Physiology and Pharmacology

Physiol Pharmacol 20 (2016) 98-107 **www.phypha.ir/ppj**

Original Article

Minocycline did not prevent the neurotoxic effects of amyloid β on intrinsic electrophysiological properties of hippocampal CA1 pyramidal neurons in a rat model of Alzheimer's disease

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Abstract

Introduction: Although aging is the most important risk factor for Alzheimer's disease (AD), there is evidence indicating that neuroinflammation may contribute to the development and progression of the disease. Several studies indicated that minocycline may exert neuroprotective effects in rodent models of neurodegenerative diseases. Nevertheless, there are also other studies implying that minocycline has no positive beneficial effects. Thus, the aim of the present study was to assess the preventive effect of minocycline against Aβ-induced changes in intrinsic electrophysiological properties in a rat model of AD.

Methods: The present study extended this line of research by examining whether inhibition of microglial activation may alter the intrinsic electrophysiological properties of CA1 pyramidal neurons in a rat model of Aβ neurotoxicity, using whole cell patch clamp.

Results: Findings showed that bilateral injection of the Aβ (1-42) into the prefrontal cortex caused membrane hyperpolarization, action potential (AP) narrowing and after hyperpolarization (AHP) amplitude enhancement. It was also resulted in a faster decay time of AP, higher rheobase current, lower firing frequency and smaller post stimulus AHP amplitude. Administration of minocycline (45mg/kg, i.p) not only failed to prevent Aβ-induced alterations in the intrinsic electrophysiological properties, but also enhanced the effects of Aβ on neuronal firing behavior.

Conclusion: It can be concluded that minocycline, as a microglial inhibitor, may enhance the disruption of electrophysiological properties of CA1 pyramidal neurons induced by Aβ neurotoxin, including AP parameters and intrinsic neuronal excitability.

Keywords:
Amyloid Beta (Aβ);
Neurotoxicity;
Minocycline;
Microglial Cells;
CA1 Pyramidal Neurons;
Intrinsic properties

Received: 27 Mar 2016
Accepted: 1 Jul 2016

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Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease which is characterized by pathological changes including neuroinflammatory processes through activation of microglia and astrocytes (Akiyama et al., 2000; Kauppinen et al., 2011). Several studies have shown the relationship between microglial-mediated inflammation and the progression of AD (McGeer and McGeer, 2002; Mandrekar-Colucci and Landreth, 2010; Cameron and Landreth, 2010). It has also been reported that microglia-mediated neuroinflammation is one of the major consequences of Aβ deposition in the brain regions contributed to cognitive function (Puli et al., 2012) and using the anti-inflammatory medicine could be useful to reduce the risk of disease development (Wyss-Coray and Rogers, 2012). Akiyama and colleagues (2000) found that neuroinflammation may facilitate the development of Aβ pathology (Akiyama et al., 2000). In addition, data from epidemiological studies suggest that anti-inflammatory agents may reduce AD incidence (Szekely and Zandi, 2010), although this is not congruent with the result of a randomized controlled trial done by Martin and colleagues (2008) that found that non-steroidal anti-inflammatory therapy was not effective on cognitive decline in AD. There are also many animal studies showing that minocycline, a known microglial activation inhibitor, not only has no beneficial neuroprotective effect, but also may exacerbated neurotoxicity (Yang et al., 2003). On the other hand, activation of microglia that is involved in the clearance of the extracellular Aβ (Morgan, 2009) around amyloid deposits has been implicated as a neuroprotective response (Puli et al., 2012; Magnus et al., 2002). Overall, although there is compelling evidence which show that glial cells contribute to AD, little, if any, information is known about the impact of inhibition of microglial activation on cellular electrophysiological alterations induced by Aβ in a rat model of AD.

In this context, the results of genetic, cellular and molecular studies have provided strong support for the involvement of glial cells activation and inflammatory processes in amyloid beta pathology in AD (Akiyama et al., 2000; Wyss-Coray and Mucke, 2002; Wyss-Coray, 2006) and phagocytosis of Aβ by microglial has been considered as a possible neuroprotective response (Wyss-Coray, 2006; Song et al., 2012). In recent years growing evidence indicate that it may be beneficial to take anti-inflammatory drugs, including minocycline to prevent or slow progression of AD (Schwartz and Shechter, 2010; Gilgun-Sherki et al., 2006; Choi et al., 2007). There are, however, controversial reports that glial inhibition may not be a neuroprotective strategy for AD (Solito and Sastre, 2012; Wisniewski et al., 1991; Streit, 2004).

There are also promising evidence indicating beneficial neuroprotective effects of minocycline in animal models of multiple sclerosis (Popovic et al., 2002), Parkinson disease (Wu et al., 2002), Huntington disease (Wang et al., 2003), brain stroke (Yang et al., 2015; Chamorro et al., 2016) and spinal cord injury (Ahmad et al., 2016). However, it has been suggested that minocycline should be given a second chance (Gámez, 2008).

Recently, in a meta- analysis, the effectiveness of minocycline on neurodegenerative diseases in rodents was studied (Li et al., 2012). It was found that moderate doses such as 45 or 50 mg/kg/day had specifically inhibitory effect on Aβ accumulation and inflammation and a suppressive effect on cognitive impairments. In the above mentioned meta-analysis it was also reported that moderate (45mg/kg/day), but not high-dosage (90 mg/kg/day) minocycline was very beneficial in the treatment of Parkinson disease. The meta-analysis conducted by Li et al. (2012) has also provided evidence indicating that minocycline has a small therapeutic window in Huntington's disease and stroke.

In the light of these observations, it seems that glial cells activation has yielded controversial and mixed results. Therefore, in the present account, it was attempted to determine whether inhibition of microglial cells may alter the intrinsic electrophysiological changes caused by Aβ neurotoxicity in rat hippocampal CA1 pyramidal neurons, using whole cell patch clamp recordings under current clamp condition.

Materials and methods

All experiments were performed on young adult Wistar rats weighting 80-100g which were housed in pairs in plastic cages and maintained on 12-hour light and 12-hour dark and constant room temperature

(21-23^{o}C) with food and water ad libitum. In the present study a rat model of AD was induced by bilateral injection of Aβ (1-42, Sigma, UK) peptide fragment into the prefrontal cortex as previously described (Haghani et al., 2012a and b). Aβ (1–42) was dissolved in sterile normal saline to the concentration of 10 ng/µl and the peptide was still perfectly soluble even in the sham group, which received equivalent volume of normal saline without Aβ. Rats were divided into five groups (5 rats in each group; at least 3 cells per rat): normal control (n=23 cells), vehicle (sham/vehicle, n=15 cells), Aβ-treated alone, Aβ plus minocycline (45mg/kg per day for 12 consecutive days, n=25 cells) and minocycline-treated alone groups (n=23 cells). Briefly, animals were anaesthetised by intraperitoneal injection of a mixture of ketamine (80 mg/kg) and xylazine (20 mg/kg), and rats were placed in a stereotaxic frame. The rats were then bilaterally received injection of Aβ (3µl /side at a rate of 0.5µl/min) into the prefrontal cortex with a 5µl Hamilton syringe. The injection coordinates were as follows: 3.2 mm AP, 2 mm ML relative to the bregma; depth 3 mm. Minocycline (45mg/kg) was also dissolved in normal saline and was intraperitoneally administered six hours after injection of Aβ and was repeated for 12 days (Mengzhou et al., 2010). The dose of 45mg/kg was selected because it has been proven to afford neuroprotection in several studies on rats (Diguet et al, 2004; Li et al., 2012) and with using this dosage the concentration of minocycline in the 10 µl of blood has been reported to be 10 µg/ml (Haghani et al., 2012b). Then, on the 12th day after drug injection, electrophysiological recordings were performed. Analysis indicated no significant difference between values from control and sham/vehicle groups, thus results were pooled and considered as one control group. All experimental procedures were approved by the Ethic Committee of Animal Use for Research of Shahid Beheshti University of Medical Sciences, and attempts were made to minimize to number of rats used and their suffering.

Whole cell patch clamp recording

The functional consequences of microglial inhibition on the electrophysiological properties of hippocampal CA1 pyramidal neurons in control rats and in rats received either intrafrontal injection of Aβ (1-42) peptide alone or Aβ + minocycline were studied using whole-cell patch clamp recordings under current clamp condition. Briefly, 12 days after treatment with Aβ alone or Aβ plus minocycline, the animals were anesthetized with ether and decapitated with a guillotine. Brains were quickly removed and transverse hippocampal slices (300 µm) were cut by using a vibroslicer (752 M, Campden Instruments, Lough-borough, U.K) in ice-cold slice preparation solution containing (in mM): 206 sucrose, 2.8 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 2 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 D-glucose and bubbled with carbogen gas (95% O_2 / 5% CO_2, pH 7.4); measured osmolarity 295 mOsm. Then, the hippocampal slices were transferred and incubated for at least 1 hour at 35^{o}C before recording in oxygenated ACSF that consisted of (in mM): 124 NaCl, 2.8 KCl, 2 $CaCl_2$, 2 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 D-glucose (pH 7.4, and 295 mOsm).

Hippocampal slices were then transferred into a recording chamber on an upright microscope (BX51W1, Olympus, JP) equipped with infrared differential interference contrast (DIC) optics and X60 water-immersion objective and pyramidal cell bodies in the CA1 area were identified and patched at room temperature (23-25°C). The patch pipette (3-5MΩ) were filled with internal solution consisted of (in mM): 135 potassium methylsulfate (KMeSO4), 10 KCl, 10 Hepes, 1MgCl2, 2 Na2ATP, and 0.4 Na2GTP, pH 7.3 with KOH, and osmolarity was adjusted to 290 mOsm. In the presence of fast synaptic blockers (1 mM *kynurenic* acid and *100µM picrotoxin),* somatic action potential recordings were obtained in current clamp mode using a MultiClamp 700B amplifier (Axon instrument), filtered at 10 kHz and digitized at 20 kHz and collected using a DigiData 1322A 16 bit A/D converter (Axon instruments) on a Pentium 4-based personal computer (Haghani et al., 2012a and b). Current-clamp protocols were controlled using the pClamp 9 software and the following parameters were analysed offline to measure excitability: resting membrane potential, membrane input resistance, AP half-width, AHP amplitude, rheobase (threshold current), AP decay time, and instantaneous firing frequency, post stimulus AHP amplitude.

Input resistance was calculated as the slope of the current–voltage plot. AP half-width was measured as the AP width at the half-maximal voltage. The rheobase was measured as the amount of depolarizing ramp current (0.43pA/ms, 800ms from

0pA to 200pA) at the firing threshold. The action potential decay time defined as the duration between peak amplitude and threshold during repolarization. The instantaneous firing frequency was calculated as the reciprocal of the interspike interval (ISI) for the first and second spikes in response to a depolarizing current pulse. Post stimulus AHP amplitude was measured after a 660ms depolarizing current steps of 100pA and 500pA as the maximum hyperpolarizing voltage deflection relative to the resting potential. The soma of CA1 pyramidal neurons were reliably and visually identified using DIC optic or electrophysiological criteria including regular spiking behaviour in response to direct somatic depolarizing current injection and a relatively long action potential latency of onset.

Statistical analysis

All data were expressed as means ± SEM and were analysed using one-way ANOVA followed by Tukey's HSD post hoc test. Differences were considered statistically significant when $p<0.05$.

Results

Twelve days after bilateral microinjection of Aβ into the prefrontal cortex, the following electrophysiological changes were found in pyramidal neurons when compared to the control condition: the cell's membrane potential was significantly shifted to more negative levels (Fig. 1A, $P<0.01$), the duration of action potential was significantly shortened (Fig. 1 B, $P<0.01$), the AHP amplitude was significantly increased (Fig. 1C, $P<0.0001$) and the action potential decay time was significantly reduced (Fig. 1D, $P<0.001$). Intra-prefrontal injection of Aβ was also associated with a significant increase in the rheobase current (Figs. 2A&B, $P<0.001$) and a significant decrease in the firing frequency (Fig. 2C, $P<0.01$). The number of evoked APs in response to somatic

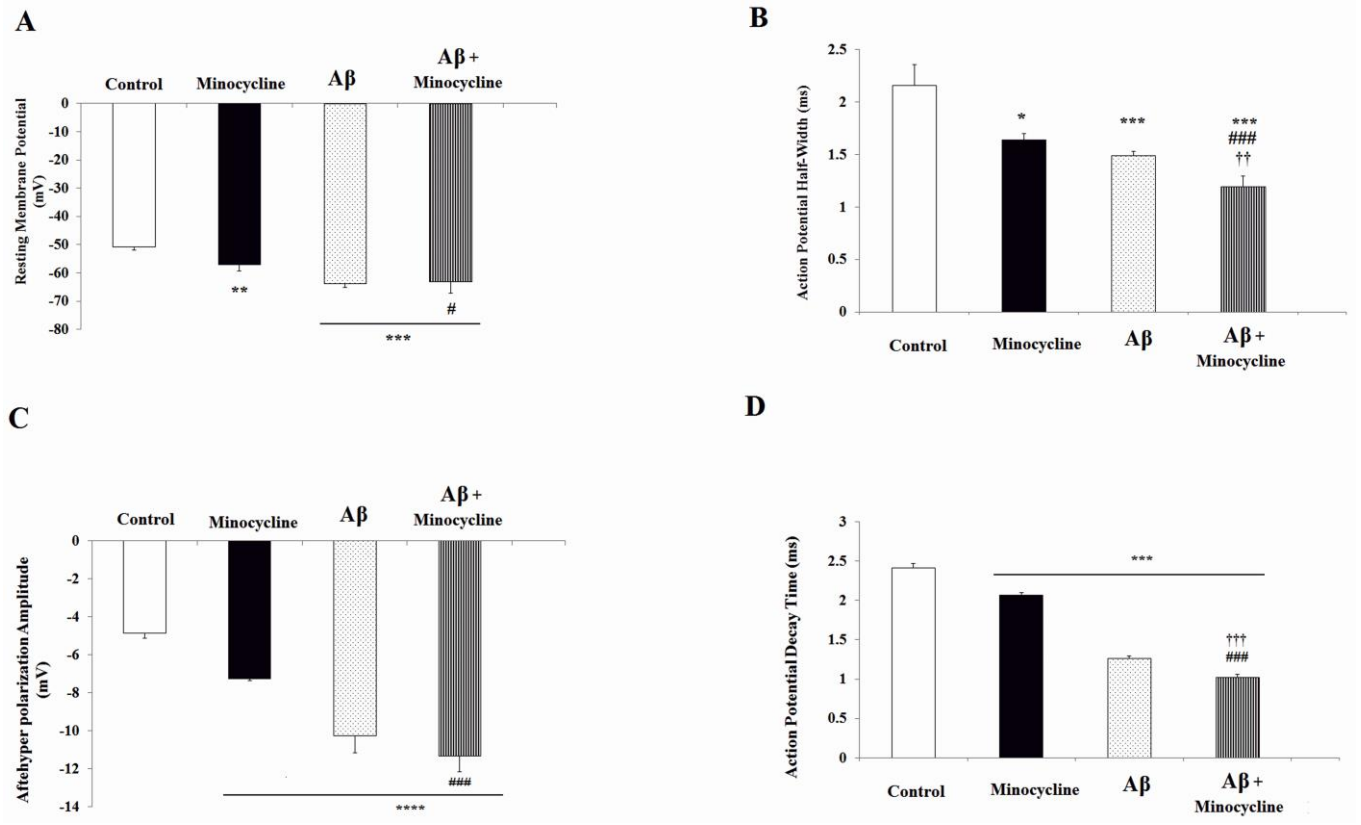

Fig.1. Effects of intrafrontal injection of Aβ and Aβ plus intraperitoneal injection of minocycline on resting membrane potential and action potential parameters. Histograms represent the mean values of resting membrane potential (A), action potential half width (B), AHP amplitude (C) and action potential decay time (D) in control, following intraperitoneal administration of minocycline (45 mg/kg) alone and after i.p. administration of minocycline pulse intra-medial frontal injection of Aβ. Minocycline treatment significantly affected the action potential characteristics both when applied alone or along with Aβ. Comparisons were made by one way ANOVA; *p<0.05, **p<0.01 and ***p<0.001 compared to control, ††p<0.01 and †††p<0.001 compared to Aβ, #p<0.05 and ###p<0.001 compared to Minocycline group.

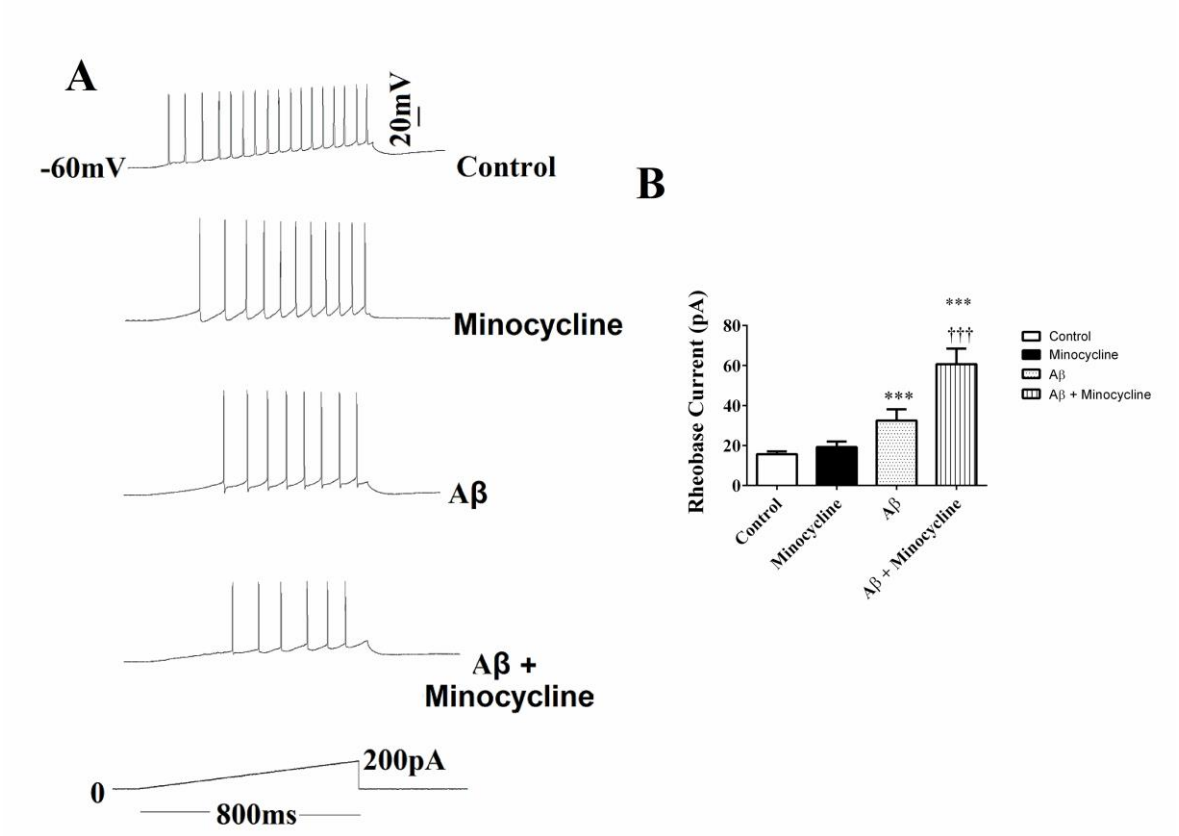

Fig.2. Alterations in the intrinsic active properties of CA1 pyramidal neurons following Aβ (1-42) injection into the frontal cortex and after treatment with Aβ plus minocycline

(A) Representative voltage traces showing response to a depolarizing ramp current injection (200pA, 0.43pA/ms, 800ms) in different experimental groups. Average histograms of rheobase current (B) and instantaneous firing frequency (C) for different experimental groups. **p<0.01 and ***p<0.001 compared to control, †††p<0.001 compared to Aβ, #p<0.05 compared to Minocycline group.

depolarizing current pulses (100pA and 500pA) were significantly lower in CA1 pyramidal neurons from Aβ treated rats compared to control cells (Figs. 3A-C). In addition, the amplitude of the post-burst AHP was significantly smaller in neurons from Aβ-treated rats compared to neurons from control rats (Fig. 3D).

Electrophysiological consequences of the inhibition of microglial function in rats CA1 pyramidal neurons following injection of Aβ neurotoxin into the medial prefrontal cortices

Administration of minocycline (45mg/kg, i.p.) six hours after injection of Aβ and continued for 12 days, did not cause further change in the membrane potential (Fig. 1A) when compared to control animals, but led to more significant decrease in the duration of AP (Fig. 1 B), an insignificant increase in the AHP amplitude (Fig. 1C) and a further significant increase in the decay time of AP (Fig. 1D, P<0.001).

In addition, minocycline treatment plus intrafrontal Aβ injection produced a significant increase in the rheobase current (P<0.001) when compared to both control and Aβ-treated alone groups (Figs. 2A&2B), but did not significantly affect the firing frequency (Fig. 2C).

In rats given injection of minocycline alone (45mg/kg, i.p.) for 12 days, CA1 pyramidal neurons exhibited more hyperpolarized membrane potential when compared to control neurons, but it was significantly depolarized than Aβ+minocycline treated groups (Fig.1 A). Its application also led to a significant reduction in AP duration (Fig. 1B, P<0.05), a significant increase in AHP amplitude (Fig. 1 C, P<0.001) and a significant decrease in both AP decay time (Fig. 1D, P<0.001) and firing frequency (Fig. 2C, P<0.001), but insignificantly increased the rheobase current (Fig. 2B) when compared to control condition. Moreover, the number of APs elicited in response to

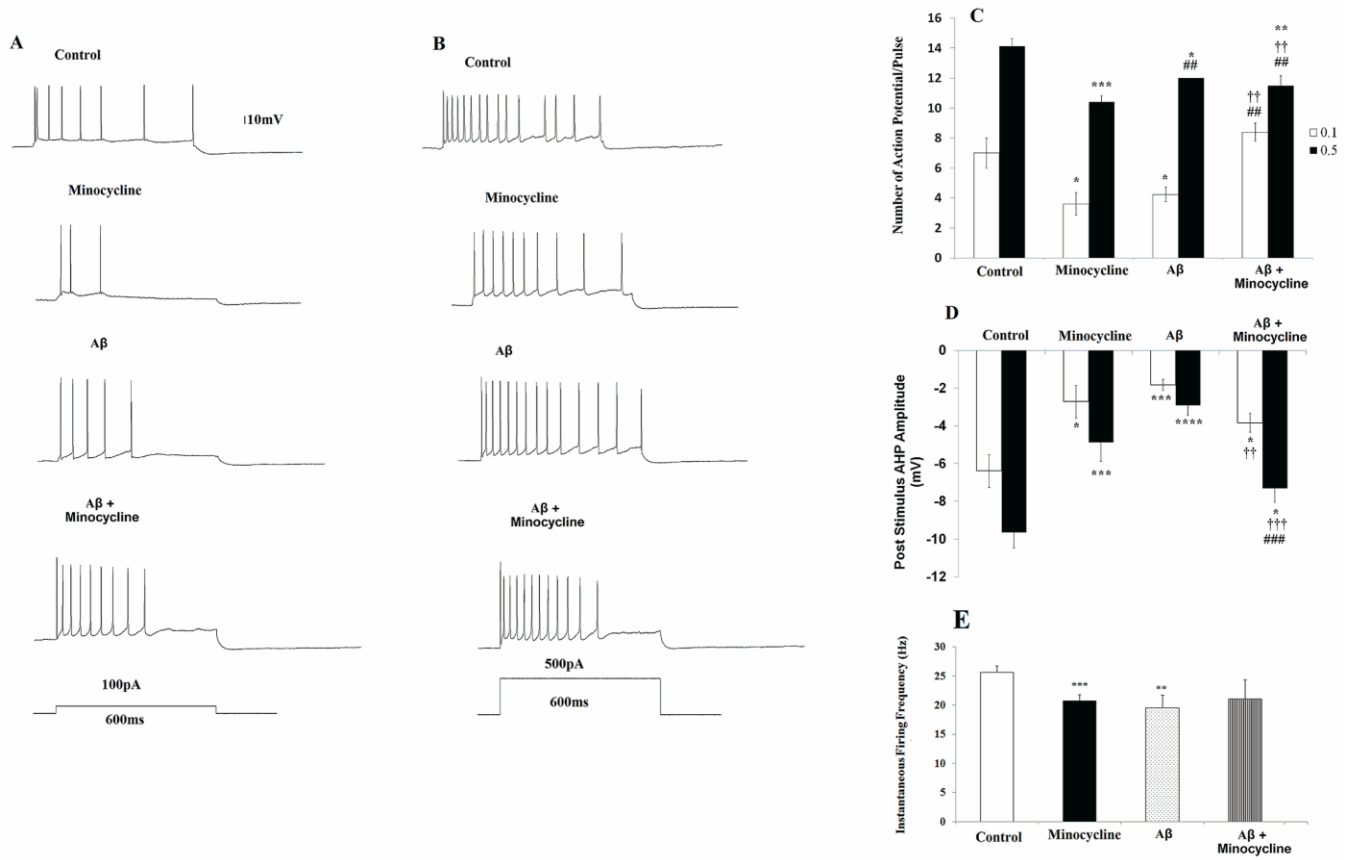

Fig.3. Effect of inhibition of microglial activation on changes in evoked action potential and post stimulus AHP amplitude induced by Aβ (1-42).

(A) Representative voltage responses to depolarizing current pulses (100 and 500pA) applied to CA1 pyramidal neurons in different groups of rats. Comparing the number of evoked action potentials (B) and the amplitude of post stimulus AHP (C) in response to depolarizing current pulses (100pA and 500pA, 660ms) in hippocampal CA1 pyramidal neurons among control and treated rats.

current injection of 100pA, but not 500pA, significantly increased in the group receiving Aβ and minocycline, as compared with the group treated Aβ alone ($p<0.001$; Figs. 3A&B). However, pyramidal neurons from Aβ plus minocycline treated rats showed a lower and higher number of APs per pulse when compared with control and minocycline-treated alone rats, respectively (Figs. 3A&B). Minocycline treatment plus Aβ was also caused a significant increase in the post AHP amplitude compared to either Aβ or minocycline treatment alone, but not control group (Fig. 3C).

Discussion

Intrafronatl injection of Aβ has been established as an animal model of AD (Haghani et al., 2012a and b; Van der Stelt et al., 2006; Eslamizade et al., 2015). There are several lines of evidence indicating direct and indirect interplay between frontal cortex and hippocampus (Thierry et al., 2000; Saint Marie et al., 2010). Intra-frontal injection of Aβ has been shown to induce neuronal loss (Eslamizade et al., 2015; Gonzalo-Ruiz et al., 2005; Gonzalo-Ruiz et al., 2006; Sun et al., 2013), this in turn may affect the function of pyramidal neurons. Intra-frontal injection of Aβ (1-42) after 12 days has been also reported to cause neuronal damage in the CA1 area that was far from the injection site (Van der Stelt et al., 2006; Eslamizade et al., 2015). In addition, it has been shown that that amyloidosis facet of AD is usually initiated from frontal cortices and then progresses neuron-to-neuron toward other regions including entorhinal cortex and the hippocampus (Harris et al., 2010; Nath et al., 2012). Hence, here Aβ (1-42) peptide was bilaterally injected into the deep frontal cortex.

In our previous study we demonstrated that bilateral

injection of Aβ (1-42) into the medial prefrontal cortex impaired hippocampal dependent learning and memory and altered the neuronal excitability (Haghani et al., 2012a and b). The findings of the present study indicate that minocycline, a microglial inhibitor, may enhance the neurotoxic effects of Aβ on the action potential shape and firing properties of CA1 pyramidal neurons. Bilateral injection of Aβ into the medial prefrontal cortex resulted in membrane hyperpolarization, decrease in the AP half-duration, AP decay time and instantaneous firing frequency, but it caused a significant increase in the AHP amplitude, AP rheobase current and post train AHP amplitude. The extent of changes in AP characteristics and firing activity were even significantly greater when minocycline was intraperitoneally administered for 12 days started 6 hours after Aβ injection than those observed in Aβ-treated alone. Therefore, it can be postulated that minocycline, which has been reported to be a microglial inhibitor, by either suppression of microglial activation or directly acting on ion channels may worsen the neurotoxic effects of Aβ on the firing properties of CA1 pyramidal neurons. Hickman and colleagues (2008) have provided evidence suggesting that microglia may play a role of double-edged sword in neuroinflammation induced by Aβ neurotoxicity. On the one hand, early their recruitment and accumulation in the site of Aβ deposition causes clearance of these neurotoxic peptides by generating anti- Aβ antibodies (Cai et al., 2014) and thereby delays Alzheimer's disease progression (Hickman et al., 2008). On the other hand, in aged animal model old AD, microglia become dysfunction and they are unable to degradate Aβ, but they are still able to produce proinflammatory cytokines which promote Aβ production and/or reduce Aβ clearance (Cai et al., 2014). Therefore, it can be postulated here that suppression of microglia activation by minocycline prevents Aβ clearance and thereby may enhance neurotoxic effects of Aβ on CA1 pyramidal neurons. Chronic elevation of Aβ In consistent with this assumption, some studies have shown a neuroprotective role for microglial activation against Aβ toxicity (Monsonego and Weiner, 2003; Puli et al., 2012). However, in contrast to this, others have shown that inhibition of microglial may have neuroprotective action against AD (Kim and Suh, 2009; Garwood et al., 2010; Parachikova et al., 2010). It has also been shown that minocycline, a microglial inhibitor, not only has no beneficial neuroprotective effect, but also may exacerbated neurotoxicity (Diguet et al., 2004; Yang et al., 2003).

The main findings of the present study were significant changes in action potential parameters and reduction of intrinsic excitability both in Aβ and Aβ+minocycline treated groups compared to control rats. These changes could be due to alterations in ion channels function. González and colleagues (2007) found that in vitro application of 100μM minocycline by blocking voltage-gated Na^+ channels caused a decreased neuronal excitability in cultured dorsal root ganglion neurons. Consistent with this, in the present work it was shown that neurons from Aβ and Aβ plus minocycline treated rats displayed a significantly higher rheobase current when compared with minocycline treated alone and control groups. This was associated with mitigation of neuronal excitability. The intrinsic neuronal excitability and input/output properties of neurons have been shown to be largely determined by distribution and function of voltage-dependent Na^+ channels (Scheff and Price, 2006). Therefore, one possible reason for the decreased excitability here observed in treatment groups could be the direct of minocycline on Na^+ channel. There is, however, another possible explanation to be considered. Inhibition of microglial function by in vivo treatment of rats received Aβ with minocycline, may cause neuronal hypoactivity through chronic elevation of Aβ (Plant et al., 2006) due to microglial dysfunction for amyloid clearance.

More recently, it has also been reported that minocycline suppresses the neuronal excitability in rat substantia gelatinosa neurons through inhibition of Ih channel current (Liu et al., 2015).

Furthermore, comparing the effect of either Aβ treatment alone or Aβ + minocycline with control group showed a significant increase in the AHP amplitude associated with a decrease in AP half width and decay time. The amplitude of post stimulus AHP was also increased in both treatment groups. One possible reason for these alterations could be that Aβ increases outward K^+ currents, which contribute to the AHP and to AP repolarization. Consistent with this explanation, several previous studies suggest that Aβ can alter the neuronal K^+ channels at both molecular and functional level.

Amyloid beta peptide has been shown to upregulate the expression of transient A-type K^+ channels in neuronal cells (Yu et al., 1998), which play a critical role in excitability and controlling repetitive firing. Furthermore, it has also been reported that Aβ enhances an outward current through delayed rectifier K^+ channels in cortical neurons and thereby induces neuronal death (Li et al., 2012).

On the light of the above discussion, it can be suggested that bilateral injection of Aβ into the medial prefrontal cortex possibly leads to a significant increase in the voltage-gated K^+ channel current and thereby decreases the firing frequency after 12 days of treatment; however, this needs to be further studied using voltage clamp technique in combination with pharmacological agents.

Another finding in the present investigation was that inhibition of microglial activation by intraperitoneal injection of minocycline before intracortical administration of Aβ not only did not prevent the effects of Aβ on neuronal firing properties, but also caused further significant increase in almost all electrophysiological parameters. In consistent with this finding, Li and colleagues reported that pretreatment of minocycline cannot protect the ventral cochlear nucleus neurons against bilirubin-induced hyperexcitation. It has also been shown that minocycline does not afford protection against the neurotoxicity caused by malonate or NMDA (Goñi-Allo et al., 2005).

Based on the present results, it can be concluded that minocycline, at least under our experimental conditions, could not exert neuroprotective effect against changes induced by Aβ neurotoxin in intrinsic electrophysiological characteristics.

Acknowledgments

This work was supported by Neuroscience Research Center and the international branch of Shahid Beheshti University of Medical Sciences.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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