

## NICOTINE AND ESTROGEN SYNERGISTICALLY EXACERBATE CEREBRAL ISCHEMIC INJURY

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**Abstract**—The greater incidence of myocardial infarction, cardiac arrest, and ischemic stroke among women who smoke and use oral contraception (OC) compared to women who do not smoke and who do or do not use OC may be due in part to how nicotine influences endocrine function in women. For example, we recently demonstrated that chronic exposure to nicotine, the addictive agent in tobacco smoke responsible for the elevated risk of cardiac arrest, abolishes the endogenous or exogenous 17 $\beta$ -estradiol-conferred protection of the hippocampus against global cerebral ischemia (a potential outcome of cardiac arrest) in naive or ovariectomized female rats. In the current study we examined the hypotheses that (1) a synergistic deleterious effect of nicotine plus oral contraceptives exacerbates post-ischemic hippocampal damage in female rats, and (2) nicotine directly inhibits estrogen-mediated intracellular signaling in the hippocampus. To test first hypothesis and to simulate smoking behavior-induced nicotine levels in the human body, we implanted osmotic pumps containing nicotine in the female rats for 16 days. Furthermore, we mimicked the use of oral contraceptives in females by administering oral contraceptives orally to the rat. Rats exposed to either nicotine alone or in combination with oral contraceptives were subjected to an episode of cerebral ischemia and the resultant brain damage was quantified. These results showed for the first time that nicotine with oral contraceptives did indeed exacerbate post-ischemic CA1 damage as compared to nicotine alone in naive female rats. In *ex vivo* hippocampal slice cultures, we found that nicotine alone or with 17 $\beta$ -estradiol directly hinders estrogen receptors-mediated phosphorylation of cyclic-AMP element binding protein, a process required for neuronal survival and also exacerbates ischemic damage. Thus, nicotine can affect the outcome of cerebral ischemia by influencing brain endocrine function directly rather than through indirect systemic effects. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

**Key words:** cardiac arrest, hippocampus, organotypic slice culture, oral contraceptives, estrogen receptor-beta.

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**Abbreviations:** Akt, serine/threonine protein kinase; CA, cardiac arrest; CREB, cyclic-AMP response element binding protein; ER- $\beta$ , estrogen receptor-beta; MAPK, mitogen-activated protein kinase; NOC, nicotine+oral contraceptives; OC, oral contraceptives; OGD, oxygen-glucose deprivation; pAkt, phosphorylated Akt.

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Smoking increases the incidence of ischemic stroke among women (Tanis et al., 2001; Goldstein et al., 2006; Girolami et al., 2008). The effect of smoking may be, in part, due to how nicotine, the addictive component of cigarette smoke (1988 Surgeon General's Report), influences endocrine function in women. In particular, nicotine exposure in women modulates estrogen metabolism, reduces circulating estrogen levels, disturbs the normal periodicity of the menstrual cycle and ultimately leads to early onset of menopause (Jensen et al., 1985; Cramer et al., 1995; Mueck and Seeger, 2005). Nicotine's impact on estrogen metabolism has potential far-reaching consequences for the cerebrovascular health of women, because endogenous circulating estrogen helps prevent cerebrovascular diseases in pre-menopausal women (Zhang et al., 1998; McCullough and Hurn, 2003). In particular, 17 $\beta$ -estradiol (E<sub>2</sub>; a potent estrogen) protects the brain from damage from cerebral ischemia (Alkayed et al., 2000; Jover et al., 2002; McCullough and Hurn, 2003; Raval et al., 2009b).

We recently demonstrated that chronic nicotine exposure overrides this protection of the hippocampus by endogenous or exogenous estrogen in naive or ovariectomized female rats exposed to cerebral ischemia (Raval et al., 2009a). However, we did not determine in that study how nicotine abolishes the beneficial effects of estrogen on the hippocampus. Secondly, while we looked at the contributions of 17 $\beta$ -estradiol to ischemic protection in the female rat hippocampus, we did not examine the possible role of progesterone, another important female sex hormone, in ischemic protection. Thirdly, we wanted to investigate the possibility that the components of oral contraceptives (17 $\beta$ -estradiol and progesterone) might exacerbate hippocampal damage following cerebral ischemia. This possible outcome is suggested by findings that young women on oral contraception (OC) who are smokers increase their risk for cardio- and cerebrovascular events by 30-fold as compared to non-smoking non-OC users (Tanis et al., 2001; Girolami et al., 2008). Thus, in the current study we examined the hypotheses that (1) chronic nicotine exposure and oral contraceptive work synergistically to exacerbate post-ischemic hippocampal damage in female rats, and (2) nicotine directly inhibits estrogen-mediated intracellular signaling in the hippocampus.

### EXPERIMENTAL PROCEDURES

All protocols were approved by the Animal Care and Use Committee of the University of Miami.

## In vivo study

Female Sprague-Dawley rats weighing  $290 \pm 20$  g were used for this study. To monitor stages of the estrous cycle, we collected vaginal smears from rats daily between 9:00 and 10:00 AM and identified cell types microscopically as described previously (Raval et al., 2009b). Only rats showing at least three consecutive normal (4 day) estrous cycles were used for experiments. Since we demonstrated earlier that the higher endogenous estrogen levels seen during proestrus protect CA1 neurons against cerebral ischemia (Raval et al., 2009b), we exposed female rats in proestrus to cerebral ischemia in the experiments below. In those instances where rats were not in the proestrus stage on the last scheduled day of treatment, we extended the treatment by 1 day:

**Group 1: Sham.** Rats were exposed to a sham surgical procedure.

**Group 2: Saline.** Osmotic pumps containing normal saline were implanted into rats for 16 days. Briefly, rats were anesthetized (isoflurane), an area between the scapulas shaved and an incision (1 cm) made to permit s.c. implantation of an osmotic pump (type 2ML2, Alzet Corp., Palo Alto, CA, USA). The incision was sutured and the rats were placed in cages for recovery.

**Group 3: Nicotine.** Osmotic pumps containing nicotine hydrogen tartrate dissolved in normal saline were implanted in rats for 16 days. The pumps delivered a dose of 4.5 mg/kg/d of nicotine hydrogen tartrate. Previous studies have shown that this method maintains nicotine and cotinine plasma levels comparable to those observed in chronic heavy smokers (Murrin et al., 1987; Wang et al., 1997). Habitual smokers regulate their smoking behavior to sustain a constant level of nicotine in their blood (Jarvik et al., 2000), so a paradigm of continuous nicotine delivery via an osmotic pump will mimic this aspect. Also, rats trained to self-administer nicotine sustain plasma nicotine and cotinine levels in this range (Shoaib and Stolerman, 1999). We used an ELISA kit (Calbiotech, Spring Valley, CA, USA) to measure plasma cotinine-nicotine metabolites so that we could determine the efficacy of our chronic nicotine delivery method over the 16 day period. Blood was collected from a femoral artery on the last day of treatment.

**Group 4: Oral contraceptive (OC).** Rats were treated with an OC for 16 days (approximately four estrous cycles). The dose was scaled to mimic a woman's daily OC dose based on a diet of 1800 cal/d. Since rats need an average of approximately 32 cal/100 g of body weight/day, a  $290 \pm 20$  g rat needed an OC dose based on an intake of 96 cal/d. The rats were given OC by an oral gavage for three consecutive days and placebo (placebo tablet given along with OC pills; administered orally) on the fourth day based on the 4-day estrous cycle of rat, and to resemble OC administration in women (Eleftheriades et al., 2005).

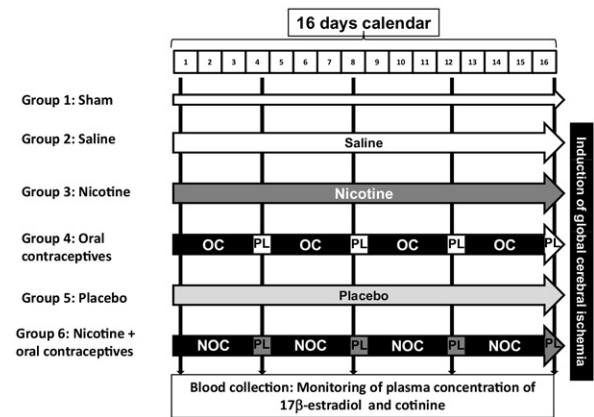
**Group 5: Placebo.** Rats were treated with placebo for 16 days.

**Group 6: Nicotine+OC (NOC).** Rats were treated with nicotine and OC for 16 days.

On the 16th day, at the end of the above treatments rats were exposed to the cerebral ischemia (Fig. 1).

## 17 $\beta$ -estradiol measurement

To study the effects of nicotine or OC on circulating estradiol, we measured the plasma concentration of estradiol with a radioimmunoassay that used a "Coat-a-Count" kit (Diagnostic Products Corporation, CA, USA). We collected blood (femoral artery) every fourth day (on the day of the placebo treatment for the OC-group; Fig. 1) during the treatment period of 16 days.



**Fig. 1.** A schematic showing 16 days calendar (number in boxes represent days) indicating saline; nicotine; OC; placebo (boxed label) and combination of nicotine plus OC. Vertical arrows indicate days of blood collection for plasma  $E_2$  monitoring. At the end of treatment rats belong to all the groups except group 1 was exposed to cerebral ischemia. Group 1 rats were exposed to sham surgical procedure without actual ischemic episode.

## Production of cerebral ischemia

Rats were anesthetized with 4% isoflurane and a 30:70 mixture of  $O_2$  and nitrous oxide, and were then intubated endotracheally. Isoflurane was subsequently lowered to 1.5–2% for endovascular access. A femoral artery and vein were cannulated for blood pressure monitoring and blood gas analysis (PE-50 polyethylene catheter, BD Biosciences, Bedford, MA, USA). Pancuronium (2 mg/kg) was injected i.v. into the rat, and the rat was mechanically ventilated (60 breaths/min) with a gas mixture containing 0.5–1.0% isoflurane. Physiological parameters including  $pCO_2$ ,  $pO_2$ , pH,  $HCO_3^-$ , and arterial blood pressure were maintained within normal limits. A rectal temperature probe (YSI, OH, USA) was gently inserted into the rectum of the rat to monitor its body temperature. A thermocouple 33-gauge needle temperature probe (Omega, Stamford, CT, USA) was implanted into the temporalis muscle of the rat to monitor its head temperature. Body and head temperatures were maintained at  $37^\circ C \pm 0.2$  throughout the experiment with assistance of lamps placed above the animal's body and head. Before each ischemic insult, blood was gradually withdrawn into a heparinized syringe to reduce the systemic blood pressure to 48–50 mmHg. Cerebral ischemia was then produced by tightening carotid ligatures bilaterally and maintaining the mean arterial blood pressure (MABP) at 48–50 mmHg for a period of 10 min. To allow post-ischemic reperfusion, we removed the carotid ligatures, and the shed blood was reinjected to restore the MABP to 100–120 mmHg. The vessels were visually inspected to verify establishment of reperfusion.

## Histopathology

At the end of 7 days of reperfusion, rats were anesthetized with isoflurane and perfused with formalin: acetic acid and methanol (FAM) as described (Raval et al., 2009b). The head was removed and immersed in FAM at  $4^\circ C$  for 1 day. The brains were then removed from the skull, and coronal brain blocks were embedded in paraffin; 10- $\mu m$  coronal sections were stained with hematoxylin and Eosin. Hematoxylin and Eosin stained sections were visualized at  $40\times$  magnification under a Nikon microscope equipped with a Sony CCD camera coupled to an MCID image analyzer (Imaging Research, St. Catherines, Ontario, Canada). For each animal, normal neurons were counted in the CA1 region of each hippocampus by an investigator

blinded to the experimental conditions. Coronal brain sections were made at the level of 3.8 mm from bregma. For each section, 18 fields per sections were obtained, and three slides per rat were counted (Raval et al., 2009a). The data are presented as the mean count from three slides.

### Ex vivo study

Organotypic hippocampal slice cultures were prepared as described previously (Raval et al., 2003). Briefly, Sprague-Dawley female rats (9–11 days old) were anesthetized by i.p. injection of ketamine (1.0 mg). Animals were decapitated and the brains quickly removed. The transverse slices (400  $\mu$ m) were dissected from the hippocampi and placed in Gey's Balanced Salt Solution (Sigma) supplemented with 6.5 mg/ml glucose at 4 °C. After 1 h, two slices were placed onto one 30 mm diameter membrane insert (Millicell-CM, Millipore) and inserts were transferred to six-well culture plates with 1 ml of culture medium per well (Raval et al., 2003). The slice cultures were incubated (equilibrated at 36 °C, 95% O<sub>2</sub>, 5% CO<sub>2</sub>, humidity 100%) for 14–15 days before being placed into one of the following experimental groups:

**Group 7: Nicotine.** Slices were exposed to nicotine (100 ng/ml) or saline-vehicle for 15 days. The dose of nicotine was chosen based on previous human and rat studies (Henningfield et al., 1993; Abbruscato et al., 2004).

**Group 8: 17 $\beta$ -estradiol.** Slices were exposed to estradiol-17 $\beta$  (1 nM; 4 h). The slices were then exposed to oxygen-glucose deprivation (OGD) 48 h after estrogen treatment. This concentration and duration of estrogen (4 h), was neuroprotective in our previous study (Raval et al., 2006). In some experiments, slices were exposed to vehicle (oil) only as a control.

**Group 9: 17 $\beta$ -estradiol+progesterone (E<sub>2</sub>+P).** Slices were exposed to 17 $\beta$ -estradiol plus progesterone (1 nM) treatment as described in Group 8.

**Group 10: Nicotine+ovarian hormones (N+E<sub>2</sub>/N+E<sub>2</sub>+P).** Slices were exposed to nicotine (described in Group 7). On 13th day of nicotine exposure, slices were transiently exposed to 17 $\beta$ -estradiol/17 $\beta$ -estradiol+progesterone (described in Group 8). The slices were then exposed to OGD 48 h after estrogen treatment.

**Group 11: Estrogen receptors (ERs) inhibitor.** Slices were exposed to the ER inhibitor ICI 182780 (1  $\mu$ M) or vehicle (DMSO) for 24 h prior to OGD.

### Induction of ischemia (oxygen glucose deprivation—OGD)

As described previously, slices were washed three times with aglycemic Hank's Balanced Salt Solution (Raval et al., 2003). The slice cultures were then transferred into an anaerobic chamber (PROOX model 110, Biospherix, Ltd. Redfield, NY, USA) which was placed in a water-jacketed incubator gassed with 95% N<sub>2</sub>/5% CO<sub>2</sub> at 37 °C (Kim et al., 2007). Then, the chamber was sealed and incubated for 40 min (ischemic insult). Following OGD, slices were placed back in the incubator in plates containing normal culture medium.

### Assessment of neuronal cell death by Propidium iodide (PI) staining technique

As described previously, the slices in all the experimental groups were incubated in culture medium supplemented with 2  $\mu$ g/ml PI for 1 h prior to imaging (Raval et al., 2003). Images of the cultured slices were taken (1) as baseline; (2) 24 h after the OGD insult to assess ischemic damage; and (3) 24 h after

NMDA treatment to assess maximum damage to neuronal cells. Fluorescence images were obtained using a SPOT CCD camera and were digitized using SPOT advanced software. Percentage of relative optical intensity was used as an index of cell death.

### Western blot analysis

The hippocampi (Groups 2–6) and slices (Groups 7–10) were collected at the end of treatment as described and stored at –80 °C. At the time of Western blot analysis, hippocampal organotypic slice cultures were separated from the supporting membrane (described previously (Raval et al., 2003)). For one sample, 40 slices were pooled. Thus,  $n=1$  for Western blot analysis represents 40 slices. Also, each slice was obtained from different pups. At the time of Western blot analysis, hippocampi or pooled slices were washed once with cold PBS. Hippocampi or slices were homogenized and lysed in a buffer containing 1% Nonidet P-40, 20 mM Tris (pH 8.0), 137 mM NaCl, 0.5 mM EDTA, 10% glycerol, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 1  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 1 mM sodium vanadate, and 1 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged further to acquire the various cell fractions, as described previously (Kim et al., 2007; Raval et al., 2009b). The resultant cell fractions were analyzed for protein content using the Bio-Rad protein assay kit. The proteins were separated on 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). Equal amounts of protein from each group were run on the same gel and analyzed at the same time. Protein bands were transferred to Immobilon-P (Millipore, MA, USA) membranes, which were then probed with the following antibodies: rabbit anti-pCREB (at Ser 133; 1:1000; Cell Signaling); monoclonal anti-lamin (1:10,000; Sigma); and rabbit polyclonal anti-ER- $\alpha$ /ER- $\beta$  (1:500; Santa Cruz Biotech, CA, USA). Immunoreactivity was detected using enhanced chemiluminescence (ECL Western blotting detection kit, Amersham-Pharmacia Biotech, UK). Western images were digitized at 8-bit precision by means of a charge-coupled-device-based (CCD) camera (8–12 bit, Xilix Technologies Corp., Vancouver, Canada) equipped with a 55 mm Micro-Nikkor lens (Nikon, Japan). The camera was interfaced to an advanced image-analysis system (MCID Model M2, Imaging Research, Inc., St Catherines, Ontario, Canada). The digitized immunoblots were subjected to densitometric analysis using MCID software.

### Confirmation of immunoreactivity of ER- $\beta$ antibody

Both ER- $\alpha$  and ER- $\beta$  antibodies are commercially available. However, ER- $\beta$  antibodies are for their non-specificity. Thus, we tested the immunoreactivity of the ER- $\beta$  antibody in hippocampal tissue of ER- $\beta$  knockout mice. Brain tissue samples of knockout/wild type mice were obtained from Dr. Kenneth Korach's laboratory at the National Institute of Environmental Health Science at Research Triangle Park, North Carolina.

### Statistical analysis

The data are presented as mean value  $\pm$  SEM. An analysis of variance (ANOVA) followed by a multiple comparison procedure (Bonferroni's test) was used to analyze the statistical differences among groups.

## RESULTS

### Chronic nicotine exposure plus OC exacerbated post-ischemic CA1 neuronal loss in female rats

First, we tested the hypothesis that a synergistic deleterious effect of NOC on post-ischemic hippocampal

**Table 1.** Summarizes the physiologic variables in each animal group

Groups (Body weight 290±20/g)	Glucose (mg/ml)	pH	pCO <sub>2</sub> (mmHg)	pO <sub>2</sub> (mmHg)	MABP (mmHg)
Group 1: Sham ischemia	146.5±6.2	7.4±0.01	38.0±3.1	126.9±6.4	119.8±5.3
		7.4±0.05	36.2±2.0	109.8±7.7	126.4±4.6
Group 2: Saline+ischemia	145±14.4	7.4±0.02	37.5±2.5	119.6±11.5	128.8±7.5
		7.4±0.02	34.8±3.5	115.3±11.4	115.0±6.5
Group 3: Nicotine+ischemia	148.8±3.5	7.4±0.03	36.3±0.9	119.1±12.8	128.5±4.5
		7.4±0.03	36.8±0.7	120.9±10.9	127.5±2.5
Group 4: Oral contraceptive+ischemia	147.8±3.4	7.4±0.02	35.9±3.7	123.7±10.4	124.3±4.5
		7.4±0.03	37.1±4.5	114.7±5.7	129.3±2.8
Group 5: Placebo+ischemia	136.3±21.4	7.4±0.01	38.1±1.2	121.4±6.36	123.3±3.8
		7.4±0.03	36.4±3.6	126.0±7.73	134.5±3.6
Group 6: Nicotine plus OC+ischemia	141.2±5.7	7.4±0.02	37.5±0.9	125.4±6.4	129.0±4.3
		7.4±0.01	38.9±1.7	124.0±10.5	125.3±7.3

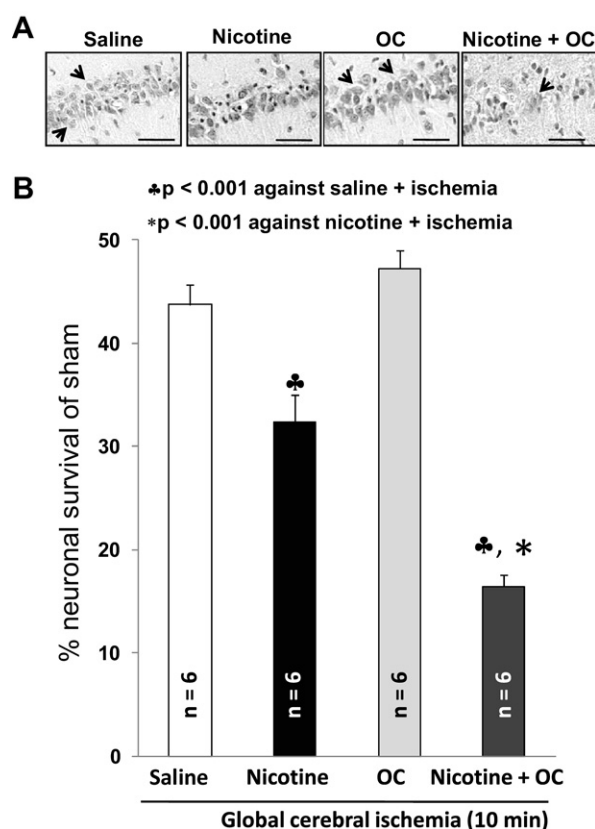
Cells with gray background denote values after ischemia. The plasma nicotine concentration in Groups 3 and 6 was 197±4.1 and 200±5.3 ng/ml, respectively.

damage in female rats. Rats were subjected to global cerebral ischemia at the end of nicotine±OC treatment (16th day; Fig. 1). Physiological variables (plasma glucose concentration, pH, pCO<sub>2</sub>, pO<sub>2</sub>, and MABP) monitored before induction of cerebral ischemia did not differ significantly among all experimental groups (Table 1). Global ischemia reduced the number of normal neurons by 56% per section in the CA1 hippocampal region of saline-treated females at the proestrus stage compared to the number seen in sham-treated controls (1204±105; n=6; P<0.001; Fig. 2). Nicotine exposure followed by ischemic insult at the proestrus stage decreased the normal neuron count to 368±56 (n=6), or 32% of the sham count. Oral contraceptive treatment alone did not significantly affect the post-ischemic number of normal neurons (567±21; n=6) as compared to the saline group (525±22; n=6). In contrast to the preceding results, only 16% (197±13; n=6) of neurons in NOC rats appeared normal as compared to sham. Interestingly, we observed a significant (P<0.001) difference in the post-ischemic number of normal neurons between the nicotine alone group and the NOC group. These results showed that nicotine and OC in combination exacerbate post-ischemic hippocampal damage as compared to nicotine alone in female rats. A placebo control experiment did not show differences from the saline group (data not shown).

#### Nicotine plus OC reduced circulating 17β-estradiol plasma levels in female rats

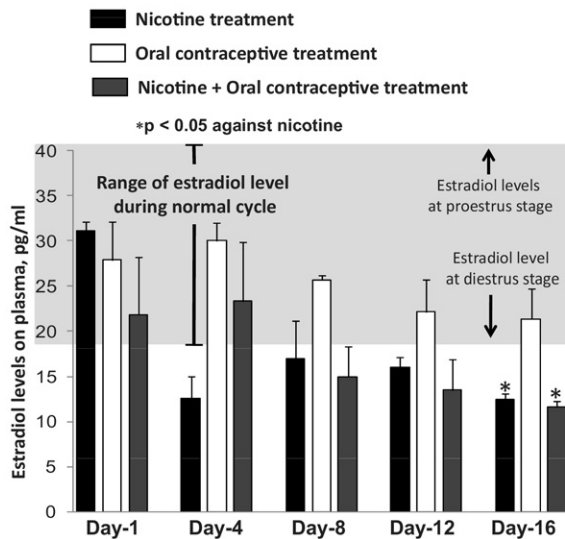
Nicotine dependence is known to modulate estrogen metabolism, reduce levels of endogenous estrogen, disturb the normal periodicity of the menstrual cycle and induce early onset of menopause in women (Jensen et al., 1985; Cramer et al., 1995; Mueck and Seeger, 2005). Thus, we tested the hypothesis that nicotine alone or in combination with OC reduces circulating 17β-estradiol levels in normally cycling rats by monitoring plasma 17β-estradiol levels in saline or nicotine or NOC-exposed rats. As can be seen in Fig. 3, nicotine alone or NOC significantly decreased circulating estradiol levels. A placebo control ex-

periment did not show any differences from saline group (data not shown).



**Fig. 2.** The severity of ischemic brain damage is far greater in females simultaneously exposed to oral contraceptives than to nicotine only. (A) Representative histological images in the hippocampal CA1 region 7 d after induction of cerebral ischemia in female rats exposed to (a) saline, (b) nicotine, (c) OC and (d) nicotine plus OC. Arrow shows normal neurons (Scale bar=20 μm). (B) The number of normal neurons in the CA1 region of hippocampus which includes the middle, medial, and lateral sub-region. Data expressed as a percentage of sham (100% normal neurons) in rat hippocampus 7 d after induction of cerebral ischemia.

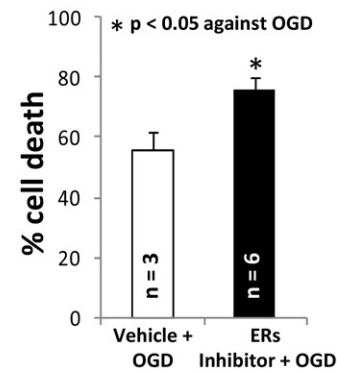




**Fig. 3.** Nicotine plus OC reduces circulating  $17\beta$ -estradiol plasma levels in female rats. This figure presents estradiol levels in plasma of nicotine/+ OC-treated rats. Blood was collected at beginning of experimental treatment and on every fourth days (day of placebo treatment in OC groups) during treatment. Gray box represents the normal range of plasma estradiol levels during normal estrous cycle. Note the decline on estradiol levels in nicotine- and nicotine plus OC-exposed groups.

### Nicotine inhibits intracellular $E_2$ -signaling and exacerbates ischemic damage in an *ex vivo* model

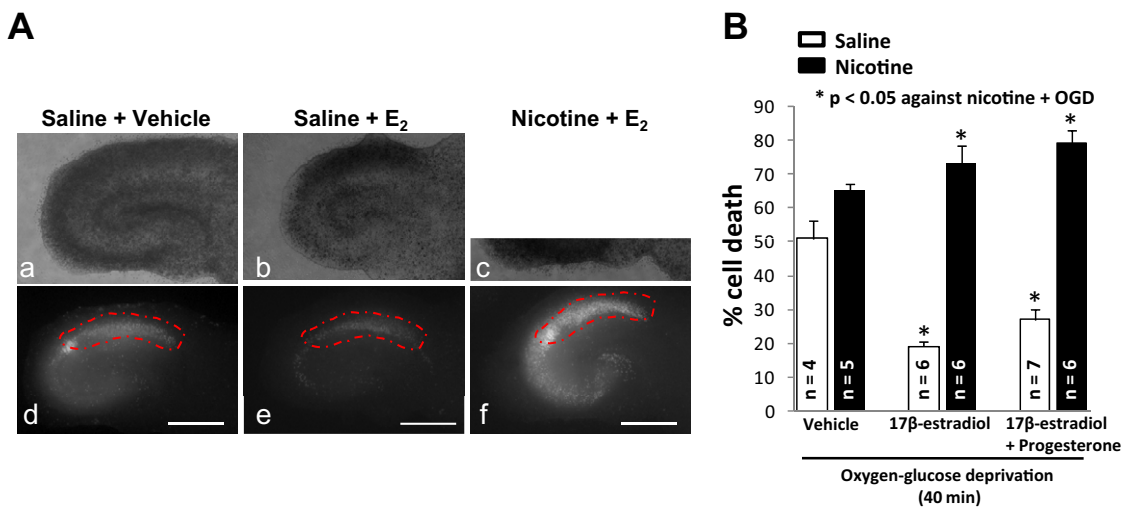
Next, we tested the hypothesis that nicotine exacerbates post-ischemic CA1 damage via inhibition of  $E_2$ -mediated intracellular signaling by exposing slices to nicotine and  $E_2$  as described in the Experimental procedures section. These slices were exposed to lethal ischemia (OGD; 40 min) 48 h later. Count of cell death of saline-,  $17\beta$ -estra-



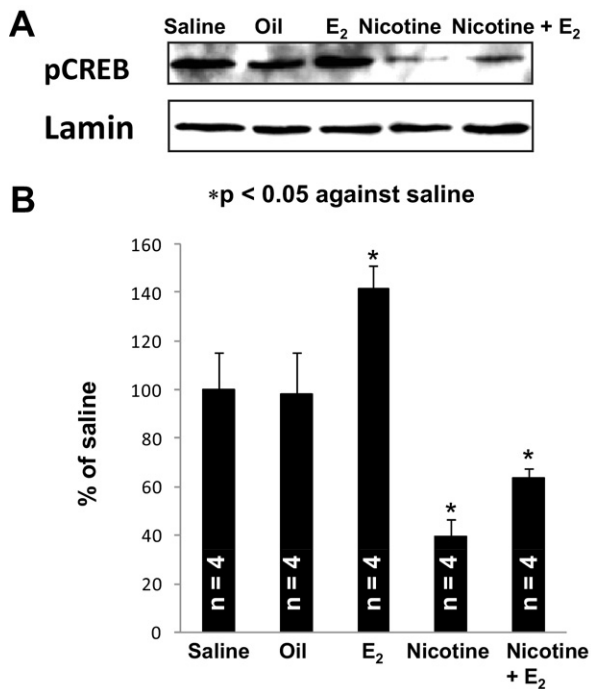
**Fig. 5.** Estrogen receptors (ERs) are required to protect CA1 neurons after OGD in *ex vivo* model. The % cell death measured in the CA1 region 24 h after OGD, demonstrating that the inhibition of estrogen receptors prior to OGD increased CA1 neuronal loss.

diol-, nicotine-, N+ $E_2$ - and N+ $E_2$ +P-treated samples were  $50 \pm 5$  ( $n=4$ ),  $19 \pm 1$  ( $n=6$ ),  $65 \pm 2$  ( $n=5$ ),  $72 \pm 5$  ( $n=6$ ), and  $79 \pm 4$  ( $n=6$ ), respectively (Fig. 4).

The neuroprotective effects of  $E_2$  against ischemia are mediated through its  $\alpha$  and  $\beta$  receptors (Lebesgue et al., 2009; Noppens et al., 2009; Zhang et al., 2009). Therefore, we tested whether ERs activation is required for post-ischemic protection, by exposing slices to the ERs inhibitor ICI 182780 ( $1 \mu M$ ) for 24 h before induction of OGD. The quantification of cell death demonstrated that inhibition of ERs significantly increased CA1 neuronal loss as compared to OGD. The PI fluorescence values for OGD, and ERs inhibitor+OGD were  $55 \pm 6$  ( $n=3$ ) and  $76 \pm 4$ , respectively ( $n=5$ ;  $P < 0.05$  against OGD). These results confirmed that ERs activation was required to rescue CA1 neurons after ischemia (Fig. 5). Control experiments using ERs inhibitor without an OGD episode did not show any



**Fig. 4.** Nicotine inhibits intracellular  $E_2$ -signaling and exacerbates ischemic damage in an *ex vivo* model. (A) Typical images of hippocampal slice cultures. (a–c); Bright-field images of saline+vehicle, saline+ $17\beta$ -estradiol, and nicotine+ $17\beta$ -estradiol groups, respectively. (d–f); PI fluorescence images showing neuronal death in the saline+vehicle, saline+ $17\beta$ -estradiol, and nicotine+ $17\beta$ -estradiol groups taken 24 h after the lethal ischemic (40 min of OGD) insult. Marked area denotes region of interest for relative optical intensity quantification. Scale bars=2.25 mm. (B) The percent of cell death measured in the CA1 region 24 h after OGD, demonstrating that nicotine plus  $17\beta$ -estradiol exposure increased neuronal loss after OGD. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.



**Fig. 6.** Nicotine inhibits E<sub>2</sub>-induced cyclic-AMP response element binding protein phosphorylation (pCREB). (A) Representative immunoblots showing pCREB content in hippocampal slices after various treatments. Note the decreased pCREB protein levels in nicotine alone or nicotine plus 17 $\beta$ -estradiol groups as compared to the 17 $\beta$ -estradiol group. (B) Densitometric analysis of scanned Western blots revealed a relative decrease of phosphorylated CREB after nicotine alone or in combination with 17 $\beta$ -estradiol exposure. Significance as compared to saline, \*  $P < 0.05$ .

neuronal death in CA1 region of hippocampus (data not shown).

#### Nicotine inhibits E<sub>2</sub>-induced cyclic-AMP response element binding protein phosphorylation (pCREB)

In hippocampal neurons, estrogen receptor activation phosphorylates the CREB (Boulware et al., 2007; Luoma et al., 2008) and E<sub>2</sub>-mediated post-ischemic neuroprotection requires activation of the CREB pathway (Raval et al., 2006, 2009b). We tested the hypothesis that nicotine inhibits E<sub>2</sub>-mediated CREB activation by exposing slices to nicotine or nicotine+E<sub>2</sub> as described in the Experimental procedures section. Western blotting demonstrated decreased pCREB in the nicotine+E<sub>2</sub> group as compared to the E<sub>2</sub> group, suggesting that nicotine inhibits the protective effects of E<sub>2</sub>-signaling in the hippocampus (Fig. 6).

#### Nicotine plus OC reduces CREB phosphorylation in the hippocampus of female rats

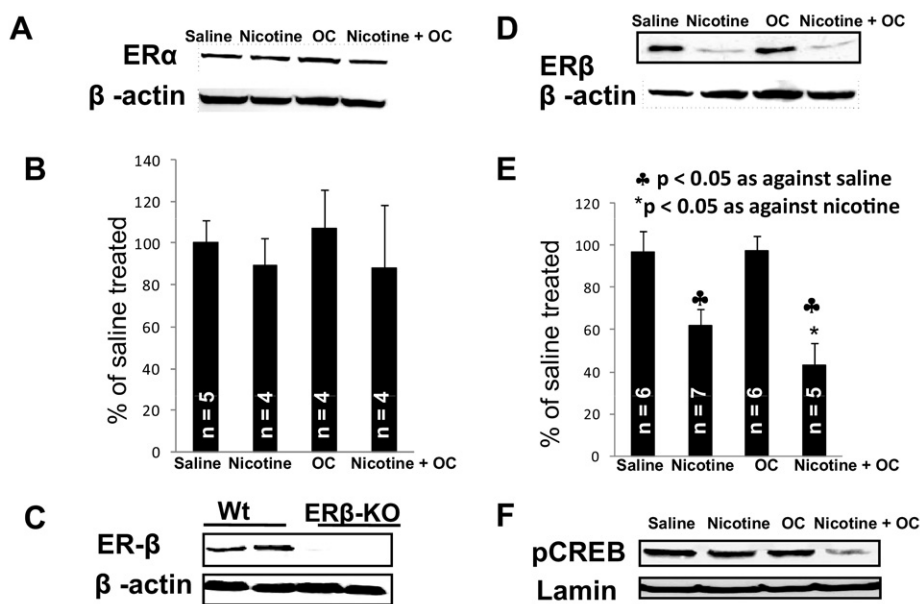
To confirm the results of our *ex vivo* experiments which found that nicotine inhibits the protective effects of E<sub>2</sub>-signaling in the hippocampus, we performed an *in vivo* experiment. The rapid effects of intracellular E<sub>2</sub>-signaling causing phosphorylation of CREB are mediated by membrane-bound ERs; thus, we tested the hypothesis that

NOC decreases ERs availability at the membrane. The results of the Western blot analysis demonstrated no change in ER- $\alpha$  protein level in the membrane fraction among various experimental groups (Fig. 7A, B). Surprisingly, the results demonstrated that NOC significantly reduced ER- $\beta$  protein levels in the membrane fraction as compared to the saline group (Fig. 7D). Here we also present data using ER- $\beta$  knockout mouse hippocampal tissue, confirming the specific immunoreactivity of ER- $\beta$  antibody that we used for our study. Analysis of Western blots of nicotine- and NOC-treated samples demonstrated a 35% ( $0.39 \pm 0.04$ ;  $n=7$ ), and 54% ( $0.27 \pm 0.06$ ;  $n=5$ ;  $P < 0.05$ ), respectively, reduction in membrane ER- $\beta$  levels as compared to saline-group ( $0.61 \pm 0.06$ ;  $n=6$ ) (Fig. 7E). We did not observe any difference between OC treatment ( $0.62 \pm 0.04$ ;  $n=6$ ) alone and the saline group. The ER- $\beta$  protein level did not change in the nuclear fraction (data not shown). Further, we observed a significant difference in membrane-bound ER- $\beta$  protein level between nicotine- vs. NOC-treated samples ( $P < 0.05$ ), which suggests that a combination of NOC has a more intense effect than nicotine. Finally, we tested the hypothesis that NOC reduces CREB phosphorylation in hippocampus of female rats. Western blotting demonstrated decreased pCREB in the nuclear fraction of the NOC group as compared to the saline/OC groups, suggesting that, similar to *ex vivo* model nicotine also inhibits the protective effects of E<sub>2</sub>-signaling in the hippocampus of female rats (*in vivo*) (Fig. 7F).

## DISCUSSION

In this study, we demonstrate for the first time that greater post-ischemic damage in the hippocampus of normally cycling female rats related to the presence of nicotine is further exacerbated in the presence of oral contraceptives. We also show that nicotine inhibits the estradiol-mediated upregulation of pCREB, which may explain at least in part how nicotine counteracts the ischemic protection normally provided by estradiol. Furthermore, we observed a significant difference in membrane-bound ER- $\beta$  protein level between nicotine vs. NOC treatment *in vivo*, which suggests that a combination of nicotine plus OC has a more intense effect than nicotine alone.

By using slice cultures, we were able to demonstrate that this action of nicotine was through a direct effect on intracellular signaling in the hippocampus and not to an indirect systemic effect (e.g. (Lilienberg and Venge, 1998; Powell, 1998; Hioki et al., 2001; Abbruscato et al., 2002; Hawkins et al., 2004; Lindenblatt et al., 2007; Rahman and Laher, 2007)). Furthermore, the exacerbation of post-OGD hippocampal damage was due to 17 $\beta$ -estradiol, given that we did not observe any significant difference when progesterone was present in slices treated with nicotine+17 $\beta$ -estradiol. On the other hand, the caveat of the slice culture approach is that hippocampal cells are not exposed to natural fluctuations of ovarian hormones. Thus, this preparation may have a different (developmental) response to ovarian hormones than tissue in mature, cycling females. Having stated this, we also mention that our recent studies



**Fig. 7.** Nicotine plus OC reduces CREB phosphorylation in the hippocampus of female rats. (A, D) Representative immunoblots showing the protein levels of estrogen receptor  $\alpha$  and  $\beta$  in the hippocampal membrane fraction for different experimental conditions. (B, E) Densitometric analysis of scanned Western blots revealed that chronic nicotine or nicotine plus oral contraceptive exposure decreased the levels of ER- $\beta$  protein in hippocampus. In contrast, ER- $\alpha$  protein levels were not changed following nicotine treatment. (C) This representative Western blot image shows the lack of immunostaining for ER- $\beta$ -knockout hippocampus confirming the specificity of the ER- $\beta$  rabbit polyclonal antibody. (F) Representative immunoblots showing pCREB content in hippocampus of female rats that underwent various treatments. Note the decreased pCREB protein levels in nicotine alone or nicotine plus OC groups as compared to the saline group.

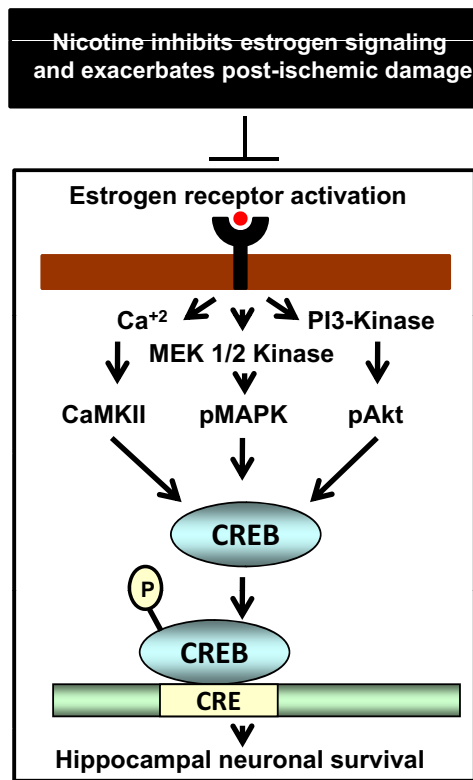
testing the efficacy of a  $17\beta$ -estradiol bolus in both *in vitro* and *in vivo* demonstrated a neuroprotective effect via activation of CREB (Raval et al., 2006, 2009b). Therefore, we conjecture that in nicotine plus ovarian hormone(s)-treated slices, increased hippocampal CA1 neuronal damage after oxygen-glucose deprivation is comparable with the *in vivo* effect of nicotine plus OC. Additionally, among *in vitro* models, the organotypic slice culture proves to be advantageous, as it retains tissue-specific cell connections and local neuronal circuits with the appropriate innervation patterns (Strasser and Fischer, 1995; Gahwiler et al., 1997) and expresses estrogen receptor subtypes as well (Gahwiler et al., 1997; Sato et al., 2002).

Estrogen-induced intracellular signaling is mediated via estrogen receptors (ER- $\alpha$  and ER- $\beta$ ) (Heldring et al., 2007; Zhao et al., 2008). Our recent study demonstrated that nicotine decreased ER- $\beta$  but not ER- $\alpha$  protein levels in the hippocampus of estrogen-treated ovariectomized female rats, suggesting that nicotine might hinder the ER- $\beta$ -mediated intracellular signaling of estrogen (Raval et al., 2009a). The role of ER- $\beta$  in ischemic neuronal survival is also supported by a recent study from Traystman and his group that demonstrated that estradiol treatment was neuroprotective and mediated through ER- $\beta$  in a mouse model of global cerebral ischemia (Noppens et al., 2009). Previous studies from various laboratories including ours have demonstrated that  $17\beta$ -estradiol-mediated post-ischemic CA1 neuroprotection requires activation of the CREB pathway (Lebesgue et al., 2009; Raval et al., 2009b). In hippocampal neurons ER- $\beta$  activation regulates phosphorylation of CREB (Boulware et al., 2007; Luoma et al., 2008).

Here data demonstrated that a nicotine-induced loss of ER- $\beta$  correlates to decreased phosphorylation of CREB and to greater post-ischemic CA1 damage after nicotine plus OC exposure in female rats (Fig. 7).

Greater post-ischemic damage owing to a disrupted ER- $\beta$ -CREB signaling pathway is particularly important given the role of this pathway in memory formation and cognition. In the hippocampus, CREB phosphorylation is required for maintenance of synaptic plasticity and for long-term potentiation (LTP) (Barco et al., 2002). Global cerebral ischemia causes the loss of LTP in the rat hippocampus (Dave et al., 2004). In female mice, activation of ER- $\beta$  potentiates LTP in CA1 neurons and improves hippocampus-dependent cognition (Liu et al., 2008). Furthermore, the use of selective ER- $\beta$  agonists increases the levels of key synaptic proteins such as the post-synaptic density, synaptophysin and the AMPA-receptor in the hippocampus of mice (Liu et al., 2008). These changes in synaptic proteins mediated by ER- $\beta$  activation are related to morphological changes such as increased dendritic branching and increased density of mushroom-type spines in hippocampal neurons (Liu et al., 2008). Also, ER- $\beta$  knockout mice treated with estradiol show impaired acquisition of a spatial reference memory, which suggests a role for ER- $\beta$  in hippocampus-dependent cognition (Rissman et al., 2002).

Overall, the preceding literature emphasizes that ER- $\beta$  plays a key role in how estrogen affects synaptic plasticity, memory, and cognition in female experimental animals. On the other hand, it has also been demonstrated that nicotine administration to female adolescent rats alters neuropro-



**Fig. 8.** Schematic diagrams depicting the main hypothesis that nicotine plus OC inhibits ER-signaling and exacerbates cerebral ischemic damage. It has been demonstrated that the estrogen-mediated activation of CREB occurs via CaMKII, MAPK, and Akt pathways (Lebesgue et al., 2009; Raval et al., 2009b). Abbreviations: Akt, serine/threonine protein kinase; CREB, cyclic-AMP response element binding protein; MAPK, mitogen-activated protein kinase; pAkt, phosphorylated Akt. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

teins in the hippocampus and contributes to neurobehavioral deficits (Xu et al., 2003). Based on this information and our previous findings that synaptic dysfunction precedes actual histopathological abnormalities after global cerebral ischemia in rats (Dave et al., 2004), we speculate that the greater disruption of the ER- $\beta$  signaling pathway seen with nicotine+17 $\beta$ -estradiol than with nicotine alone causes greater post-ischemic synaptic dysfunction, thus exacerbating post-ischemic CA1 damage in female rats. However, future studies are needed to see how nicotine affects ER- $\beta$ -mediated synaptic plasticity. It is possible that nicotinic acetylcholine receptors may be involved in observed deleterious effects of nicotine as the homomeric  $\alpha 7$ -nicotinic acetylcholine receptor ( $\alpha 7$ -nAChR) is widely expressed on hippocampal pyramidal neurons and warrants further investigation (Fabian-Fine et al., 2001; Gaimarri et al., 2007). In addition, the question of how, or even if, ER- $\alpha$  receptors participate in post-ischemic neuroprotection needs further investigation. Our results suggest that ER- $\alpha$  could be involved since the general estrogen receptor inhibitor ICI 182780 inhibited estrogen-mediated neuroprotection of the CA1 region. Our past and present studies suggest a prominent role for ER- $\beta$  in post-ischemic

neuroprotection and not for ER- $\alpha$  (Raval et al., 2009a), however a role for ER- $\alpha$  cannot be totally excluded.

## CONCLUSION

In conclusion, we found that a significant deleterious interaction between nicotine and E<sub>2</sub> that directly inhibits estrogen receptor-mediated phosphorylation of CREB in the hippocampus of female rats may help explain post-ischemic damage in the female hippocampus (Fig. 8). These findings could explain why women addicted to nicotine might respond differently to hormone replacement therapy (HRT) or oral contraceptives. Further knowledge regarding how nicotine interacts with female endocrinology is essential for understanding the effects of nicotine addiction on HRT and for understanding why the Women's Estrogen for Stroke Trial and the Women's Health Initiative failed to show any benefit of HRT (Kernan et al., 1998; Viscoli et al., 2001; Shumaker et al., 2003; Wassertheil-Smoller et al., 2003; Anderson et al., 2004). More studies like the current one are needed to understand the perils or benefits of estrogen for women's cardio-/cerebrovascular health.

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