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Maturation alterations in constitutive activity of medial prefrontal cortex κ -opioid receptors in Wistar rats

Sunil Sirohi^{1,2} and Brendan M Walker^{1,2,3,4,*}

¹Laboratory of Alcoholism and Addictions Neuroscience, Department of Psychology, Washington State University, Pullman, WA

²Department of Integrative Physiology & Neuroscience, College of Veterinary Medicine, Washington State University, Pullman, WA

³Graduate Program in Neuroscience, Washington State University, Pullman, WA

⁴Translational Addiction Research Center, Washington State University, Pullman, WA

Abstract

Opioid receptors can display spontaneous agonist-independent G-protein signaling (basal signaling/constitutive activity). While constitutive κ -opioid receptor (KOR) activity has been documented *in vitro*, it is unknown if KORs are constitutively active in native systems. Using [³⁵S] GTP γ S coupling assay that measures receptor functional state, we identified the presence of medial prefrontal cortex (mPFC) KOR constitutive activity in young rats that declined with age. Furthermore, basal signaling showed an age-related decline and was insensitive to neutral opioid antagonist challenge. Collectively, the present data are first to demonstrate age-dependent alterations in the mPFC KOR constitutive activity in rats and changes in the constitutive activity of KORs can differentially impact KOR ligand efficacy. These data provide novel insights into the functional properties of the KOR system and warrant further consideration of KOR constitutive activity in normal and pathophysiological behavior.

Keywords

Constitutive activity; kappa-opioid receptors; negative-efficacy antagonist; nor-binaltorphimine; dynorphin; age

*Corresponding author: Brendan M. Walker, Ph.D., Laboratory of Alcoholism and Addictions Neuroscience, Department of Psychology, Washington State University, 100 Dairy Road; Mail Code 647620, Pullman, WA 99164-7620, 509-335-8526 (office), 509-335-5324 (FAX), b_walker@wsu.edu.

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Conflicts of interest: none

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INTRODUCTION

G protein-coupled receptors (GPCRs) constitutive activity is well documented in receptor pharmacology. First demonstrated in delta-opioid receptors (DORs), constitutive activity is thought to occur in most GPCRs systems (Costa and Herz 1989;Costa and Cotecchia 2005;Bond and Ijzerman 2006). Antagonists block agonist-induced effects; however, it has been shown that antagonists can range from zero efficacy to negative efficacy depending on the ligands ability to alter receptor constitutive activity (Strange 2002;Kenakin 2004;Bond and Ijzerman 2006). Neutral antagonists (zero efficacy antagonists) inhibit agonist-induced effects without affecting basal signaling, whereas negative efficacy antagonists (possessing inverse agonist properties) attenuate receptor constitutive activity (Strange 2002;Kenakin 2004). Interestingly, ~ 85% of antagonists for known neurotransmitter systems can function as negative-efficacy antagonists (Milligan 2003;de Ligt *et al.* 2000;Greasley and Clapham 2006). It is prudent to mention that in addition to efficacy of a given ligand, the state and physiological environment of the receptor system (e.g., receptor basal signaling state, certain receptor mutations, availability of G-proteins, intracellular ionic milieu, etc.) are all critical determinants of the magnitude and direction of the actual response elicited by a given ligand (Kenakin 2002;Perez and Karnik 2005;Sirohi *et al.* 2007;Sirohi *et al.* 2009;Navani *et al.* 2011). In the absence of constitutively active receptor system, negative efficacy antagonists and neutral antagonists are indistinguishable.

Like many GPCRs, constitutive activity has been documented for opioid receptors. Following the seminal discovery of constitutively active DORs (Costa and Herz 1989), studies have reported basal mu-opioid receptor (MOR) signaling activity (Wang *et al.* 2001). Basal kappa-opioid receptor (KOR) signaling has been documented *in vitro* (Wang *et al.* 2007); however, it is not yet known if KORs are constitutively active in native systems. As identified above, only negative efficacy antagonists can assess constitutive activity and one extensively studied KOR antagonist (nor-binaltorphimine; nor-BNI) has shown evidence of inverse agonist properties *in vitro* (Wang *et al.* 2007) and given our previous experience with the compound, nor-BNI was selected for use in the present investigations as a putative negative efficacy antagonist. We previously demonstrated a KOR specific effect of nor-BNI in the GTP γ S assay (Kissler and Walker, 2015) and initiated research on KOR involvement in executive function that implicated the KOR as being an important regulator of impulse control specific to stopping an already-initiated action (Walker and Kissler, 2013). Converging lines of evidence suggested that KORs in the mPFC could be important regulators of executive function: 1) The mPFC has been implicated as a substrate for stop-signal reaction time performance (e.g., Bari *et al.* 2011), 2) KOR-mediated regulation of neurotransmitters within the mPFC could promote maladaptive behavioral regulation (Tejeda *et al.* 2013;Tejeda *et al.* 2015), 3) dynorphin (DYN; an endogenous KOR ligand) and KOR mRNA are upregulated in the PFC of deceased human alcoholics (Bazov *et al.* 2013), a report that nicely parallels our published data showing a 'one-two punch of alcoholism' due to increases in both dynorphin (DYN; an endogenous KOR ligand) A-like immunoreactivity and KOR function in the central amygdala. Such dysregulation could be characteristic of dynorphin (DYN; an endogenous KOR ligand) / KOR dysregulation in

distinct brain nuclei that mediate executive function and contribute to normal goal-directed behavior.

Given our established and sustained interest in identifying the nature and extent of DYN / KOR dysregulation that could contribute to alcohol dependence-induced cognitive impairment, and considering observations of age-dependent KOR agonist effects in rodents (e.g., Goodwin and Barr 2005; Anderson *et al.* 2014), we evaluated the hypothesis that ontogenic alterations in mPFC KOR function are sensitive to maturation in a manner consistent with age-dependent KOR agonist effects observed in the literature. To this end, we evaluated the functional state of KORs in mPFC tissue obtained from 60-150 day old rats.

MATERIAL AND METHODS

Animals

Male Wistar rats 60-150 days were obtained from our breeding colony at WSU that is semi-annually restocked with breeders from Charles River Laboratory (Hollister, CA) and housed in an environmentally controlled vivarium on a reverse light cycle (lights off at 6 a.m.). Food and water was available ad libitum. All work adhered to National Research Council's Guide for the Care and Use of Laboratory Animals (2011) and Institutional Animal Care and Use Committee guidelines.

General Procedure

Brain tissue was collected from 60-150 days old rats and the mPFC microdissected. First, the effect of nor-BNI (negative-efficacy KOR antagonist) and CTOP (neutral mu-opioid antagonist) on basal signaling was determined in the presence or absence of DYN A (endogenous KOR agonist) and DAMGO (exogenous MOR agonist), respectively using 60 days old rat brain tissue. Next, in order to examine the impact of age on the nor-BNI negative efficacy, mPFC brain tissue obtained from 60-150 days old rats was incubated with nor-BNI in the GTP γ S signaling assay.

[³⁵S]GTP γ S Signaling Assay

The assay was conducted as described previously (Kissler *et al.* 2014). It is a functional assay that measures the guanine nucleotide exchange at G proteins as a result of ligand binding to G protein-coupled receptors (GPCRs), by monitoring the binding of a radiolabeled, [³⁵S]GTP γ S, in the presence of unlabeled GDP and is described in detail elsewhere (Harrison and Traynor 2003). In short, an agonist will increase GTP γ S binding; whereas, a negative efficacy antagonist will reduce GTP γ S binding, if receptors are constitutively active (Bond and Ijzerman 2006). A neutral antagonist has no efficacy and thus does not alter GTP γ S binding. Therefore, a negative efficacy antagonist can serve as a tool to detect constitutive receptor activity.

Rat mPFC tissue was micro-dissected and homogenized (45 strokes; on ice) in 1.5ml of membrane buffer (pH 7.4, 50.0mM Tris-HCl, 3.0mM MgCl₂, and 1.0mM EGTA). The homogenate was centrifuged (21004g, 4°C for 30 min), re-suspended in 1.5ml of membrane

buffer, homogenized (12-15 strokes) and centrifuged again. The pellet was re-homogenized (12-15 strokes) in 1.5ml of assay buffer (pH 7.4, 50.0mM Tris-HCl, 3.0mM MgCl₂, 0.2mM EGTA, 100.0mM NaCl). Protein estimation was conducted using a BCA protein assay (Pierce). Samples were homogenized (12-15 strokes) prior to the addition of the protein homogenate (3µg). DYN A (0.0-1.0 µM), nor-BNI (0.0-10.0µM), CTOP (0.0-10.0µM) or DAMGO (0.0-1µM), were incubated in assay buffer in triplicates (90 min; 25°C) with 10µM GDP and 0.05nM [³⁵S] GTPγS in 1.0ml total volume. The reaction was terminated by filtration using a cell harvester, and washed 3x in ice cold phosphate buffer (pH 7.2). Bound radioactivity on the filter discs was quantified by liquid scintillation spectrophotometry.

Data Analysis

One-way repeated-measure of ANOVA was utilized to examine the impact of nor-BNI or CTOP on basal or agonist-stimulated GTPγS coupling. Data from the GTPγS coupling assay was analyzed using a mixed-model two-way ANOVA to compare antagonist-mediated changes in GTPγS signaling for the 60-150 days old rats. The within-subject variable was nor-BNI concentration and the between-groups variable was age. If a main effect was identified, one way repeated measure of ANOVA was done.

RESULTS

First the effect of nor-BNI (a negative efficacy antagonist) and CTOP (a neutral antagonist) on basal and agonist-stimulated GTPγS coupling was examined in the 60 days old rat mPFC. Nor-BNI concentration-dependently decreased ($F_{3,15}=10.60$, $p=0.001$, $\text{power}=0.992$) basal GTPγS coupling, whereas CTOP was ineffective in altering the basal GTPγS coupling (Figure 1a). Nor-BNI and CTOP concentration dependently blocked DYN [1.0µM] and DAMGO [1.0 µM]-stimulated GTPγS coupling, respectively (Figure 1b).

Next, we examined age-dependent differences in the basal signaling. The one-way ANOVA revealed a main effect of age on basal signaling ($F_{3,80}=6.248$, $p=0.001$, $\text{power}=0.957$) showing a decline in the basal signaling activity with age. Post-hoc analyses revealed significant differences in basal signaling of 120 ($p=0.005$) and 150 ($p=0.001$) days group compared to 60 days old rat mPFC tissue (Figure 2).

In order to examine age-dependent changes in nor-BNI negative efficacy, mPFC tissue from 60-150 days old rat brain was incubated with nor-BNI [0–10 µM] in the GTPγS coupling assay. Nor-BNI concentration-dependently decreased ($F_{2,7,215}=36.27$, $p=0.000$, $\text{power}=1.0$) basal GTPγS coupling in all age groups. A main effect of age was observed for norBNI-induced decrease in basal GTPγS coupling ($F_{3,80}=11.11$, $p=0.000$, $\text{power}=1.0$) with greatest decrease observed in 60 days old rat mPFC (Figure 3).

DISCUSSION

Negative efficacy antagonists are critical for evaluating receptor constitutive activity and studies have reported this phenomenon for many GPCRs, including opioid receptors (Costa and Herz 1989; Wang *et al.* 2004; Wang *et al.* 2007). While basal signaling activity at MOR and DOR have been documented repeatedly (Liu and Prather 2001; Liu and Prather

2002;Wang *et al.* 1994;Wang *et al.* 2004;Costa and Herz 1989), constitutive activity at KORs has remained relatively undocumented, at least *in vivo* (Becker *et al.* 1999;Wang *et al.* 2007). Nor-BNI, a classical KOR-selective antagonist with no detectable affinity (K_i 10 μ M) for most non-opioid receptors and transporters (Munro *et al.* 2013;Metcalf and Coop 2005), has been shown to act as a negative-efficacy antagonist *in vitro* (Wang *et al.* 2007). Here we show that nor-BNI reduced basal signaling in rat mPFC brain tissue and this effect declines with age.

The GTP γ S coupling assay has been successfully utilized for *in vitro* and *in vivo* assessment of constitutive opioid receptors activity (Wang *et al.* 2004;Bilsky *et al.* 2010;Barreda-Gomez *et al.* 2010). Membranes for the GTP γ S assay were prepared from the rat brain tissue by utilizing an approach similar to that used previously to measure constitutive activity of opioid receptors (Raehal *et al.* 2005;Wang *et al.* 2004). By definition, both neutral and negative-efficacy antagonists can block agonist-stimulated receptor activity but only a negative-efficacy antagonist is capable of reducing agonist-independent receptor signaling. To ensure that altered basal signaling in response to nor-BNI was mediated by negative efficacy and not the result of competitive displacement of an agonist, it was imperative that the effects of the neutral antagonist were tested under identical experimental conditions. As neutral antagonists for KORs are not well characterized, we tested the effect of CTOP (a neutral mu-opioid antagonist; Hawkins *et al.* 1989;Meye *et al.* 2012) on basal signaling. Using this assay, first we examined the effect of nor-BNI on basal signaling in 60 day old rat mPFC tissue. Overall, nor-BNI and CTOP blocked DYN and DAMGO-stimulated GTP γ S coupling, respectively (Figure 1b). However, only nor-BNI significantly decreased basal binding (Figure 1a) which supports the concept of agonist-independent KOR constitutive activity. As shown in Figure 1a, nor-BNI concentration dependently decreased basal signaling with highest concentration decreasing basal signaling by ~20%. nor-BNI has been previously shown to reduce basal signaling in the cell line expressing KORs (Wang *et al.* 2007), however this is the first demonstration of KOR constitutive activity in the native rat brain tissue. CTOP was ineffective in altering basal signaling under identical assay conditions. As such, these data support the hypothesis that KORs display constitutive activity in the rat mPFC.

Nor-BNI's reduction in basal signaling was specific to 60 days old rat brain tissue and this effect was absent in older rat brain region (unpublished observations). An age-dependent decline in KOR density in the rat brain (Bhargava *et al.* 1994) suggests that KOR constitutive activity could decline as a function of age-dependent changes in KOR density with support provided by the demonstration that relatively high expression of the receptors is critical for observations of constitutive activity *in vitro* (Costa and Cotecchia 2005;Parra and Bond 2007;Bond and Ijzerman 2006;Greasley and Clapham 2006;Herrick-Davis *et al.* 1999). Therefore, we evaluated the effect of nor-BNI in the mPFC tissue collected from 60-150 day old rats. Basal signaling declined with the age (Figure 2) and importantly, there was an age-dependent decline in nor-BNI alteration of basal signaling (Figure 3). These data suggest high KOR constitutive activity in the mPFC of young rats decreases with age and explains the age-dependent decline in the nor-BNI negative efficacy. An age-dependent decline in DYN concentration could account for the observed differences in nor-BNI

efficacy and norBNI-induced effects merely reflect blockade of endogenous agonist-stimulated signaling. However, age-dependent increases in the DYN peptide content have been reported in the rat mPFC brain region which negates this concern (Nguyen *et al.* 2005). It is also important to note that the negative efficacy profile of nor-BNI was relatively abolished in 150 day old rat brain tissue which further speaks against endogenous agonist contamination in our assay conditions.

One characteristic of nor-BNI is an unusually long duration of action (Endoh *et al.* 1992) related, in part, to its interactions with intracellular signaling cascades that raises the interesting question of whether reduced nor-BNI negative efficacy in the older animals of the current study is representative of some cascade-mediated generalized lack of effect for nor-BNI in older animals. To be sure, that is not the case as studies have confirmed nor-BNI efficacy in older rodents using techniques that range from gene to behavior (e.g., Kissler *et al.* 2014; Menard *et al.* 2014). The two competing hypotheses to explain the long-term effects of nor-BNI relate to 1) abnormal c-Jun N-terminal kinase (JNK) activation by nor-BNI (Bruchas *et al.* 2007; Bruchas and Chavkin 2010; Melief *et al.* 2010) that is required for long-term effects in a manner unrelated to non-competitive antagonism and 2) nor-BNI's pharmacokinetic properties prolong elimination and allow for continued competitive antagonism (Patkar *et al.* 2013). Neither theory is exclusionary of the other nor do they lack support for their claims which suggests that there is a combined contribution to the long-term effects of nor-BNI.

Receptor basal signaling has been best characterized in artificially transfected systems *in vitro* through mutagenesis or pharmacology, but observing constitutive activity *in vivo* has traditionally been difficult (Costa and Cotecchia 2005; Parra and Bond 2007; Bond and Ijzerman 2006; Greasley and Clapham 2006; Herrick-Davis *et al.* 1999; Lam *et al.* 2011). The present data are first to demonstrate that KORs are constitutively active in the rat mPFC in an age-dependent manner providing additional mechanisms whereby dysregulation of the DYN / KOR system could occur to promote maladaptive behavioral regulation.

The physiological importance of GPCRs constitutive activity is increasingly becoming apparent and antagonists with negative efficacy could have clinical utility (de Ligt *et al.* 2000; Parra and Bond 2007). These current results indicate the presence of KOR constitutive activity in one brain substrate mediating impulse control and strategic behavior (Kim and Lee 2011; Bechara and Van Der Linden 2005; Boes *et al.* 2009). We have recently reported KOR activation-induce impulsive phenotypes in rats (Walker and Kissler 2013) and, well documented in both human and animal models, younger organisms engage in more impulsive and risky behaviors than their adult counterparts (Green *et al.* 1994; Doremus-Fitzwater *et al.* 2012; Whelan R *et al.* 2012). Considering the possibility that age-related declines in impulsivity are a function of maturational changes in KOR constitutive activity, dysregulated constitutive activity under pathological conditions such as alcohol dependence and withdrawal could contribute to symptoms such as heightened impulsivity and impaired executive control.

In conclusion, for the first time, KORs constitutive activity was identified to be age-dependent in the rat mPFC. Maturational changes in KOR constitutive activity could

differentially impact KORs ligand efficacy. Moreover, dysregulation of constitutive activity in addictive and neuropsychiatric disorders such as alcohol dependence could underlie many of the symptoms that contribute to maladaptive behavioral regulation.

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ABBREVIATIONS

GTPγS	(guanosine 5'-O-[gamma-thio] triphosphate)
GPCR	(G-protein-coupled receptor)
Nor-BNI	(nor-binaltorphimine)
DAMGO	([D-Ala ² , NMe-Phe ⁴ , Gly-ol ⁵]-enkephalin)
KOR	(κ -opioid receptor)
DOR	(delta-opioid receptor)
MOR	(mu-opioid receptor)
CTOP	(D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH ₂)

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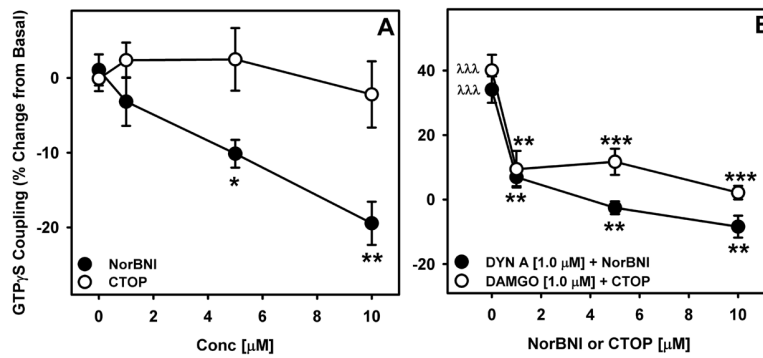


Figure 1. Effect of nor-BNI and CTOP on basal and agonist-stimulated GTP γ S coupling in the rat mPFC

A) Nor-BNI (negative efficacy opioid receptor antagonist) decreased ($F_{3,15}=10.60$, $p=0.001$, power=0.992) basal GTP γ S coupling in a concentration dependent manner, whereas a neutral opioid receptor antagonist (CTOP) failed to alter basal GTP γ S coupling (* $p<0.05$, ** $p<0.01$ compared to zero concentration). **B)** As an internal control for assay responsiveness, opioid receptor agonists (i.e., DYN A and DAMGO) significantly stimulated GTP γ S coupling that was blocked by nor-BNI and CTOP, respectively, $\lambda\lambda\lambda p<0.001$ when compared to basal signaling. ** $p<0.01$, *** $p<0.001$ compared to the respective agonist-stimulated GTP γ S coupling. Mean (\pm SEM) is plotted from six experiments ran in triplicates.

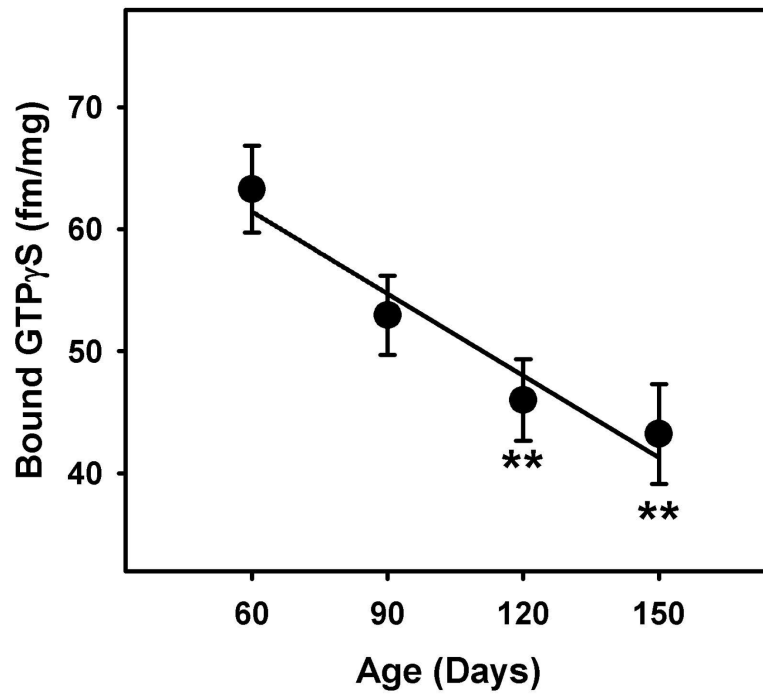


Figure 2. Maturational changes in the basal GTP γ S coupling in the rat mPFC

A oneway ANOVA revealed an age-dependent decline in the basal GTP γ S signaling.

** $p < 0.01$ ($F_{3, 80} = 6.248$, $p = 0.001$, power = 0.957), ** $p < 0.01$ compared to 60 days old group.

Mean (\pm SEM) is plotted from seven experiments ran in triplicates.

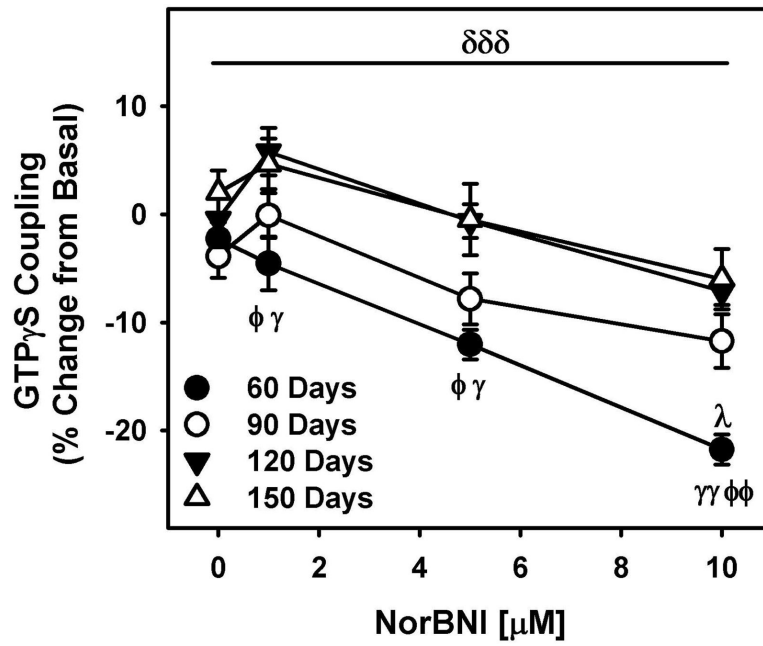


Figure 3. Maturation changes in nor-BNI negative efficacy in the rat mPFC

Nor-BNI concentration-dependently decreased ($F_{2,7,215}=36.27$, $p=0.000$, power=1.0) basal GTP γ S coupling as a function of age with greatest decrease observed in 60 days old rat mPFC. This effect was attenuated in an age-dependent manner. $\delta\delta\delta$ Main effect of age ($F_{3,80}=11.11$, $p=0.000$, power=1.0), $\lambda p<0.05$ when compared with same nor-BNI concentration of 90 days old group, $\phi p<0.05$, $\phi\phi p<0.01$ with 120 days old group and $\gamma p<0.05$, $\gamma\gamma p<0.01$ with 150 days old group. Mean (\pm SEM) is plotted from seven experiments conducted in triplicates.