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Estradiol regulates large dense core vesicles in the hippocampus of adult female rats

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Abstract

Previous work has shown that the steroid hormone estradiol facilitates the release of anticonvulsant neuropeptides from inhibitory neurons in the hippocampus to suppress seizures. Because neuropeptides are packaged in large dense core vesicles, estradiol may facilitate neuropeptide release through regulation of dense core vesicles. In the current study, we used serial section electron microscopy in the hippocampal CA1 region of adult female rats to test three hypotheses about estradiol regulation of dense core vesicles: (1) Estradiol increases the number of dense core vesicles in axonal boutons, (2) Estradiol increases the size of dense core vesicles in axonal boutons, (3) Estradiol shifts the location of dense core vesicles toward the periphery of axonal boutons, potentially lowering the threshold for neuropeptide release during seizures. We found that estradiol increases the number and size of dense core vesicles in inhibitory axonal boutons, consistent with increased neuropeptide content, but does not shift the location of dense core vesicles closer to the bouton periphery. These effects were specific to large dense core vesicles (>80 nm) in inhibitory boutons. Estradiol had no effects on small dense core vesicles or dense core vesicles in excitatory boutons. Our results indicate that estradiol suppresses seizures at least in part by increasing the potentially releasable pool of neuropeptides in the hippocampus, and that estradiol facilitation of neuropeptide release involves a mechanism other than mobilization of dense core vesicles toward sites of release.

Keywords

Serial section electron microscopy; CA1; Neuropeptide; Seizure; Catamenial epilepsy

Introduction

Estrogens modulate seizures in epilepsy. For about onethird of women with epilepsy, seizures fluctuate with the menstrual cycle, termed catamenial epilepsy (Herzog 2008), which is related to fluctuating levels of the ovarian steroids, estradiol and progesterone (Backstrom 1976; Guille et al. 2008). Metabolites of progesterone are generally

anticonvulsant (Majewska et al. 1986; Kaminski et al. 2004), whereas estradiol can be proconvulsant or anticonvulsant depending on the dose and duration of treatment, the type of seizure investigated, and which aspect of seizures is measured (Veliskova 2006).

In a previous study (Ledoux et al. 2009), we used acute kainic acid injection to investigate how 24 h of estradiol treatment affects hippocampus-dependent seizures in adult female rats. We found two distinct effects. First, estradiol reduced the latency to seizure onset, likely due to decreased GABAergic inhibition (Rudick and Woolley 2001; Rudick et al. 2003; Ledoux and Woolley 2005). Second, however, estradiol-treated animals were less likely to progress to the most severe stages of seizure behavior and severe seizures that did occur were shorter in duration. Further experiments indicated that the seizure-suppressing effect of estradiol was mediated, at least in part, through regulation of neuropeptide release. In the hippocampus, anticonvulsant neuropeptides such as neuropeptide Y (NPY; Milner and Veznedaroglu 1992), cholecystokinin (CCK; Harris et al. 1985), and somatostatin (Feldman et al. 1982; Kunkel et al. 1994) are synthesized by subsets of GABAergic interneurons, packaged in large dense core vesicles (DCVs; Bean et al. 1994), and are released during high frequency firing (Sørensen et al. 2008) as well as during seizures (Manfridi et al. 1991; Husum et al. 1998; Marti et al. 2000). We found that estradiol increased the NPY content of GABAergic axonal varicosities in hippocampal CA1 and facilitated the release of NPY from GABAergic varicosities during seizures. Furthermore, blocking NPY action in the hippocampus eliminated the estradiol-induced decrease in seizure duration (Ledoux et al. 2009).

The aim of the current study was to investigate whether estradiol regulates DCVs in the hippocampus in a way that could account for increased neuropeptide content of GABAergic varicosities and facilitation of neuropeptide release during seizures. We tested three specific hypotheses: (1) Estradiol increases the number of DCVs in axonal boutons, (2) Estradiol increases the size of DCVs in axonal boutons, (3) Estradiol shifts the location of DCVs toward the periphery of axonal boutons, potentially lowering the threshold for neuropeptide release during seizures. We used serial section electron microscopy to reconstruct both inhibitory and excitatory axonal boutons in the cell body layer and stratum radiatum of hippocampal CA1 in adult female rats that were ovariectomized and treated with estradiol or oil vehicle for 24 h. We analyzed the number and size of DCVs in each bouton type as well as the distance of each DCV to the nearest plasma membrane. The results showed that estradiol regulates DCV number and size within inhibitory but not excitatory boutons. The location of DCVs within boutons was not affected by estradiol in either bouton type.

Methods

Animals

All animal procedures were performed in accordance with the National Institutes of Health Guide to the Care of Laboratory Animals and were approved by the Northwestern University Institutional Animal Care and Use Committee. Eight 58–60 day old adult female Sprague–Dawley rats (Harlan, Indianapolis, IN) were used. Animals were kept on a 12 h light:dark cycle with soy-free rat chow and water freely available. Rats were ovariectomized under ketamine (85 mg/kg)/xylazine (13 mg/kg, i.p.) anesthesia and 3 days later were given

a single subcutaneous injection of either 10 μg of 17β -estradiol benzoate ($n = 4$) or oil vehicle ($n = 4$). Twenty-four hours after oil or estradiol treatment, animals were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and perfused with 2 % paraformaldehyde/2 % glutaraldehyde in phosphate buffer (PB).

Electron microscopy

The preparation of tissue for EM was performed as described previously (Hart et al. 2007). Briefly, coronal blocks of tissue containing the dorsal hippocampus were postfixed overnight at 4 °C, cryoprotected with 30 % sucrose in PB, and sectioned (100 μm) using an SM2000R freezing microtome (Leica, Bannockburn, IL). Sections were stained with 1 % osmium tetroxide and flat embedded in Eponate resin (Ted Pella, Redding, CA). The embedded tissue from each animal was then coded to ensure that experimenters were blind to treatment group during all phases of sectioning, imaging, and analysis. Tissue blocks containing the CA1 region were dissected from flat embedded sections and mounted on BEEM capsules. Series of 24–35 ultrathin (~75 nm) sections from each of 2 blocks per animal were cut using a Reichert Ultracut S ultramicrotome (Leica), collected onto formvar-coated slot grids, stained with 3 % uranyl acetate followed by 2.66 % Reynold's lead citrate, and imaged with a JEOL 1230 transmission electron microscope equipped with a CCD camera. For each block, a low magnification image (400 $\times$) was taken of the first section and used to select four areas (two in the cell body layer and two in the stratum radiatum) for serial imaging using a random systematic approach. Images of each selected area were then digitally captured at 30,000 $\times$ through the entire series of sections. This resulted in 4 stacks of 16.5 μm^2 images per brain for the cell body layer and four for the stratum radiatum.

Three-dimensional reconstruction

Electron micrographs from each image stack were imported into RECONSTRUCTTM software (available for free download at <http://synapses.clm.utexas.edu>) and aligned using software tools. Our analysis focused on axonal boutons and their contents. For each axonal bouton contained completely within a series, profiles of the plasma membrane, mitochondria, synapses and any DCVs were traced manually on aligned images. DCVs were typically circular with an electron dense core separated from an outer membrane by an electron lucent space. The diameter of all DCVs, (i.e., in both complete and incomplete boutons) was measured using RECONSTRUCTTM. Based on previous studies (Zhai et al. 2001; Sorra et al. 2006; Pickel et al. 1995; Salio et al. 2006), we divided DCVs into those 80 nm or larger, which are most likely to be neuropeptide-containing secretory vesicles, versus those smaller than 80 nm, which may contain presynaptic active zone material. Also, boutons were divided into those forming asymmetric (presumptive excitatory) versus symmetric (presumptive inhibitory) synapses. Boutons with presumptive excitatory synapses contained numerous round clear vesicles and formed synapses with dendritic spines and occasionally with dendritic shafts. Boutons with presumptive inhibitory synapses were much more prevalent in the cell body layer, contained both round and pleomorphic vesicles, and formed synapses with cell bodies and occasionally with dendritic shafts. Boutons with these characteristics are referred to as excitatory and inhibitory boutons in the remainder of this report. DCVs were found in both bouton types, but were most common in inhibitory boutons in the cell body layer. We used the cylindrical diameters method (Fiala and Harris 2001) to

obtain section thickness for accurate three-dimensional distance and volume measurements. RECONSTRUCT™ was then used to calculate the volume of each bouton as well as to measure the distance from each DCV to the nearest plasma membrane and synapse. A total of 2,330 complete boutons (1,119 in OVX + O and 1,211 in OVX + E) were reconstructed using this approach.

Statistics and data display

Quantitative data are expressed as mean $\pm$ SEM. Statistical comparisons were made using unpaired, two-tailed Student's *t*-tests with *n* as the number of animals. EM images were viewed in Photoshop (Adobe Systems, San Jose, CA), cropped, and levels adjusted to enhance contrast when necessary.

Results

Estradiol increases the number of DCVs in perisomatic inhibitory boutons

We reconstructed 110 perisomatic inhibitory boutons in oil-treated control animals (27.5 ± 2.9 boutons in $176 \pm 12 \mu\text{m}^3$ tissue volume per animal) and 109 in estradiol-treated animals (27.3 ± 4.4 boutons in $180 \pm 11 \mu\text{m}^3$ tissue volume per animal). The average volume of inhibitory boutons was similar between the two groups ($0.28 \pm 0.03 \mu\text{m}^3$ oil, $0.34 \pm 0.02 \mu\text{m}^3$ estradiol, $p > 0.10$). From reconstructed boutons, we quantified the number of DCVs per inhibitory bouton, the proportion of inhibitory boutons containing DCVs, and the average number of DCVs per bouton considering only boutons that contained any DCVs. In addition, because the size of DCVs may be related to their cargo, with small DCVs (< 80 nm diameter) contributing presynaptic active zone contents (Zhai et al. 2001; Sorra et al. 2006) and large DCVs (≥ 80 nm diameter) containing neuropeptides (Pickel et al. 1995; Salio et al. 2006), we considered both types of DCVs together and large DCVs separately. The vast majority of DCVs in inhibitory boutons (86 %) were large DCVs.

Figure 1 shows representative electron micrographs and corresponding 3D reconstructed images of DCV-containing perisomatic inhibitory boutons in an oil- (Fig. 1a) and estradiol-treated (Fig. 1b) rat. Quantitative analysis showed that estradiol significantly increased the number of DCVs in inhibitory boutons, driven by a change in the number of large DCVs. Considering all-size DCVs, the average number of DCVs per inhibitory bouton was 0.17 ± 0.04 in oil-treated compared to 0.45 ± 0.09 in estradiol-treated animals ($p < 0.05$, not shown). Considering large DCVs specifically, there were 0.15 ± 0.03 large DCVs per bouton in oil-treated animals compared to 0.38 ± 0.08 in estradiol-treated animals ($n = 4$, $p < 0.05$; Fig. 1c); in contrast, the number of small DCVs was unchanged (0.02 ± 0.02 in oil vs. 0.06 ± 0.04 in estradiol, $p = 0.4$). Estradiol also significantly increased the proportion of inhibitory boutons that contained any DCVs, from 15 ± 3 % in oil-treated controls to 27 ± 3 % in estradiol-treated animals ($p < 0.05$, not shown) and specifically large DCVs, from 13 ± 2 % in oil-treated controls to 26 ± 2 % in estradiol-treated animals ($p < 0.01$; Fig. 1d). Considering only those boutons that contained any DCVs, the average number of large DCVs per bouton was 1.10 ± 0.06 in oil-treated animals compared to 1.49 ± 0.23 in estradiol-treated animals, but this was not a significant difference ($p = 0.16$, Fig. 1e). The distribution of DCV sizes in each group is shown in Fig. 1f.

Taken together, these results show that estradiol more than doubled the number of large DCVs per inhibitory bouton in the CA1 cell body layer, driven primarily by an increase in the proportion of boutons containing DCVs rather than an increase in the number of DCVs per bouton that contained them. The effects of estradiol were specific to large DCVs (> 80 nm), which are those most likely to contain neuropeptides. Thus, increasing the DCV content of perisomatic inhibitory boutons is one way that estradiol may increase the pool of neuropeptides that could be released during seizures.

Estradiol increases the size of DCV cores in perisomatic inhibitory boutons

Next, we asked whether estradiol affected the size of DCVs in perisomatic inhibitory boutons. We measured DCV diameter in two ways, from outer membrane to outer membrane and specifically of the cargo-containing core of each DCV. The sample for this analysis included both DCVs in completely reconstructed inhibitory boutons (19 DCVs oil and 54 DCVs estradiol) as well as in boutons that were incomplete but that could be positively identified as inhibitory boutons based on forming a symmetric synapse within the imaged series (an additional 30 DCVs oil and 23 DCVs estradiol). Interestingly, whereas DCV diameter measured from outer membrane to outer membrane was not different (90 ± 2 nm oil vs. 90 ± 4 nm estradiol), estradiol significantly increased the diameter of DCV cores (Fig. 2a–f). Average DCV core diameter for all-size DCVs was 52 ± 2 nm in controls compared to 60 ± 2 nm in estradiol-treated animals ($p < 0.05$, Fig. 2g). The same was true considering large DCVs only, in which core diameters were 55 ± 1 nm in oil-treated controls versus 63 ± 2 nm in estradiol-treated animals ($p = 0.01$, Fig. 2f). This suggests that, in addition to increasing DCV number, estradiol may increase the neuropeptide content of individual DCVs. Consistent with a greater protein density, DCV cores in the estradiol group were typically stained more darkly than in controls (Fig. 2a–f), but this was not quantified.

Although there were no effects of estradiol on the size of inhibitory boutons, inhibitory boutons that contained DCVs differed in two ways from those that did not. Considering both treatment groups together ($n = 8$), inhibitory boutons that contained DCVs tended to be larger than boutons without DCVs ($0.41 \pm 0.07 \mu\text{m}^3$ with DCVs, $0.27 \pm 0.01 \mu\text{m}^3$ without DCVs, $p = 0.07$) and they were significantly more likely to contain mitochondria ($94 \pm 4\%$ with DCVs, $75 \pm 5\%$ without DCVs, $p < 0.01$). These differences may reflect different populations of neurons that synthesize and release neuropeptides compared to those that do not, or differing energetic demands to support neuropeptide release.

Estradiol does not affect the location of DCVs within perisomatic inhibitory boutons

Our previous results indicated that estradiol facilitates NPY release from inhibitory boutons during seizures (Ledoux et al. 2009). One possibility is that estradiol increases DCV release probability by mobilizing DCVs closer to potential release sites at the bouton periphery. To investigate this, we measured the shortest distance from each DCV to the bouton membrane, considering only DCVs in completely reconstructed boutons. This analysis showed no effect of estradiol on DCV location within boutons. The average minimum distance of DCVs to the nearest plasma membrane was not different between control (49 ± 2 nm) and estradiol-treated animals (53 ± 8 nm, $p > 0.10$; not shown). Similarly, there was no difference in the

average minimum distance to the nearest synapse between control (457 ± 79 nm) and estradiol-treated animals (440 ± 36 nm, $p > 0.10$; not shown). Thus, it is unlikely that estradiol facilitates neuropeptide release by mobilizing DCVs closer to potential release sites.

Estradiol does not regulate DCVs in excitatory boutons

Image stacks from the cell body layer also contained excitatory boutons, which typically formed asymmetric synapses with the proximal dendrites of CA1 pyramidal cells. We reconstructed these boutons as well, for comparison with inhibitory boutons. The same four image stacks per animal used for inhibitory bouton reconstruction contained an average of 35 ± 9.3 excitatory boutons in oil-treated control animals and 35 ± 9.5 in estradiol-treated animals. Excitatory boutons in the cell body layer were approximately half the size of inhibitory boutons in the same area ($0.12 \pm 0.02 \mu\text{m}^3$ oil, $0.15 \pm 0.05 \mu\text{m}^3$, estradiol).

In contrast to results for inhibitory boutons, none of the DCV parameters in excitatory boutons were affected by estradiol (all p values > 0.10). The average number of DCVs per excitatory bouton in both groups (0.18 ± 0.05 oil, 0.17 ± 0.02 estradiol) was similar to values for inhibitory boutons in oil-treated controls. DCVs in excitatory boutons tended to be smaller than in inhibitory boutons with about half (52 %) falling into the small (< 80 nm) category. This suggests either that a greater fraction of DCVs in excitatory boutons contains presynaptic active zone contents rather than neuropeptides, and/or that neuropeptide-containing DCVs in excitatory boutons are smaller than in inhibitory boutons.

To further investigate DCVs in excitatory boutons, we reconstructed excitatory boutons in the stratum radiatum in four image stacks per animal, similar to analysis in the cell body layer. There were too few inhibitory boutons in our stratum radiatum images to perform a statistical analysis. Like for DCVs in excitatory boutons in the cell body layer, estradiol had no effects on DCVs in the stratum radiatum (all p values > 0.10). Excitatory boutons in the stratum radiatum were significantly smaller than those in the cell body layer ($p < 0.02$), but like in the cell body layer, did not differ between groups ($0.06 \pm 0.005 \mu\text{m}^3$ oil, $0.07 \pm 0.004 \mu\text{m}^3$ estradiol). Although DCVs were much less common in stratum radiatum excitatory boutons (0.03 ± 0.01 DCVs per bouton oil, 0.06 ± 0.01 DCVs per bouton estradiol) than in cell body layer excitatory boutons, stratum radiatum DCVs were similarly small in size; over half (65 %) fell into the small (< 80 nm) category. Finally, unlike for inhibitory boutons, neither bouton volume nor the frequency of mitochondria differed between excitatory boutons containing DCVs versus lacking them in the cell body layer or stratum radiatum.

Discussion

The current study investigated estradiol-induced changes in large, presumably neuropeptide-containing, DCVs in the hippocampus with the aim of explaining previous observations that estradiol increases the NPY content of inhibitory boutons in CA1 and facilitates NPY release during seizures. We found that estradiol increases the number of large DCVs and the size of DCV cores in perisomatic inhibitory axonal boutons, but that it does not shift the location of these DCVs closer to the plasma membrane of boutons. The effects were specific

to large DCVs in inhibitory boutons; in the same tissue, estradiol had no effects on small DCVs or DCVs in excitatory boutons.

These ultrastructural observations build on previous findings. In Ledoux et al. (2009), we found that the same estradiol treatment used here increases the NPY content of inhibitory axonal boutons measured with double-label immunofluorescence and EM immunocytochemistry. Here, we investigated all DCVs, rather than those labeled for a specific neuropeptide. That estradiol increased the number of DCVs and diameter of DCV cores generally suggests that estradiol might regulate neuropeptides in addition to NPY. NPY is only one of several neuropeptides synthesized and released by inhibitory neurons in hippocampal CA1 (Jinno and Kosaka 2003; Baraban and Tallent 2004). Other neuropeptides found in inhibitory neurons and that also might be regulated by estradiol include CCK (Harris et al. 1985; Wyeth et al. 2010), vasoactive intestinal polypeptide (VIP; de Lanerolle et al. 1995; Acsady et al. 1996a, b), and somatostatin (Feldman et al. 1982, Kunkel et al. 1994; Tallent and Qiu 2008). Based on the density of axonal projections of each interneuron subtype in the CA1 cell body layer, NPY, CCK, and VIP are those most likely to be regulated by estradiol.

That estradiol increased the diameter of DCV cores suggests that estradiol may promote DCV maturation and increase the neuropeptide content of individual DCVs. DCVs are synthesized in stages, beginning with formation of immature secretory granules in the trans-Golgi network at the soma; immature granules are then thought to coalesce and develop into mature secretory granules (Morvan and Tooze 2008) as they are transported toward sites of release. As DCVs mature, they become larger, their cargo condenses, and they undergo membrane remodeling that promotes efficient exocytosis. The larger and apparently denser DCV cores we observed after estradiol treatment may reflect a greater population of mature DCVs available for release of neuropeptides upon stimulation. Interestingly, several proteins have been shown to influence DCV size and maturation, including the Rho guanine nucleotide exchange factors Kalirin and Trio (Ferraro et al. 2007) as well as vesicle membrane-associated adaptor proteins (Grabner et al. 2006). Isoforms of Kalirin (Ma et al. 2011) and membrane-associated adaptor proteins (Lee et al. 2004) are regulated by estradiol, but so far, no studies have addressed a connection between these effects of estradiol and DCV maturation.

We also found that more inhibitory axonal boutons contained DCVs in estradiol-treated than control animals. Theoretically, these additional DCVs could result from increased somatic synthesis and transport of DCVs into axons and/or through mobilization of existing DCVs from non-bouton axonal segments into boutons. On the one hand, 24 h estradiol treatment has been shown to increase NPY mRNA and peptide levels in hippocampal CA1, suggesting increased NPY synthesis (Nakamura and McEwen 2005). However, the same study also showed that estradiol does not affect protein levels or mRNA for CCK, indicating that estradiol does not upregulate neuropeptide synthesis in the hippocampus generally. Given this, transport of DCVs to boutons from neighboring axonal compartments likely contributed to the general increase in DCVs per bouton that we found. Interestingly, large DCV transport in axons has been shown to be activity-dependent. Stimulation of cultured trigeminal neurons with KCl (to depolarize them) increased the fraction of DCVs moving in

an anterograde direction, though it also decreased their instantaneous velocity (Sobota et al. 2010). Additional studies will be necessary to determine whether estradiol regulates the factors that control DCV maturation and/or their movements within axons.

Some DCVs in the hippocampus contain the α form of the estrogen receptor (ER α ; Ledoux et al. 2009; Tabatadze et al. 2013) suggesting that they could be directly responsive to estradiol. By analogy to ER α -positive small vesicles in inhibitory boutons, which are mobilized toward synapses by estradiol (Hart et al. 2007), we investigated whether estradiol mobilizes large DCVs toward potential release sites at the bouton periphery. Such an effect might account for facilitation of neuropeptide release during seizures. However, we found no evidence that estradiol shifts the location of DCVs closer to the bouton periphery. Thus, while estradiol mobilization of DCVs from axons into boutons is a likely possibility, estradiol does not appear to mobilize DCVs within boutons themselves. The mechanism(s) by which estradiol facilitates NPY release during seizures therefore remains to be explained. This could be an effect specific to NPY-containing DCVs that was not detectable in the general DCV analysis done here, or estradiol might regulate activity-dependent Ca^{++} influx or the coupling between Ca^{++} influx and DCV exocytosis (Verhage et al. 1991; Xia et al. 2009). These possibilities remain to be tested.

In summary, we have found that the same estradiol treatment shown previously to suppress hippocampus-dependent seizures through an NPY-dependent mechanism also regulates large, presumably neuropeptide-containing DCVs in inhibitory neurons in the hippocampus. The effects that we observed suggest mobilization of DCVs from non-bouton sites in axons into axonal boutons and increased neuropeptide content of DCVs, both of which could increase the quantity of neuropeptide released from inhibitory neurons during seizures. Estradiol does not appear to mobilize DCVs within inhibitory boutons, however, or to regulate DCVs in excitatory boutons.

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Fig. 1. Estradiol increases the number of dense core vesicles (DCVs) in perisomatic inhibitory axonal boutons. Representative electron micrographs (**a1**, **b1**) and 3D reconstructions (**a2**, **b2**) showing DCVs in inhibitory boutons from an oil-treated control (**a**) and estradiol-treated (**b**) rat. Inset in **b1** shows a second DCV in the same bouton in the estradiol-treated rat. For 3D reconstructions, axonal boutons are *green*, somatic membranes are *gray*, and DCVs are *black*. The same DCVs are indicated by arrowheads in (**a**) and (**b**). Scale bar in (**b1**) indicates 100 nm and applies to **a1** and **b1**. Bar graphs showing mean $\pm$ SEM number of

large DCVs (L-DCVs) per inhibitory bouton in oil- vs. estradiol-treated rats (**c**), proportion of inhibitory boutons containing large DCVs (**d**), and number of large DCVs per inhibitory bouton considering only boutons with any DCVs (**e**). (**f**) Distribution of DCV diameters in inhibitory boutons in oil- and estradiol-treated rats. $*p < 0.05$, $**p < 0.01$, $n = 4$ rats per group

Fig. 2. Estradiol increases the diameter of dense core vesicle (DCV) cores in perisomatic inhibitory axonal boutons. (a–f) Representative electron micrographs showing DCVs in oil- (a–c) and estradiol-treated (d–f) rats. Scale bar in f = 100 nm and applies to a–f. Bar graphs showing mean $\pm$ SEM diameter of DCVs cores for all DCVs (g) and large DCVs specifically (h). * $p < 0.05$, $n = 4$ rats per group