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Research report

Age-dependent evaluation of long-term depression responses in hyperthyroid rats: Possible roles of oxidative intracellular redox status

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HIGHLIGHTS

- Aging leads to weakened antioxidant defense and increased lipid peroxidation.
- Hyperthyroidism increases GPx activity and decreases NOS levels at young ages.
- Hyperthyroidism favors LTD at young ages, but LTP at old ages.
- In hyperthyroidism, age-associated plasticity is modulated by a mechanism involving NOS.
- Plasticity modulation explains the increased memory deficit in old age that occurs with hyperthyroidism.

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ABSTRACT

Background: According to the free radical theory, a gradual accumulation of the free radicals normally produced in the body underlies the changes associated with aging. Thyroid hormones (THs) are related to oxidative stress not only due to their stimulation of metabolism but also due to their effects on antioxidant mechanisms. Thyroid dysfunction increases with age; thus, changes in TH levels in elderly individuals could be a factor affecting the development of neurodegenerative diseases. However, the relationship is not always clear, based on current evidence regarding synaptic plasticity.

Methods: Hippocampal long-term depression (LTD) and oxidative status in the hippocampus were evaluated at two different ages (2–3 and 12–14 months) in male rats. Rats were administered 0.2 mg/kg/day of L-thyroxine for 21 days starting at postnatal day 40 to induce hyperthyroidism. LTD was induced in the dentate gyrus using low frequency stimulation of the perforant pathway. Spectrophotometry was performed to measure catalase (CAT), superoxide dismutase (SOD), and malondialdehyde (MDA) levels, glutathione peroxidase (GPx) activity, and total nitrite/nitrate (tNOx) and nitric oxide synthase (NOS) levels.

Results: A reliable LTD was elicited in young rats with hyperthyroidism, while the same protocol could induce a small magnitude of synaptic LTD in the absence of spike-LTD in control rats. In aged rats, controls did not express LTD, but a significant LTP of spike was induced in the absence of synaptic LTD in hyperthyroid rats. While CAT levels were significantly decreased, MDA levels were increased in the aged groups compared to the corresponding young groups. Young rats with euthyroidism had significantly lower GPx activity than each of the hyperthyroid groups. There was no significant difference in SOD levels among the groups. Compared with aged rats, young rats exhibited a hyperthyroidism-induced decrease in NOS levels. Nevertheless, neither the main effects of age and thyroxine administration nor the interaction between these factors reached significance for tNOx.

Conclusion: These results indicate that hyperthyroidism-related changes in synaptic plasticity are modulated by aging. This modulation may explain the increased cognitive impairment in this disease at older ages, which probably depends on alterations in NOS levels.

Abbreviations: LTP, long-term potentiation; LTD, long-term depression; THs, thyroid hormones; MWM, Morris water maze; NO, nitric oxide; NOS, nitric oxide synthase; CAT, catalase; MDA, malondialdehyde; fT4, free T4; fEPSP, field excitatory postsynaptic potential; PS, population spike; LFS, low frequency stimulation; NO_{2-}, nitrite; NO_{3-}, nitrate

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1. Introduction

Long-term depression (LTD), the opposing process to long-term potentiation (LTP), is an activity-dependent reduction in the efficacy of neuronal synapses that lasts hours or longer following a long patterned stimulus. LTD, working together with LTP, has been proposed as the cellular substrate of information processing and memory formation (Bliss and Cooke, 2011). LTD occurs in many areas of the CNS through varying mechanisms depending upon brain region (Linden, 1994) and developmental progress (Foster, 2007) and serves to selectively weaken specific synapses to make constructive use of synaptic strengthening caused by LTP. Therefore, a decrease in the ability to induce LTD would cause the synapses to reach maximal efficiency, which would inhibit the encoding of new information. In contrast, the facilitation of LTD may significantly determine the efficacy of learning and memory by limiting acquisition and favoring the decline of memory (Genoux et al., 2002; Malleret et al., 2001; Roman et al., 1999). In particular, the computational ability of LTD to properly counterbalance LTP may be essential to maintaining synaptic strengths in the linear range (Stanton, 1996).

Although the general roles of thyroid hormones (THs) in the mammalian body are well known, THs have key roles in the central nervous system, such as axonal and dendritic growth and the formation of new synaptic connections. In particular, the formation of synaptic plasticity in the hippocampal region of the brain is related to learning and memory, and many studies have shown the effect of thyroid hormones in young and old age (Bernal, 2007; Rivas and Naranjo, 2007). In accordance with this information, these hormones mediate the regulation of both LTP and LTD in the hippocampus. In our laboratory, we have shown impaired LTP in the hippocampal dentate gyrus in hyperthyroid rats (Taşkın et al., 2011). Additionally, reduced learning and memory performance in the Morris water maze (MWM) accompanied this result (Bitiktaş et al., 2016). These findings are consistent with the findings of other researchers (Ge et al., 2012; Gerges et al., 2004). However, LFS-induced LTD plasticity was more durable under conditions of increasing levels of free L-thyroxine in adult hyperthyroid rats in our previous study (Tan et al., 2016b). These results suggest that hyperthyroidism alters the direction of plasticity and promotes LTD over LTP.

One of the possible mechanisms underlying the thyroid dysfunction-induced impairment of LTP and LTD may be increased oxidative stress because thyroid hormones are related to oxidative stress not only due to their stimulation of metabolism but also due to their effects on antioxidant mechanisms (Venditti and Di Meo, 2006). In addition, it is known that oxidative stress increases with age. Numerous studies have reported correlations between age and the accumulation of oxidative damage to cellular macromolecules (Floyd and Hensley, 2002; Moskovitz et al., 2001). For example, aged rats exhibit enhanced lipid peroxidation in their brains (Barton et al., 2004; Gupta et al., 1991; Kiran et al., 2004; Murray and Lynch, 1998; O'donnell and Lynch, 1998).

Several studies in healthy older individuals have revealed an age-dependent decline in serum TSH and free T3 along with an increase in reverse T3 (rT3) and the maintenance of stable serum free T4 levels (van den Beld et al., 2005). Considering that aging is expected to be associated with a low antioxidant defense capacity derived from thyroid dysfunction, we aimed to determine the contribution of aging to hyperthyroidism-induced alterations in LTD and the indices of oxidative stress (catalase, glutathione peroxidase, superoxide dismutase). Because lipids are one of the first targets of free radicals once they are produced and nitric oxide (NO) is able to protect the cells from oxidative damage by Sharpe et al. (2003), we also investigated the role of lipid peroxidation and NO in mediating some aspects of the aging process in hyperthyroidism.

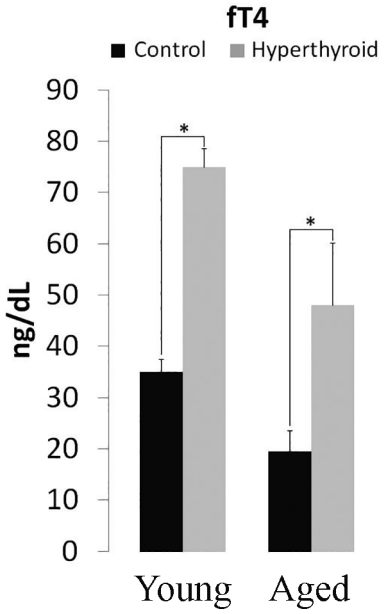

Fig. 1. Plasma free T4 (fT4) levels in rats treated with L-thyroxine at two different ages. fT4 levels decrease with age in control (black) and hyperthyroid rats (gray). Data are presented as the mean and standard error of the mean of six rats in each group. * Depict significant differences.

2. Results

2.1. Plasma free T4 (fT4) levels

For fT4 levels, a 2 × 2 ANOVA showed a significant age effect ($F_{1.22} = 6.670$; $P = 0.017$) and treatment effect ($F_{2.22} = 12.453$; $p < 0.001$) but no significant interaction between these factors ($p > 0.05$). These data showed that plasma fT4 levels decreased with age. A post hoc analysis also indicated that plasma fT4 levels were higher in hyperthyroid rats than in control rats at both the young ($p = 0.02$) and old ($p = 0.038$) time points (Fig. 1).

2.2. Electrophysiology

2.2.1. Input/output curve

To address the question of whether thyroid status and age, alone or together, have an effect on baseline synaptic strength, we compared the I/O curves generated before LFS application. As shown in Fig. 2, the PS amplitude and the EPSP slope steadily increased as a function of the PP stimulus intensity in all subgroups. An analysis of the raw data with repeated-measures ANOVA yielded no significant interaction of stimulus intensity with age group and thyroid status on PS amplitude ($p > 0.05$) or on EPSP slope ($p > 0.05$). These data indicate that the strength of the synaptic connections and the ability of the granule cells to produce an action potential were comparable between the four groups. Therefore, there was no difference in the baseline function among the four groups that might confound the interpretation of the other measures of synaptic function.

2.2.2. Long-term depression

Fig. 3 shows the time course of the fEPSP slope (Fig. 3A) and PS amplitude (Fig. 3B) before and after 1-Hz stimulation. We expected from this type of stimulation that the 5-min average of the fEPSP slope and PS amplitude must be significantly lower than the baseline value (i.e., 100%) at 55–60 min after the end of LFS application, inducing an early phase of LTD (E-LTD). As expected, only a small magnitude of LTD was elicited in young control rats (78.1 ± 8.7% of baseline, $p = 0.045$, one sample t-test), which was characterized by the absence of PS-LTD

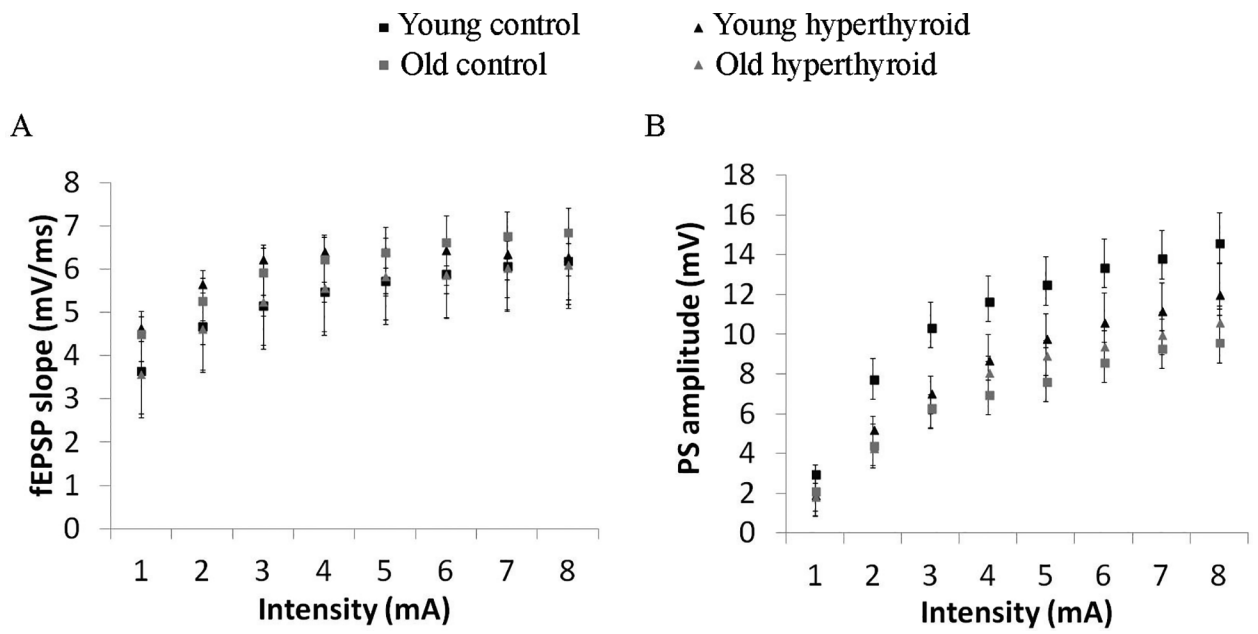

Fig. 2. The input-output (I/O) curves of rats treated with L-thyroxine at two different ages. The I/O curves were constructed by plotting the excitatory postsynaptic potential (EPSP) slope (a) and the population spike (PS) amplitude (b) versus the increasing magnitudes of stimulation of the perforant pathway in the groups (n = 7) before the induction of long-term depression.

(114.2 ± 15.0% of baseline, $p > 0.05$). Interestingly, in the young hyperthyroid rats, the same LFS protocol induced a reliable LTD in the DG of the hippocampus, as evidenced by marked depression exclusively in the fEPSP slope (fEPSP slope: 41.7 ± 9.1% of baseline, $p = 0.001$; PS amplitude: 129.4 ± 15.3% of baseline, $p > 0.05$). Aged control rats did not express LTD of fEPSP (control: 85.3 ± 11.8% of baseline, $p > 0.05$) or LTD of PS (132.2 ± 13.8% of baseline, $p > 0.05$), but a significant LTP of PS amplitude was induced in aged hyperthyroid rats (241.3 ± 24.8% of baseline, $p = 0.001$) in the absence of fEPSP-LTD (115.1 ± 10.4% of baseline, $p > 0.05$) in the dentate gyrus. A 2 × 2 ANOVA of these values yielded a significant main effect for age group (fEPSP: $F_{1.24} = 16.06$; $p = 0.001$; PS: $F_{1.24} = 13.40$; $p = 0.001$) and a significant interaction between thyroxine and age (fEPSP: $F_{1.24} = 10.85$; $p = 0.003$; PS: $F_{1.24} = 6.99$; $p = 0.014$). The main effect of thyroid status was significant on PS amplitude ($F_{1.24} = 12.27$; $p = 0.002$) but not on fEPSP slope ($p > 0.05$). Pairwise comparisons confirmed that young hyperthyroid rats had a significantly lower fEPSP slope than aged control ($p = 0.026$) and aged hyperthyroid rats ($p = 0.001$) and that aged hyperthyroid rats had a significantly higher PS amplitude than the other groups ($p < 0.001$ for all comparisons).

2.3. Antioxidant defenses and lipid peroxidation in the hippocampus

Fig. 4 depicts the levels of major intracellular antioxidant enzymes (CAT, SOD, GPx) in different samples obtained from the hippocampus of young and aged rats after the administration of L-thyroxine. A set of 2 × 2 ANOVAs indicated that the CAT levels were significantly lower in aged rats than in young rats, irrespective of L-Tx administration ($F_{1,18} = 17.32$; $p < 0.001$, interaction: $p > 0.05$). Pairwise comparisons revealed that young rats, with or without hyperthyroidism, had significantly higher levels of CAT than each of the aged groups ($p < 0.05$ for all comparisons, Fig. 4A).

The activity levels of GPx were higher in hyperthyroid rats than in control rats, irrespective of age ($F_{1,18} = 6.37$; $p = 0.021$, interaction: $p > 0.05$). Pairwise comparisons revealed that young rats with euthyroidism had significantly lower GPx activity than each of the hyperthyroid groups ($p < 0.05$, Fig. 4B).

There was no main effect or interaction effect of L-thyroxine administration and age on SOD levels ($p > 0.19$ for all comparisons, Fig. 4C).

Fig. 4 also includes MDA levels as an indicator of lipid peroxidation. We found that lipid peroxidation was increased in aged rats, irrespective of L-Tx administration ($F_{1,15} = 42.00$; $p < 0.001$, interaction: $p > 0.05$). Pairwise comparisons revealed that young rats, with or without hyperthyroidism, had significantly lower levels of MDA than each of the older groups ($p < 0.05$ for all comparisons, Fig. 4D).

2.4. Nitric oxide metabolites and nitric oxide synthase.

Fig. 5 depicts the levels of nNOS and nitric oxide metabolites (tNOx) in the hippocampus. A 2 × 2 ANOVA showed that aging abolished the effect of hyperthyroidism on nNOS levels ($F_{1,23} = 51.75$, $p < 0.001$), as indicated by significant interaction effects between age and thyroid hormone status ($F_{1,23} = 31.76$, $p < 0.001$). Pairwise comparisons revealed that the highest nNOS level was found in young euthyroid rats but the lowest levels were found in young hyperthyroid rats ($p < 0.01$, Fig. 5A). Nevertheless, neither the main effects of age group and Tx administration nor the interaction between these factors reached significance for tNOx ($p > 0.05$ for all comparisons, Fig. 5B).

3. Discussion

Although the maximal vulnerability of the central nervous system to thyroid hormone imbalance occurs during the earliest stages of development, the present study suggests that synaptic plasticity can also be affected by moderate elevations in TH levels during adulthood. By inducing hyperthyroidism in the early and late stages of adulthood, we found that excess thyroid hormones has an age-dependent effect on the synaptic responses to LFS and hippocampal nNOS levels and an age-independent effect on hippocampal GPx activity. In addition, this type of hyperthyroidism did not alter the age-dependent decrease in antioxidant status or the age-dependent increase in lipid peroxidation.

LTD represents the weakening of synaptic strength (Massey and Bashir, 2007) and can be divided into at least two temporally distinct phases: early-LTD (E-LTD, < 4 h) and late-LTD (L-LTD, > 4 h) (Kauderer and Kandel, 2000). To minimize the potential effects of urethane anesthesia on the stability of the field potential, the recording time scale, which is limited to 1 h after LFS administration, is

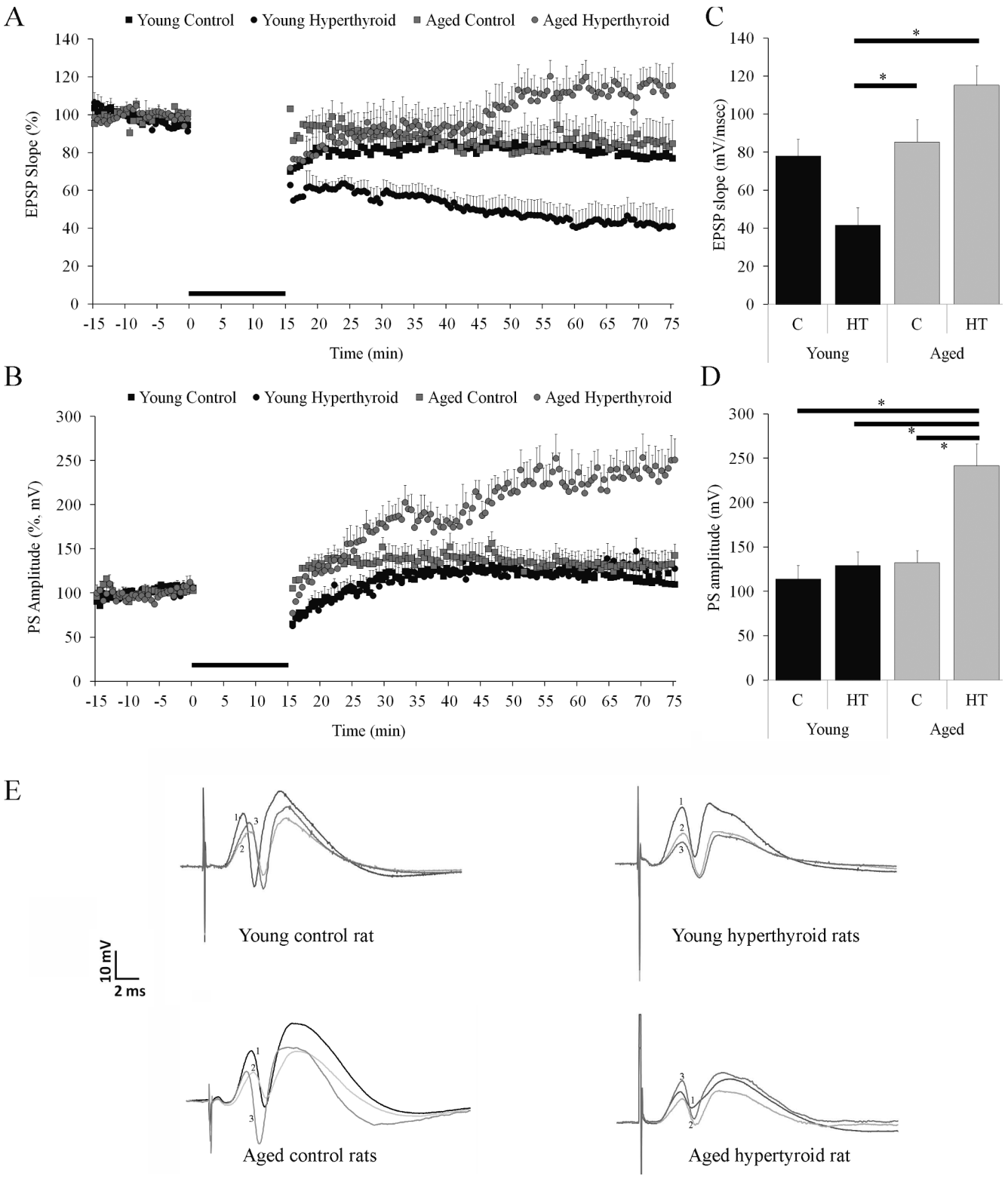

Fig. 3. Electrophysiological results of the control and experimental groups. Time course of the slope of fEPSP (A) and the amplitude of PS (B) during long-term depression. Each fEPSP and PS is expressed as a percentage of the average in the baseline period (between −15 and 0 min). LTD was measured as percentage of EPSP slope (C) and PS amplitude (D) measured 70–75 min after the onset of low-frequency stimulation (1-Hz, 900 sec, black line) to baseline. Note that young hyperthyroid rats had a significantly lower fEPSP slope than aged control and aged hyperthyroid rats and that aged hyperthyroid rats had a significantly higher PS amplitude than the other groups (*, $p < 0.05$). E: Representative traces of field potential recordings made immediately before (depicted with "1") and after application of LFS (depicted with "2") and at the end of recording (depicted with "3"). Note that the ratio of the fEPSP (first upward deviation) slope in trace depicted with "3" to those in trace depicted with "1" is reduced in young hyperthyroid rat, but increased in aged hyperthyroid rat.

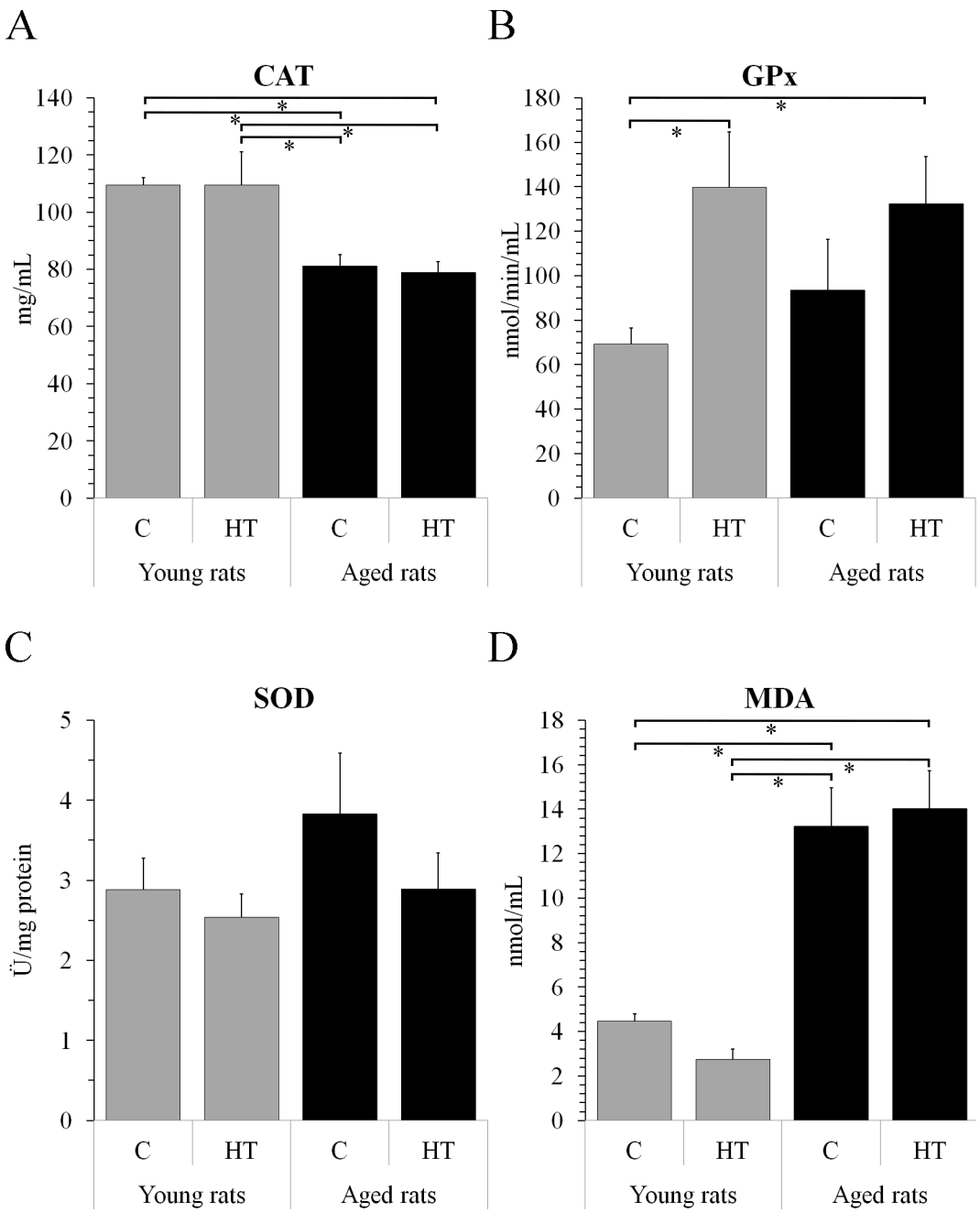

Fig. 4. Effects of aging and hyperthyroidism on antioxidant defenses and lipid peroxidation in the hippocampus. The bar graphs show activity levels of catalase (CAT, A), glutathione peroxidase (GPx, B), superoxide dismutase (SOD, C), and malondialdehyde (MDA, D). Hyperthyroidism (HT) was induced at young (postnatal days 40–61, young rats) or old age (postnatal days 330–341, aged rats). Note that hyperthyroidism has no effect on CAT, the levels of which decrease with age, and MDA, the levels of which increase with age; but hyperthyroidism increases the activity of GPx in both young and aged rats. Data are presented as the mean and standard error of the mean of five – six rats in each group. * depict significant differences ($p < 0.05$).

informative for E-LTD. Previous studies have shown that LTD can be barely induced by prolonged LFS in the dentate gyrus of young rats (Kemp et al., 2000; Norris et al., 1996b; Wong et al., 2007). In agreement with these studies, in response to the LFS protocol that we used in the present study, a tentative LTD was observed in young control rats. However, how LTD expression is affected by age remains a subject of debate. On the one hand, an increase in susceptibility to LTD induction has been reported in aged animals (Foster and Kumar, 2007; Kumar and Foster, 2005; Norris et al., 1996a); on the other hand, several studies indicate that DG-LTD is more difficult to induce with increasing age (Milner et al., 2004) (Norris et al., 1996b) or that there are no age-related changes in the magnitude of asymptotic LTD (Kumar et al., 2007). We found that aged control rats did not express LTD of fEPSP, suggesting a decreased ability of hippocampal neuronal networks to generate synaptic depression with age (Billard and Rouaud, 2007; Kollen et al., 2010).

As we have shown in our previous study (Tan et al., 2016a), the present study confirms that a durable depression of synaptic strength could be induced in young hyperthyroid rats but not control rats. This result may indicate that hyperthyroidism leads to the easy deletion of synapses in the early adulthood period. Extending this finding to aged animals, we report that aging causes the induction of an LTP characterized by increased PS amplitude following LFS in hyperthyroidism. However, accompanied increases in fEPSP slope were not observed in

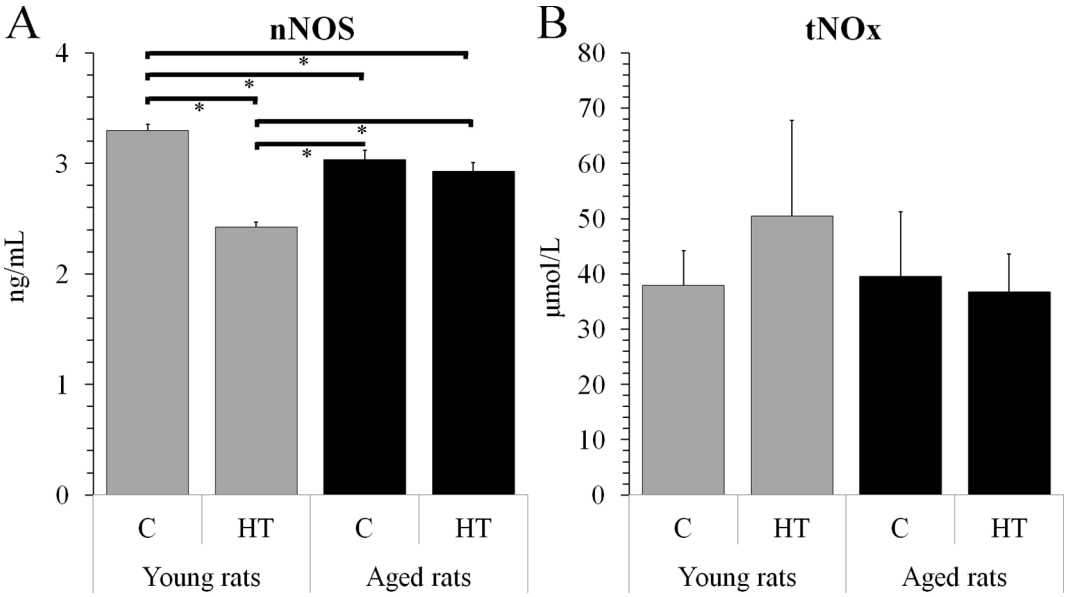

Fig. 5. Effects of aging and hyperthyroidism on neuronal nitric oxide synthase and nitric oxide metabolites in the hippocampus. The bar graphs show neuronal nitric oxide synthase (nNOS) levels (A) and nitric oxide metabolites (tNOx). Hyperthyroidism (HT) was induced at young (postnatal days 40–61, young rats) or old age (postnatal days 330–341, aged rats). Data are presented as the mean and standard error of the mean of five – six rats in each group. * Depict significant differences ($p < 0.05$).

these rats, indicating that chronic hyperthyroidism may have a more serious effect on postsynaptic cell excitability than on presynaptic neurotransmitter release. A population spike represents the synchronous discharge of a homogeneous population of neurons, and the amplitude of this spike is directly proportional to the number of active neurons (Bliss and Lomo, 1973). Therefore, we have concluded that the resulting neural output does not differ despite the weak synaptic strength in young rats and is increased independent from synaptic LTP in aged rats under the condition of excess thyroid hormones. This type of LFS-induced LTP suggests that under certain conditions, the excitatory synapses can undergo potentiation that involves non-NMDA receptors. This slow-rising potentiation may cause a failure in the loss of inappropriate synapses in old hyperthyroid rats and thus may be a reason for age-dependent memory deficits in clinical hyperthyroidism.

The brain is especially sensitive to ROS action and lipid peroxidation formation because it utilizes high levels of oxygen, contains large amounts of lipids that free radicals can readily react with, and exhibits low capacity of antioxidant enzymes, including SOD, which converts free radicals to hydrogen peroxide, and CAT and GPx, which further metabolize hydrogen peroxide to water and oxygen (Halliwell, 1992; Lewen et al., 2000). The biochemical results of this study suggest that, unlike SOD and GPx, there are age-related changes at the levels of CAT, which is another major antioxidant, and MDA, which is an indicator of lipid peroxidation. An increase in lipid peroxidation and a decrease in antioxidants has been previously observed in normal elderly people (Akila et al., 2007). This unbalanced ratio of SOD and GPx plus CAT activity is reported to be an important determinant of cellular aging (de Haan et al., 1995) and may have contributed to the failure of aged rats to produce LTD. Our results thus confirm a critical role for oxidative stress in cognitive function and for increased lipid peroxidation in the aging brain (Barton et al., 2004; Clausen et al., 2010; Devi and Kiran, 2004; Gupta et al., 1991; Hashimoto et al., 2004; Murray and Lynch, 1998; O'donnell and Lynch, 1998; Petursdottir et al., 2007; Venkataraman et al., 2013). The present study also shows that age-related changes resulting from free radical reactions are not complicated by the presence of hyperthyroidism.

The present study also indicates an increase in hippocampal GPx activity complicated by hyperthyroidism. Although the findings about the effect of hyperthyroidism on GPx activity are controversial in nonneural tissue (Asayama et al., 1987; Mano et al., 1997), the present study is the first to show an increase in GPx in the hippocampus, to the best of our knowledge. Considering hippocampal CAT levels in aged rats, one can conclude that the decreased antioxidant ability can be compensated by increased GPx in the hippocampus of aged hyperthyroid rats. The high activity of this enzyme induced in the hippocampus might contribute to protecting this structure from the neurodegenerative effects of hyperthyroidism. Nevertheless, it has been reported that synaptic NMDA receptor activity is coupled to the transcriptional control of the glutathione system (Hardingham et al., 2015). Therefore, this finding does not rule out the possibility that changes in GPx activity could be a factor for hyperthyroidism-related plasticity changes.

The results of several studies have demonstrated a stimulatory effect of TH on nNOS activity (Serfozo et al., 2008) and NOS gene transcription (Ueta et al., 1995), whereas others have reported that TH is a repressor of nNOS (Cano-Europa et al., 2008; Sinha et al., 2008). In the present study, probably due to the repressor effect, a decreased level of nNOS was found in young rats with hyperthyroidism compared with young control rats. Although the direct effect of NOS on synaptic plasticity has been less studied, it is noteworthy that the impairment of NO synthesis shifts striatal plasticity towards LTD in endothelial NOS (−/−) mice (Doreulee et al., 2003). In addition, the isoforms of NR1 in the intensely nNOS immunoreactive interneurons of the hippocampus differ from those found in most other neurons in these regions (Weiss et al., 1998), and one of eight distinct NR1 isoforms has been implicated in hippocampal LTD (Kutsuwada et al., 1996). Together with these studies, our findings indicate the possible involvement of NOS isoforms in synaptic modulation. The present study also suggests that changes in the expression of the nNOS gene may occur during aging (Colas et al., 2006). It has been suggested that such changes in nNOS may protect animals to some extent against age-associated cognitive decline in memory tasks that typically involve hippocampal regions (James et al., 2015).

In the present study, the changes in NOS levels are not supported by changes in the levels of NO end products (nitrate and nitrite), probably due to changes in the rate of metabolic removal in aging. Moreover, it has been reported that NO production declines faster than NOS expression (Herrera et al., 2006). Nonetheless, age-related changes in NO

production may be related to age-related changes in synaptic plasticity, as NO is a messenger molecule in a variety of cellular functions, including synaptic plasticity (Huang, 1997; Pigott, 2012; Son et al., 1996). NO-dependent LTD involving the activation of guanylyl cyclase and the subsequent activation of PKG in the hippocampus has been described (Reyes-Harde et al., 1999). To the best of our knowledge, since there are no data addressing the association between tissue NOx and THs, further studies are needed to investigate the detailed role of NO in LTD responses in hyperthyroidism with aging.

Various studies have implicated the role of the nonenzymatic endogenous antioxidant glutathione (GSH) in synaptic plasticity. For instance, GSH can bind to the NMDA channel as either an agonist or antagonist in particular circumstances, modulate NMDA-mediated Ca^{2+} currents (Aizenman et al., 1989; Ogita et al., 1995; Tang and Aizenman, 1993) and thereby regulate LTP (Patten et al., 2013). The oral supplementation of aged mice with N-acetylcysteine, a precursor for the formation of glutathione, reverses the L-type calcium channel-dependent LTP seen in aged animals to NMDAR-dependent LTP (Robillard et al., 2011). However, the potential mechanisms whereby GSH or other antioxidants alter synaptic plasticity in hyperthyroidism have remained undefined in this study.

In summary, these results indicate that hyperthyroidism favors LTD at young ages but LTP at old ages. Hyperthyroidism-related changes in synaptic plasticity seem to be modulated by a mechanism that involves NOS in aging. In addition, weakened antioxidant defense and increased lipid peroxidation are not complicated by hyperthyroidism, and hyperthyroidism increases GPx activity and decreases NOS levels at young ages. Together, these findings may explain why the incidence of cognitive impairment in hyperthyroidism increases with aging.

4. Experimental procedures

4.1. Animals

The experiments were conducted in accordance with the Council of the European Communities Directive of 24 November 1986 (86/609/EEC) on the protection of animals used for experimental purposes and the guiding principles for the care and use of laboratory animals approved by Erciyes University. Twenty-six young (2–3 months old) and twenty-six aged (12–14 months old) male *Wistar albino* rats were enrolled in this study. All rats were obtained from the Experimental Research and Application Center of Erciyes University (ERAC, Kayseri, Turkey) and housed in a controlled environment (20 °C and 60% humidity with lights on at 8:00 and off at 20:00 and with water and food pellets available *ad libitum*). Each age group was equally subdivided into 2 groups: control and hyperthyroid (n = 13 for each group; 3–4 rats for each cage). Seven rats were used for electrophysiological studies, and the remaining six rats were used for antioxidant enzyme studies.

4.2. Induction of hyperthyroidism by L-thyroxine

The animals in the hyperthyroid group were intraperitoneally administered L-thyroxin (CAS: 51-48-9; Sigma, St. Louis, Missouri, USA) daily at a dose of 0.2 mg/kg/mL, dissolved in a vehicle (a 5% sodium hydroxide and saline solution), for 21 days between day 40 and 60 in the young group and between day 360 and 380 in the aged group. The development of hyperthyroidism was confirmed in six rats of each subgroup by elevated plasma free T4 (fT4) levels. Blood samples were collected intracardially immediately after LTD experiments and centrifuged at 1000 × *g* for 30 min (Cat No: 75004030; SL 16R Centrifuge Series; Thermo Scientific, Langenselbold, Germany) and quickly frozen at −20 °C until it assayed by an ELISA kit (ELISA Kit for Free Thyroxine (fT4) – Catalog Number: CEA185Ge; Cloud-Clone Corporation, USA).

4.3. Electrophysiology

The details of the protocols used for the electrophysiological experiments are described elsewhere (Artis et al., 2012; Taşkın et al., 2011). Briefly, after rats were anesthetized with intraperitoneally injected urethane (1.2 g/kg), a double-barrel glass micropipette (Borosilicate, outer diameter of 1.5 mm, length of 10 cm; World Precision Instruments) was inserted into the granule cell layer of the DG in the right hemisphere (in mm, from bregma: anteroposterior: 23.5; mediolateral: 2.15; dorsoventral: 2.5–3 below the dura) to record the field potential. A bipolar tungsten electrode (stainless steel, Teflon-coated, 127 μm in diameter, insulated except at the tips) was used to stimulate the medial perforant path (PP, from bregma, in mm: anteroposterior: 28.0; mediolateral: 4.2; dorsoventral: 2–2.5 below the dura) of the right hemisphere. The depth of the recording and stimulating electrodes (dorsoventral coordinate) was adjusted to obtain a large positive field excitatory postsynaptic potential (fEPSP) followed by a negative-going population spike (PS) in response to perforant path stimulation. The positions of both electrodes were previously verified to be in the granule cell layer of the dentate gyrus and in the PP (Artis et al., 2012; Taşkın et al., 2011). The recording barrel was filled with 3 M NaCl (tip resistance: 2–10 MΩ).

After a stable fEPSP was obtained, the PP was stimulated by pulses at an intensity that ranged from 0.1 to 1.5 mA at 0.05 Hz three times and by increasing the intensities from a 0.1 mA to a 1.5 mA by 0.2 mA per step to create an input-output curve, which was stored for offline analysis. The stimulus intensity produced by half of the maximum PS amplitude was determined (test stimulus) and then used throughout the experiment. LTD was evoked using low frequency stimulation (LFS) that consisted of 900 stimuli at 1 Hz, with the test stimulus occurring after 15 min of baseline recording. Following the delivery of LFS, the test stimulus was repeated every 30 sec for up to 60 min. Data recording was restricted to 1 h following LFS application to minimize the potential influences of urethane anesthesia on the stability of field potential that might occur with prolonged anesthesia. LTD magnitude was defined as average fEPSP slope (synaptic component) and average PS amplitude (somatic component) measured at 55–60 min post-LFS.

4.4. Preparation of tissue homogenates

Animals were euthanized 1 day after the last injection. While under anesthesia (5% isoflurane), animals were exsanguinated by cardiac puncture and within 15 min the brain was removed from the skull. The brain was sectioned midsagitally, with the entire left and right hemispheres. After dissection, the right hippocampus was weighed and homogenized separately in phosphate-buffered saline (PBS: KH_2PO_4; pH: 7.0) with a tissue homogenizer (WiseTis Homogenizer – HG-15D, China). The tissue homogenate was centrifuged at 12,000 × *g* for 15 min in cold centrifuge at +4 °C. After centrifugation, the supernatants were removed and used to measure indices and biochemical markers of oxidative status. Protein content in tissue homogenate was measured using the bicinchoninic acid (BCA; Thermo Scientific, USA) method (Smith et al., 1985).

4.5. Antioxidant defenses and lipid peroxidation in the hippocampus

Catalase activity (CAT) was assessed as described in the manufacturer's instructions (Catalase Human ELISA Kit – Catalog Number: ab123456 – Abcam, UK). Briefly, after incubating of 50 μL homogenates with 50 μL catalase detector antibodies, 50 μL HRP label, and 100 μL TMB containing H_2O_2, optical density were determined spectrophotometrically at 600 nm.

Hippocampal SOD activity was determined by the method developed by Sun et al. (Sun et al., 1988). The principle of the method is based, briefly, on the inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine/xanthine oxidase system as a superoxide

generator. Activity was assessed in the ethanol phase of the supernatant after 1.0 mL of ethanol/chloroform mixture (5/3, v/v) was added to the same volume of sample and centrifuged. One unit of SOD was defined as the enzyme amount causing a 50% inhibition in the NBT reduction rate. Activity was expressed as units per milligram protein.

A GPx activity assay was performed as described in the manufacturer's instructions (Glutathione Peroxidase Assay Kit; Millipore, Cat no: 353919). Briefly, the assay was initiated by the addition of the GPx substrate cumene hydroperoxide, and the loss of absorbance at 340 nm (corresponding to the oxidation NADPH to $NADP^+$) was measured each minute for 5 min. The GPx rate was determined to be 1.0 nmol of NADPH oxidized per min at 25 °C (milliunit) per mL.

The MDA assay in hippocampal tissue was performed with the method developed by Ohkawa et al. (1979). MDA levels were measured in the samples on a UV–VIS Recording Spectrophotometer (SmartSpec 3000, Bio-Rad, USA). After the reaction of MDA with thiobarbituric acid, the reaction product was spectrophotometrically analyzed at 532 nm using tetrametoxypropane as a standard. The results are expressed as nmol/ml.

4.6. Indirect measurement of nitric oxide production

Direct measurement of NO in vivo is difficult because of the short half-life of the gas. Nitric oxide is synthesized by nitric oxide synthase (NOS), and once formed, it is rapidly oxidized to nitrite (NO_{2-}) and nitrate (NO_{3-}), which are considered inert end products of NO metabolism (NOx). Changes in the levels of these inactive compounds have been successfully demonstrated in biological fluids and tissue. The quantification of NO_{2-} and NO_{3-} is a useful means of indirectly estimating endogenously produced NO (Tuzgen et al., 2003). Therefore, nitrite, nitrate, and NOS were measured using commercially available ELISA kits (Total Nitric Oxide and Nitrate/Nitrite Assay – Catalog Number: KGE001/SKGE001/PKGE001, USA and Nitric Oxide Synthase 1 [NOS1] Rat SimpleStep ELISA™ Kit – Catalog Number: ab196266 – Abcam, UK).

To measure of tNOx levels, briefly, 50 µL of each sample was incubated for 30 min at 37 °C in a 50 µL reaction diluents containing detergent, 25 µL of NADH, and 25 µL nitrate reductase. The reaction was initiated by addition of the nitrate reductase to convert nitrate to nitrite. The reaction was terminated by the addition of 50 µL of Griess reagent I and 50 µL of Griess reagent II. After each mixture was incubated for 10 min at room temperature, optical density were determined spectrophotometrically at 540 nm.

Hippocampal homogenates were lysed using the cell extraction buffer, provided by the NOS1 Rat SimpleStep ELISA® kit (ab196266, Abcam), containing phosphatase inhibitors and protease inhibitor aprotinin for 20 min at 4 °C. The homogenate was then centrifuged for 20 min at 18,000 × g at 4 °C and the supernatant was collected. The levels of nNOS were determined according to the manufacturer's instructions. Unknown concentrations were determined from standard equivalents using NOS1 rat lyophilized recombinant protein, corrected for corresponding protein concentrations, and presented as ng per mg protein.

5. Statistics

Two-factor analysis of variance (ANOVA) was carried out by using SPSS, and the data were analyzed to determine the significance of the main effects, i.e., age and L-thyroxine administration, as well as their interactions. Tukey's multiple comparison post hoc test was performed to determine significance levels. The results are reported as the mean ± SEM with the level of significance set at $P < 0.05$.

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