et al., 1997). It is not known if internalization of opioid receptors varies constitutively, i.e. in the absence of activation by an agonist. It seems possible that oxidative stress may influence the

internalization and trafficking of MOR differently than that of DOR and KOR. In this case, more internalization of MOR under oxidative stress conditions could result in reduction in membrane bound MOR protein.

Opioid receptor proteins may become oxidized under oxidative stress conditions. Studies have shown that free oxidative radicals cause oxidation of proteins in various regions of aged mammalian brain (Oliver et al., 1987, Smith et al., 1991). Our findings suggest that the MOR protein may be more vulnerable to oxidative damage than the DOR and KOR proteins.

We focused on membrane component of the cell extracts because the opioid receptors are membrane bound G protein coupled receptors which execute their activity through G proteins and adenylyl cyclase (Ueda et al., 1988, Gilman, 1986). Specificity of antibodies used in the present study has not been established previously in membrane extracts of differentiated SK-N-SH cells but the bands obtained with these antibodies are consistent with those reported in the literature. Western immunoanalysis of MOR proteins revealed a band at approximately 55 Kd which is consistent with the previous findings that describe molecular masses of 45 Kd to 75 Kd for the MOR in humans and rodents (Roy and Loh, 1987; Ueda et al., 1988). Western immunoanalysis of DOR proteins revealed a band at approximately 58 Kd. This size is in agreement with the findings in the literature which describes molecular masses of 45 Kd to 65 Kd for the DOR in humans and rodents (Roy and Loh, 1987; Ueda et al., 1988, DeMoliou-Mason and Barnard, 1984; Gomathi and Sharma, 1993; Anand and Oommen, 1995). Western blot analysis of KOR showed a band at approximately 45 Kd which is consistent with the

previous reports (Chow and Zukin, 1983; Simon et al., 1987, Roy and Loh, 1987; Ueda et al., 1988). Western blots for β_1 Na^+-K^+ ATPase expressed single or double bands migrating between 35-60 Kd, which is in accord with the previous findings (Vagin et al., 2004, Vagin et al., 2005).

In the present study, we have not analyzed the cytosolic component of the cells for opioid receptor proteins. It is a possibility that, after 3-NPA treatment, the opioid receptor proteins might have degraded and/or migrated from cell membrane to intracellular compartment, thus, reducing the membrane content of proteins. Our results regarding the effect of 3-NPA on MOR protein content in the plasma membranes in differentiated SK-N-SH cells give an initial insight into the mechanism underlying changes in opioid receptor function.

The results of the present study support our earlier findings showing inhibition of MOR function under oxidative stress conditions (Raut et al., 2006) and suggest that this effect might be due to decrease in number of membrane bound *mu* opioid receptors in SK-N-SH neuronal cells. Thus, it seems that there is a selective impairment of the MOR proteins under oxidative stress conditions while DOR and KOR proteins remain unaffected.

In this study, we observed 17-20 % increase in KOR protein levels after 1 hr and 24 hrs exposure of 10 mM 3-NPA. This effect was statistically insignificant. This could be due large inter-experimental variability in the effects of 3-NPA on KOR proteins as suggested by large standard error of means. KOR in SK-N-SH cells has not been extensively studied, and to our knowledge, it has not been confirmed whether this cell

line expresses KOR or if this receptor subtype is functional in these cells. Only one study reported that KOR agonists inhibited the amplitude of K^+-induced $[Ca^{2+}]_i$ increase in SK-N-SH cells, but this effect was not blocked by KOR antagonist nor-binaltorphimine (Hurle et al., 1999). In the present study, in differentiated SK-N-SH cells, we obtained a band of approximately 45 Kd for KOR, which is consistent with the findings reported in the literature (Chow and Zukin, 1983; Simon et al., 1987, Roy and Loh, 1987; Ueda et al., 1988). Studies on function of KOR in differentiated SK-N-SH cells have found that spiradoline (selective KOR agonist) did not inhibit cAMP significantly (unpublished data), suggesting that if SK-N-SH cells express KOR, these receptors may not be functional, and hence, their contribution to cAMP inhibition might be insignificant. Therefore, further explorative studies on expression and function of KOR in differentiated SK-N-SH cells are necessary.

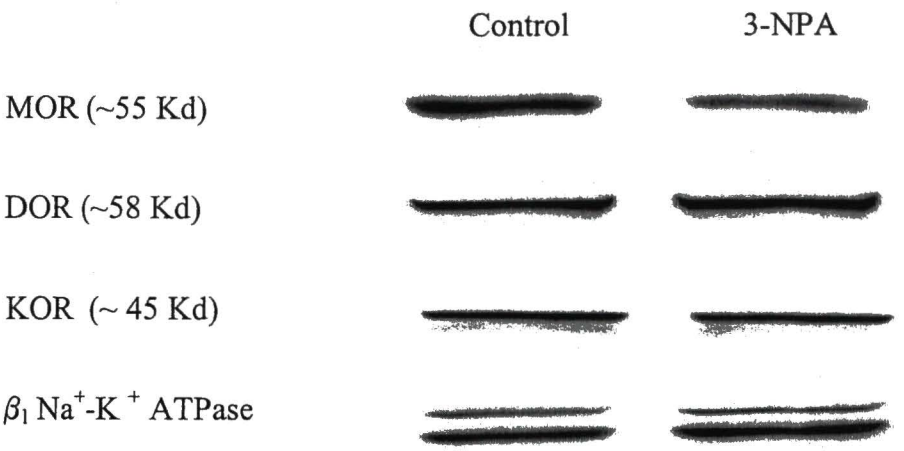
Oxidative stress may not be the only factor contributing to the alterations in MOR protein levels after 3-NPA treatments observed in our study. Irrespective of oxidative damage, other consequences of reduced mitochondrial succinate dehydrogenase activity, e.g., reduced cellular ATP, could be involved in the changes in opioid receptors. Understanding the effects of these individual elements on opioid receptors will help in identifying the specific factor(s) responsible for alterations in opioid receptor function under oxidative stress conditions.

Conclusions

In summary, the results of our study show for the first time that 3-NPA-induced oxidative stress conditions may be selectively detrimental to MOR but not DOR and KOR proteins in plasma membrane of SK-N-SH neuronal cells. The findings imply that opioid receptor subtypes differ significantly in their sensitivity to consequences of mitochondrial damage. Additional studies should be directed at elucidating the mechanism(s) underlying the differential responses of opioid receptor subtypes to mitochondrial energy impairment in neuronal cells. Amino acid residues known to be especially sensitive to oxidation, such as cysteine and methionine, are located in the extracellular loop of each of the MOR subtype (Law et al., 1999). As protein thiols can be easily oxidized and such a reversible modification is increasingly implicated in signal transduction, future studies should focus on the opioid receptor's protein thiol oxidative modification. The proteomic analysis of oxidatively modified opioid receptor proteins could give more insight into the underlying mechanism of modification of opioid receptors proteins under oxidative stress conditions. The current and future research findings will help to better understand changes in the opioid receptor system under conditions associated with neuronal damage. Ultimately, the new knowledge may be applied to assessment and treatment of pain in aging and neurodegenerative pathologies such as dementia, stroke and Alzheimer's disease.

Figure 1. Western immunoassay of MOR, DOR and KOR proteins from membranes of differentiated SK-N-SH cells after treatment with 3-NPA (10mM for 2 hrs). The wells were loaded with 30 μg of proteins. Blots were stripped and reprobed with anti- β_1 Na^+-K^+ ATPase to ensure accurate protein loading and transfer. Panel (A) shows immunoblots of MOR, DOR, KOR and β_1 Na^+-K^+ ATPase. (B) Western immunoblots were quantified using scanning densitometry. Columns depict normalized densitometric values. * $p < 0.05$ vs. control (No NPA).

(A)


Control
3-NPA
MOR (~55 Kd)
DOR (~58 Kd)
KOR (~ 45 Kd)
β_1 Na^+-K^+ ATPase

(B)

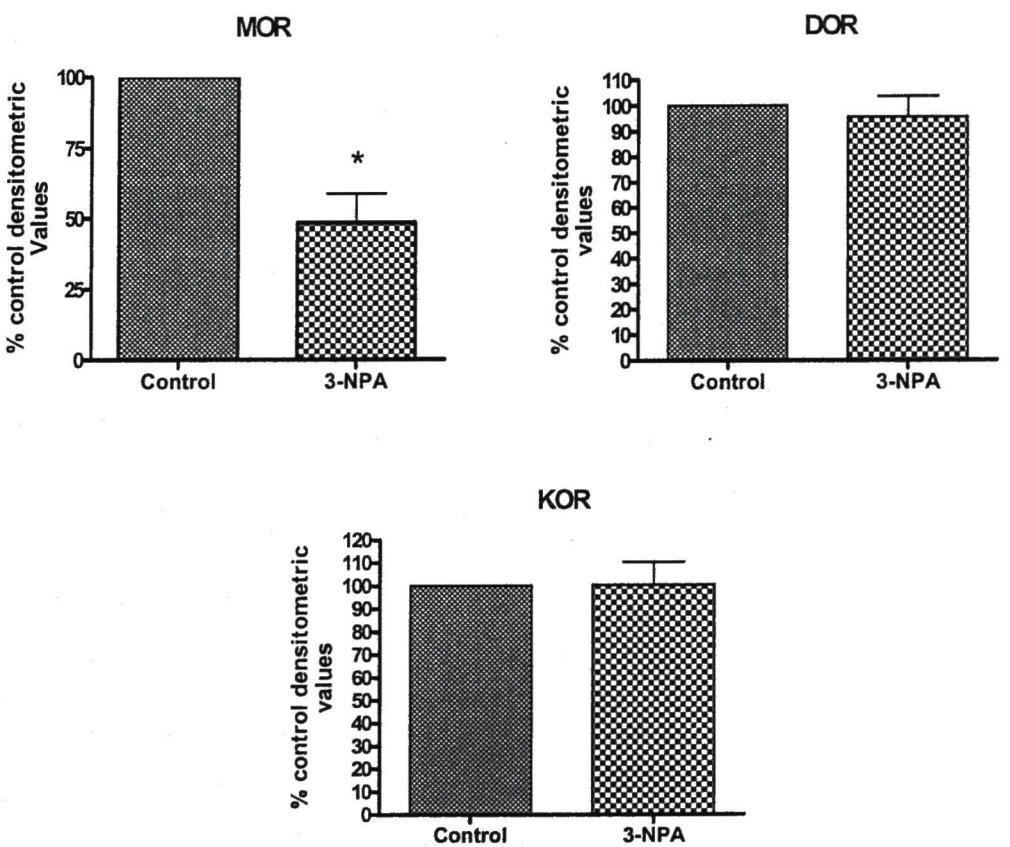
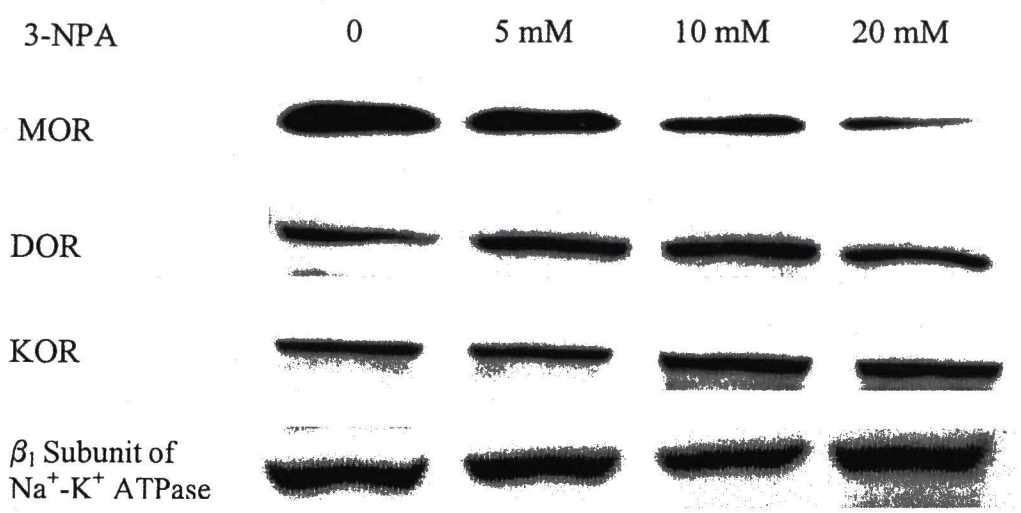

MOR
% control densitometric Values
100
75
50
25
0
*
Control
3-NPA
DOR
% control densitometric values
110
100
90
80
70
60
50
40
30
20
10
0
Control
3-NPA
KOR
% control densitometric values
120
110
100
90
80
70
60
50
40
30
20
10
0
Control
3-NPA

Figure 2. Dose-response effects of 3-NPA on MOR, DOR and KOR proteins from membranes of differentiated SK-N-SH neuronal cells. The cells were incubated with 0, 5, 10 and 20 mM of 3-NPA for 2 hrs. (A) Western immunoblots of MOR, DOR, KOR and β_1 Na^+-K^+ ATPase. All the wells were loaded with 30 μg of proteins. Blots were stripped and reprobed with anti- β_1 Na^+-K^+ ATPase to ensure accurate protein loading and transfer. (B) Western immunoblots were quantified using scanning densitometry. Columns depict normalized densitometric values. * $p < 0.05$ vs. control (0).

(A)


3-NPA
0
5 mM
10 mM
20 mM
MOR
DOR
KOR
β_1 Subunit of
Na^+-K^+ ATPase

(B)

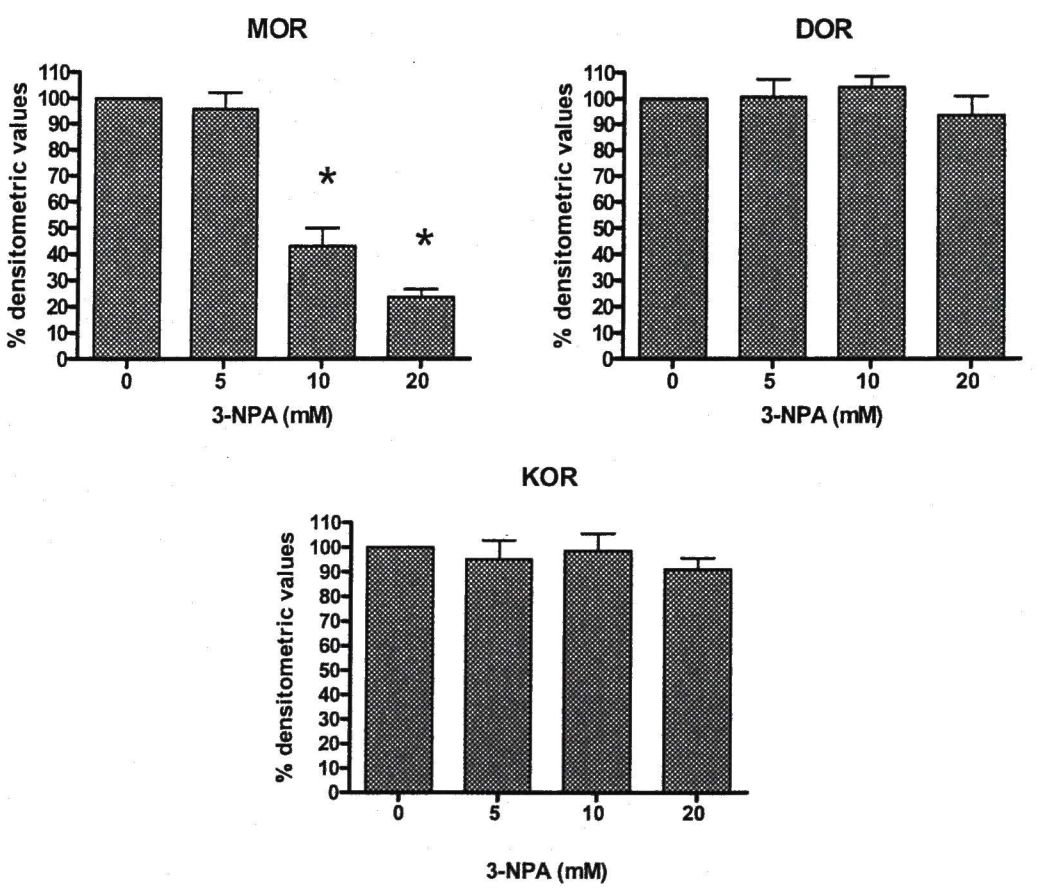
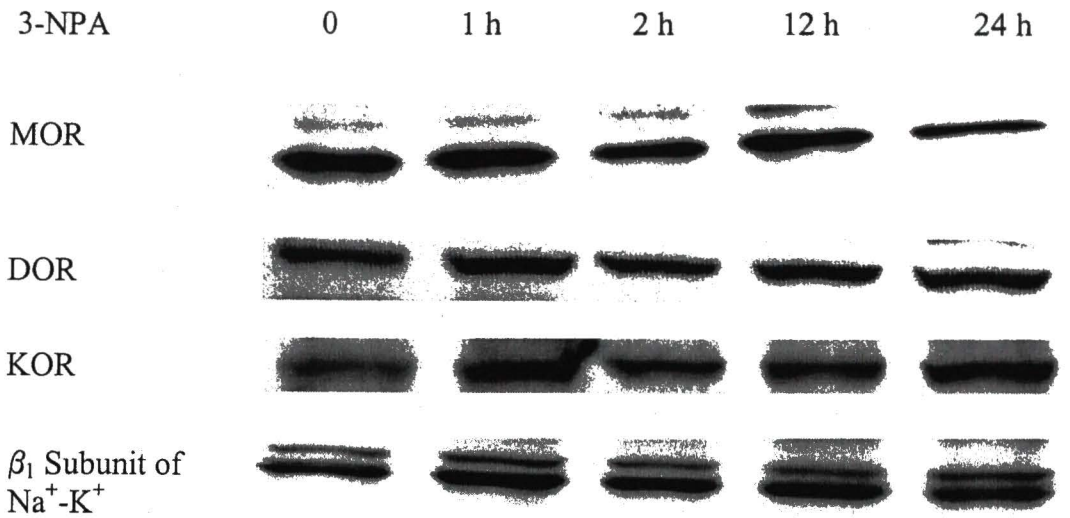
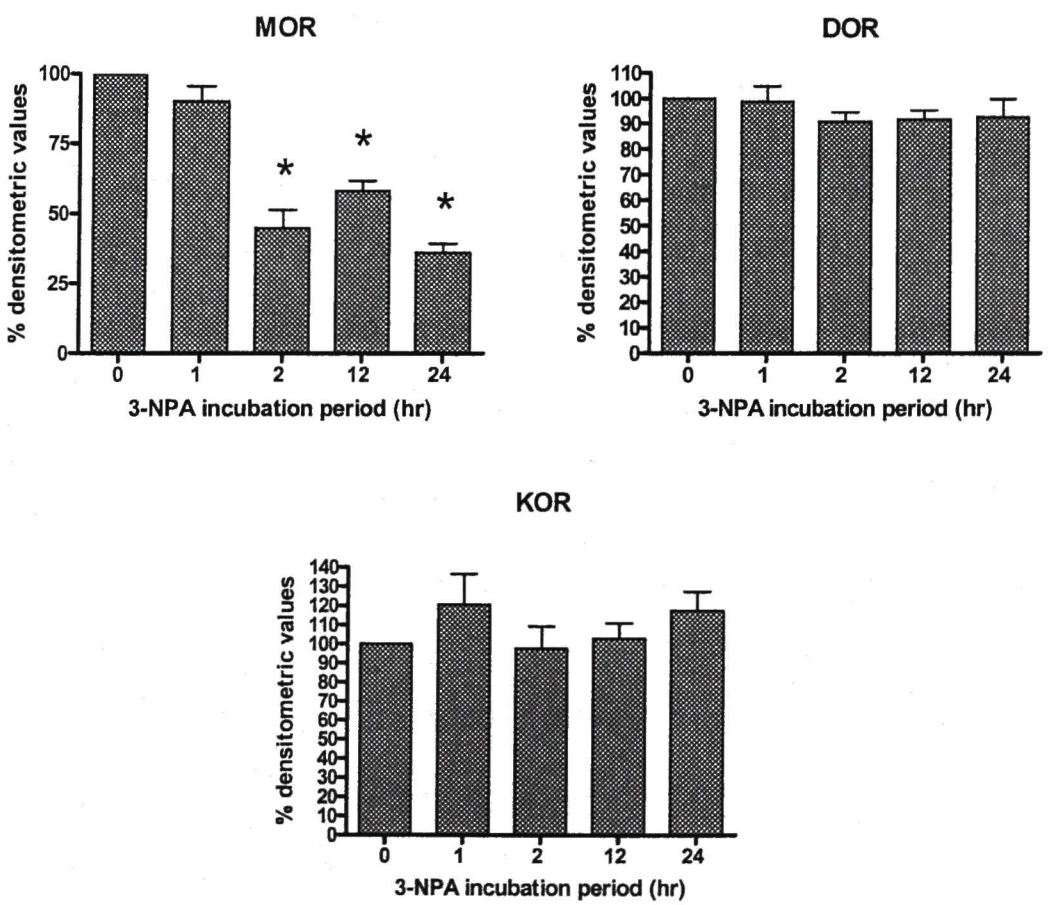

MOR
% densitometric values
110
100
90
80
70
60
50
40
30
20
10
0
*
*
0
5
10
20
3-NPA (mM)
DOR
% densitometric values
110
100
90
80
70
60
50
40
30
20
10
0
0
5
10
20
3-NPA (mM)
KOR
% densitometric values
110
100
90
80
70
60
50
40
30
20
10
0
0
5
10
20
3-NPA (mM)

Figure 3. Time-response effects of 3-NPA on MOR, DOR and KOR proteins from membranes of differentiated SK-N-SH neuronal cells. The cells were incubated with 3-NPA (10mM) for 0, 1, 2, 12 and 24 hrs. (A) Western immunoblots of MOR, DOR, KOR and β_1 Na^+-K^+ ATPase. All the wells were loaded with 30 μg of proteins. Blots were stripped and reprobed with anti- β_1 Na^+-K^+ ATPase to ensure accurate protein loading and transfer. (B) Western immunoblots were quantified using scanning densitometry. Columns depict normalized densitometric values. * $p < 0.05$ vs. control (0).

(A)


3-NPA
0
1 h
2 h
12 h
24 h
MOR
DOR
KOR
β_1 Subunit of Na^+-K^+

(B)


MOR
% densitometric values
100
75
50
25
0
*
*
*
0
1
2
12
24
3-NPA incubation period (hr)
DOR
% densitometric values
110
100
90
80
70
60
50
40
30
20
10
0
0
1
2
12
24
3-NPA incubation period (hr)
KOR
% densitometric values
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0
1
2
12
24
3-NPA incubation period (hr)

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CHAPTER V

SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

Summary and Conclusions

Inadequate treatment of pain in aged population and individuals with neurodegenerative diseases such as Alzheimer's disease, stroke and Huntington's disease is an important public health problem. Incidence and prevalence of pain in the population of elderly people is much higher than in younger individuals. Oxidative damage to neurons has been implicated in aging and neurodegenerative disorders. A number of clinical and experimental reports have shown an increase in pain sensitivity and decrease in responses to opioids in aging but the role of oxidative stress in these alterations remain unclear.

The present research project focused on two issues: (1) the effects of aging on baseline nociceptive sensitivity to pain and opioid-induced antinociception and the correlation between these factors and oxidative damage in various brain regions in male Fischer 344 rats and (2) effects of oxidative stress on the function and expression of opioid receptors in opioid-responsive SK-N-SH neuronal cells.

Pain sensitivity and opioid-induced antinociception in male Fischer 344 rats of three age groups, i.e. young (3-6 months), adult (9-12 months) and old (21-24 months) was studied with the hot plate method. Sensitivity to thermal nociceptive stimulus

increased with aging. Morphine and fentanyl-induced antinociception decreased with advancing age.

Markers of protein oxidation (protein carbonyls) and lipid peroxidation (TBARS) were estimated in cerebral cortex, hippocampus, striatum and midbrain in the three age groups of rats. With aging, the levels of these markers were found to be elevated, implying increased oxidative damage to the brain. The pilot experiments were conducted to study the effect of aging on *mu* opioid receptor (MOR) protein in cerebral cortex in the rats. The studies demonstrated reduction in MOR protein with advancing age.

To determine the possible association between oxidative damage and opioid-induced antinociception, correlation between these two factors was analyzed. There was a significant negative correlation between oxidative damage and opioid-induced antinociception. These findings suggest that oxidative damage may play a role in modulation of responses to opioids in aging. Animal studies demonstrated that age-related oxidative damage along with reduction in opioid receptors in brain may contribute to alterations in pain sensitivity and responses to opioids.

Opioid-responsive SK-N-SH neuronal cells were utilized to more fully elucidate the effects of oxidative stress at the opioid receptor level. The function and expression of *mu*, *delta* and *kappa* opioid receptors (MOR, DOR and KOR respectively) in differentiated SK-N-SH cells were studied. 3-nitroproprionic acid (3-NPA), a succinate dehydrogenase inhibitor, was used to induce oxidative stress. Generation of reactive oxygen species (ROS) was determined with 2', 7'-dichlorofluorescin diacetate assay. The changes in intracellular cyclic AMP (measured by cAMP assay) induced by MOR, DOR

and KOR selective agonists were used as a marker of opioid receptor function. Effects of oxidative stress on expression of opioid receptor proteins were studied by Western blot technique.

Studies with the SK-N-SH neuronal cells demonstrated that 3-NPA treatments resulted in an increase in ROS, and under these oxidative stress conditions, there was a significant reduction in MOR but not DOR and KOR function. Thus, under conditions of mitochondrial damage, the function of MOR was significantly decreased, while the function of DOR and KOR did not change, suggesting that the effect of 3-NPA on opioid receptors was receptor subtype-specific.

The effects of 3-NPA were assessed in the presence of an antioxidant, Trolox, an analog of vitamin E. The function of MOR was preserved in the cells treated with Trolox. These findings support the hypothesis that oxidative stress leads to decline in opioid receptors function which could be preserved/prevented by antioxidant. These results substantiate the role of oxidative damage in decline of opioid receptor function.

The effects of oxidative stress on opioid receptors protein in SK-N-SH neuronal cells were studied and it was found that, 3-NPA induced oxidative stress reduced MOR proteins while DOR and KOR proteins remained unaffected. These *in vitro* findings demonstrate selective impairment of the MOR proteins under conditions of oxidative stress.

The results from this research project showed that aging and oxidative stress affect the opioid receptor system and subsequently contribute to alterations in pain sensitivity and responses to opioids.

The present research project has provided a number of interesting findings. Our studies have shown that aging, oxidative damage and opioid receptor function and protein expression are interrelated. The results have shown a strong correlation between oxidative damage and opioid-induced antinociception. These results describing the involvement of oxidative damage in opioid analgesia may have an impact on research as well as health care and may assist in formatting the new treatment modalities which involve combination of opioids with antioxidants. Current population of the USA is approximately 300 millions (U.S. Census Bureau, 2006). According to Kinsella and Victoria (2001), approximately 14% population is above 65 years of age and this figure is going to raise upto 20.3 % in 2030. According to American Geriatric Society (2002), prevalence of pain in this aged population varies between 40-80 %. So, broadly, approximately 20-25 millions elderly people in the USA suffer from pain. Our results suggesting involvement of oxidative damage in opioid analgesia may have a huge impact on pain-related health care and may influence the economic burden on the health care system. The new treatment modalities based on these research findings will also assist in initiating more effective approaches for pain treatment in aging and neurodegenerative disorders like Alzheimer's disease and stroke.

Future Directions

The results from our project have shown that aging and oxidative stress may affect opioid receptor system and may contribute to alterations in pain sensitivity and

responses to opioids. Following research projects may help to understand the underlying mechanisms of the effects of aging and oxidative stress on opioid receptor system.

(I) Our pilot studies demonstrated a reduction in *mu* opioid receptor proteins in brain in aging rats. However, it is not clear if the reduction is due to oxidation of proteins or due to decreased synthesis of opioid receptors under oxidative stress conditions. Further studies may need to explore proteins and mRNA expression of opioid receptor subtypes in brain in aging animals. These studies will help to understand if aging affects the opioid receptors' synthesis or their post-translational modifications or it affects only membrane bound opioid receptors, for example, by oxidation of proteins. These studies will further add to knowledge of effects of aging on opioid receptors.

(II) Studies focusing on effects of antioxidant intervention on pain sensitivity and opioid-induced antinociception in aging may confirm the role of oxidative stress on opioid system. Data from our *in vivo* studies have shown a negative correlation between oxidative damage to brain and opioid-induced antinociception. The *in vitro* studies have confirmed that antioxidant pretreatment can preserve the function of opioid receptors under oxidative stress conditions. These results imply that antioxidant pretreatment or intervention can influence the responses to opioids in advanced age.

Oxidative damage has been implicated in age-related decline in neurological functions. For example, age-related decline in long term potentiation (a measure of synaptic plasticity) in hippocampus and dentate gyrus has been observed and increase in ROS has been shown to play a role in this phenomenon (Aurbach and Segal, 1997, Murray and Lynch, 1998). The long term potentiation impairment in aged rats was

reversed with treatments with antioxidants like vitamin E, vitamin C or α-lipoic acid for 8 weeks (Murray and Lynch, 1998, McGahon et al., 1999). Caloric restriction lowers oxidative stress by decreasing the generation of ROS and enhancing the antioxidant defense mechanisms (Sohal, 1996, Weindruch and Sohal, 1997). Forster et al., (2000) have shown the reversible effects of caloric restriction on protein oxidation in brain after 6 weeks of caloric restriction in aging C57BL/6 mice. It has been reported that antioxidants like vitamin E and propyl gallate can prevent amyloid β-peptide-induced oxidative damage to neuronal cells (Behl et al., 1994, Mark et al., 1995). Furthermore, caloric restriction has also been shown to improve thermotolerance and to reduce thermal damage to cells in rats (Hall et al., 2000).

Considering these findings and our data, it can be hypothesized that antioxidant pretreatment can increase the responses to opioids in rats of advanced age. The hypothesis may be tested in old rats (21-24 months). The rats will be divided into 2 groups. One group will serve as control (no antioxidant pretreatment) and other group will receive antioxidant pretreatment for 6-8 weeks. Then the opioid-induced antinociception can be measured with opioids in these rats. The rats pretreated with antioxidant will show higher levels of opioid-induced antinociception as compared to control group. If the results from the animal pilot studies provide supportive data, the hypothesis can be tested in different age groups of rats with different doses and durations of administration of antioxidants. Based on these studies, the optimum regimens of antioxidant treatment can be determined. Moreover, these studies will form the basis for future studies in humans, where, the effects of antioxidant pretreatment on opioid

analgesia in aged population can be determined. Moreover, the effects of combination of opioids with antioxidants on opioid analgesia can also be tested. These studies will substantially affect pain-related health care in elderly population and may have a huge impact on the economic burden on the health care system

(III) Additional studies should be directed at elucidating the mechanism(s) underlying the differential responses of opioid receptor subtypes to mitochondrial energy impairment in neuronal cells. The proteomic analysis i.e. detection, localization and quantification of oxidatively modified opioid receptor proteins could give more insight into the underlying mechanism of modification of opioid receptors proteins under oxidative stress conditions. The proteomic analysis will also help to identify the amino acid residues which play a critical role in oxidative modification of opioid receptors.

(IV) It is also possible that the levels of endogenous opioids such as endorphins and dynorphins change with aging that could contribute to the alterations in pain sensitivity and opioid antinociception. Further studies exploring the effects of aging and oxidative damage on endogenous opioid system will aid to more fully understand the underlying mechanisms of changes in pain sensitivity and opioid analgesia in aging.

Expansion of current research findings will help to better understand the opioid receptor system under conditions associated with neuronal damage. Ultimately, the new knowledge may be applied to assessment and treatment of pain in aging and neurodegenerative pathologies such as dementia, stroke, and Alzheimer's disease.

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