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Oestrogen effects in olivo-cerebellar and hippocampal circuits

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Abstract. 17β-oestradiol (E2) is known to exert activating effects on CNS excitability, which are in part mediated by increases in glutamate responses, as we have shown in cerebellum. In addition, this steroid is known to facilitate rapid, rhythmic limb movement. Because the inferior olive is believed to be a timer of rapid movement, we have investigated effects of E2 on patterns of discharge recorded from dorsal accessory olive (DAO) using chronically implanted microwires. E2 increases the frequency of rhythmic olivary discharge as well as the number of synchronized neurons in association with facilitation of rhythmic limb and vibrissae movement. One possible mechanism for this effect is via an increase in gap junction proteins, as olivary cells are electrotonically coupled. Levels of connexin 32 (Cx32) and the dendritic lamellar body, both markers for gap junction-associated proteins, are increased threefold after 48 h E2 exposure (2 μg, i.p.), compared to control in both ventral medulla and hippocampal neurons. Gap junction conductance has also been shown to be decreased by γ-aminobutyric acid (GABA)ergic input. For this reason, we tested effects of 48 h E2 treatment on $GABA_A$ receptor subunit proteins and GABAergic synaptic current. E2 increased levels of the α4 subunit in hippocampus via an increase in the GABA-modulatory progesterone metabolite 3α-OH-5α-pregnan-20-one. This effect was correlated with a decrease in decay time of tetrodotoxin-resistant miniature inhibitory postsynaptic currents (mIPSCs) recorded from pyramidal cells in CA1 hippocampus, an effect which would tend to reduce total GABA inhibition. In sum, these effects of E2 are consistent with the concept that E2 exerts primarily activating effects on CNS excitability.

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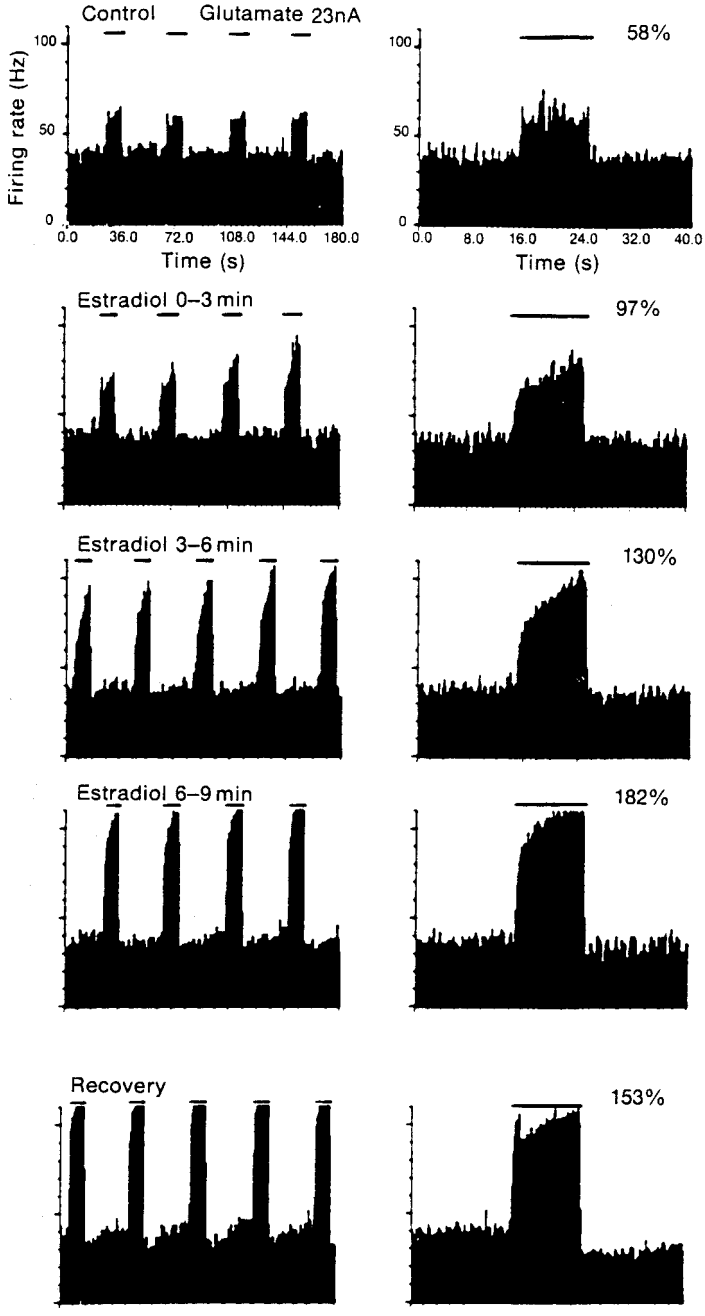
In addition to classic effects on reproductive function, 17β-oestradiol (E2) is known to be associated with a variety of excitatory effects in the CNS. Behaviourally, this is evidenced by activation of an array of sensorimotor parameters, including improved limb coordination, balance and sensory perception (Hampson & Kimura 1988, Smith & Chapin 1996a,b). The word 'oestrous', now used to denote the stage of the cycle following peak levels of

circulating E2, was initially used to describe the sensorimotor activation observed at this time, and literally means 'frenzy'. A number of clinical studies suggest that increased levels of E2 in the circulation are also correlated with 'activating effects' on mood, memory and attentional mechanisms (Ross et al 1998). Further evidence for this activating action of E2 is provided by reports of its proconvulsant action. Cyclic elevations in circulating E2 can exacerbate ongoing convulsive activity, although this effect is dependent upon the seizure subtype (Bäckström 1976, Herzog et al 1997). E2 administration can also facilitate the acquisition of kindled seizures in the dorsal hippocampus in experimental animals (Buterbaugh & Hudson 1991). This proconvulsant action appears to be direct, as local application of E2 to the surface of the cat cerebral cortex induces focal seizures characterized by 2–3 Hz spike and slow wave discharge (Marcus et al 1966).

Excitatory amino acid receptors

These excitatory effects of E2 on the motor system may be due to effects of the steroid on neurotransmitter receptor systems of relevant sensorimotor areas, such as cerebellum and basal ganglia. Early results from this laboratory demonstrated that E2, applied locally by pressure ejection or systemically at physiological concentrations, can enhance excitatory responses of neurons within the cerebellum (Purkinje cells) to excitatory amino acid (EAA) neurotransmitters, including glutamate, within minutes (Smith et al 1987a, see Fig. 1). This effect was specific for EAA receptor subtypes quisqualate and NMDA and was long-term, persisting for at least 6–8 h after exposure of the neuron to the steroid (Smith 1989). Although enhanced quisqualate responses were seen in ovariectomized animals and were not due to classic receptor activation, potentiation of NMDA responses by E2 was only observed under conditions of E2-priming (Smith 1989). Because the β form of the E2 receptor (ERβ) has been localized to the cerebellar Purkinje cell (Shughrue et al 1997), it is possible that the effects of chronic E2 are receptor-mediated. More recent studies have demonstrated that E2 can increase kainate responses of pyramidal cells in CA1

FIG. 1. Locally applied 17β-oestradiol (E2) augments Purkinje cell responses to glutamate. Strip chart records (left) and corresponding peri-event histograms (right) indicate changes in Purkinje cell response to glutamate before (upper records), during (middle records) and after (lower records) continuous pressure ejection of E2 (0.5 μmol/l in 0.01% propylene glycol-saline) at 1–2 p.s.i. Each histogram sums unit activity from 4–5 glutamate pulses (solid bar, 23 nA) of 10 s duration, occurring at 40 s intervals. Glutamate-induced excitation is indicated as a percent change in firing rate relative to spontaneous discharge (numbers next to bars). Purkinje cell responses to glutamate were significantly enhanced within seconds after the onset of E2 application, and did not recover to control levels of response by 30 min after termination of steroid application. (Reprinted with permission from Smith et al 1988, © Elsevier Science.)


Control
Glutamate 23nA
58%
Firing rate (Hz)
100
50
0
0.0 36.0 72.0 108.0 144.0 180.0
Time (s)
0.0 8.0 16.0 24.0 32.0 40.0
Time (s)
Estradiol 0–3 min
97%
Estradiol 3–6 min
130%
Estradiol 6–9 min
182%
Recovery
153%

hippocampus while longer-term treatment is also able to increase NMDA responses in this CNS site (Wong & Moss 1992, Foy et al 1999), presumably as a result of increases in NMDA binding via an increase in NR1 subunit levels (Gazzaley et al 1996, Weiland 1992a). In contrast, the rapid effects of this steroid appear to be mediated by a G protein-coupled mechanism (Moss & Gu 1999). The outcome of this increase in excitatory tone is an increase in dendritic spine density in CA1 hippocampus functionally resulting in an increase in the gain of the input/output relationship and facilitation of long-term potentiation (Woolley & McEwen 1992, Woolley et al 1997, Murphy et al 1998, Foy et al 1999). Increases in synaptogenesis in this region are seen on pro-oestrus as evidenced by the number of free postsynaptic densities (Desmond & Levy 1998). In contrast, in striatum E2 is able to increase dopamine release through novel membrane effects (Xiao & Becker 1998). Together these findings suggest a global effect of this steroid on excitatory neurotransmitter systems.

Contrast enhancement of olivo-cerebellar circuits

More recent studies in this laboratory have focused on the effects of E2 on olivo-cerebellar networks. Across the rat oestrous cycle, elevations in circulating levels of E2 are followed closely by increases in progesterone. As steroids delivered by the circulation, effects of these lipophilic molecules would be evidenced at the network level rather than at single synapses. Therefore we have examined effects of combined hormone administration on networks of neurons recorded from chronically implanted bundles of electrodes in the cerebellar Purkinje cell layer, as well as the afferents from the inferior olivary nucleus (Smith & Chapin 1996a,b). Our earlier studies (Smith et al 1987b) demonstrated that systemic injection of physiological concentrations of progesterone enhances γ-aminobutyric acid (GABA)-mediated inhibition of Purkinje cell discharge recorded extracellularly via local conversion to the GABA-modulatory metabolite, 3α,5α-THP (3α-OH-5α-pregnan-20-one). In combination with the EAA responses enhanced by E2, the dual action of both hormones would increase the contrast of neuronal responses to both excitatory and inhibitory input, an effect we have demonstrated both in the cerebellum and inferior olivary nucleus (Smith & Chapin 1996b). Circuits involving the rostral dorsal accessory olive (rDAO) are believed to signal errors or motor event changes to the cerebellum (Gellman et al 1985). Acting as a selective sensory filter, this structure gates out sensory input during active movement presumably via GABAergic inhibition. During non-movement, input, reflecting motor error or event change, is gated in via direct glutamatergic input from sensory afferents. Assessment of single unit responses to peripheral stimulation indicates that E2 and progesterone enhance both the excitatory response to afferent input during non-movement, as well as the

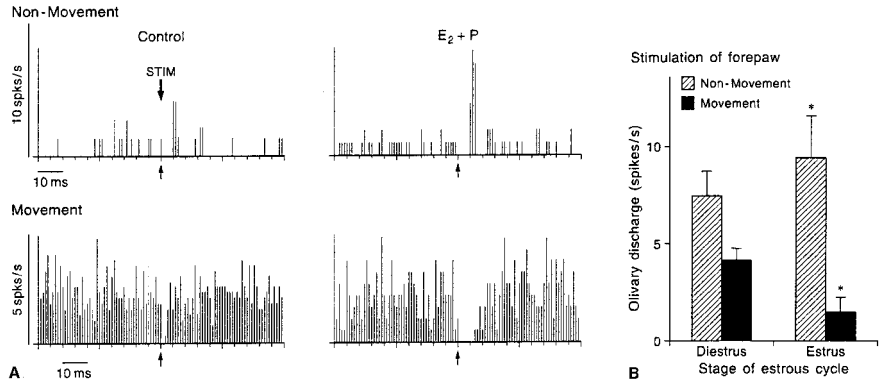


FIG. 2. Contrast enhancement of olivary responses to forepaw stimulation after administration of oestrus hormones. (A) Representative histograms from the same cell illustrate the effects of E2 and progesterone (P) on responses of rostral dorsal accessory olivary (rDAO) neurons to forepaw stimulation during movement and non-movement of this limb. Non-movement-dependent responses (upper panel) were enhanced by administration of oestrus hormones (right). During forepaw movement (lower panel), suppression of these responses lasted longer after hormone treatment (right) than in the control case (left). Hormones (30 ng E2 + 50 μg P) were administered i.p. 20 min before testing and 24 h after an initial priming dose of 2 μg E2 to replicate oestrus conditions. (B) Sensorimotor gating of the rDAO across the oestrous cycle: a summary diagram of responses to forepaw stimulation. On the night of behavioural oestrus, following elevations in circulating E2 and P, the difference or 'contrast' between responses during movement and non-movement is enhanced. ($n=15$ neurons, 3 rats). (Reprinted with permission Smith & Chapin 1996a, © Springer-Verlag.)

inhibitory response during movement, compared to results obtained during dioestrus (Smith & Chapin 1996a,b, see Fig. 2). Similar changes were observed on the night of behavioural oestrus. Such an effect would be expected to sharpen the 'contrast' of responses elicited by expected and unexpected input. Thus, oestrous-enhanced error signalling by the rDAO might be expected to increase the resolution for detection of motor errors by this structure, an effect consistent with reports of improved motor coordination associated with the night of behavioural oestrus (Smith & Chapin 1996a,b).

Rhythmic discharge

In addition to more general effects on sensorimotor performance, E2 is known to be associated with facilitation of rapid, rhythmic movements of the limbs and digits. In human studies, finger tapping frequency increases during the midcycle peak in E2 (Becker et al 1982), as does the ability to accurately perform other repetitive rapidly alternating tasks such as repeating vowels, typing and the

peg-and-board task (Broverman et al 1968, Hampson & Kimura 1988). E2-deficient girls with Turner's Syndrome experience significant improvement in the speed of both repetitive, non-guided tasks and spatially guided motor tasks following E2 therapy (Ross et al 1998). Results from the present laboratory demonstrate that following 48 h of E2 exposure (2 μg, i.p.), both limb stepping and vibrissae movement (whisking) are faster, with a more consistent frequency (Smith 1998). One potential site for E2 in producing these effects is the inferior olivary nucleus. In addition to functioning as an error signalling device, this structure is reported to act as a putative timing device for coordinated movement due to its ability to support subthreshold oscillations of membrane potential (Llinás & Yarom 1986). These oscillations may then generate rhythmic spike trains upon appropriate sensorimotor stimulation, in phase with the endogenous membrane potential fluctuations (Lang et al 1996). Membrane oscillations of individual neurons are dependent upon a low-threshold Ca^{2+} spike and can be modified by K^+ currents associated with anomalous rectification (Llinás & Yarom 1986). *In vivo*, however, oscillations are an emergent property of rhythmically firing somatotopically aligned neurons coupled via gap junctions (Lang et al 1996). For this study, we tested the hypothesis that E2 enhances synchronized, rhythmic discharge of neurons within the inferior olive in conjunction with rhythmic movement of the limbs and vibrissae. Towards this end, female rats were implanted bilaterally with two eight-microwire (25 or 50 μm) bundles into the limb and vibrissa area of the DAO (Smith 1998). In addition to determination of the sensory receptive field and characteristic waveform, cells were identified by their ability to follow high frequency antidromic stimulation from the contralateral paravermal cerebellum (Smith & Chapin 1996a). DAO discharge was recorded simultaneously from up to 48 neurons during treadmill locomotion or spontaneous whisking (rhythmic protraction and retraction of the vibrissae) before and after administration of E2, either systemically or locally applied.

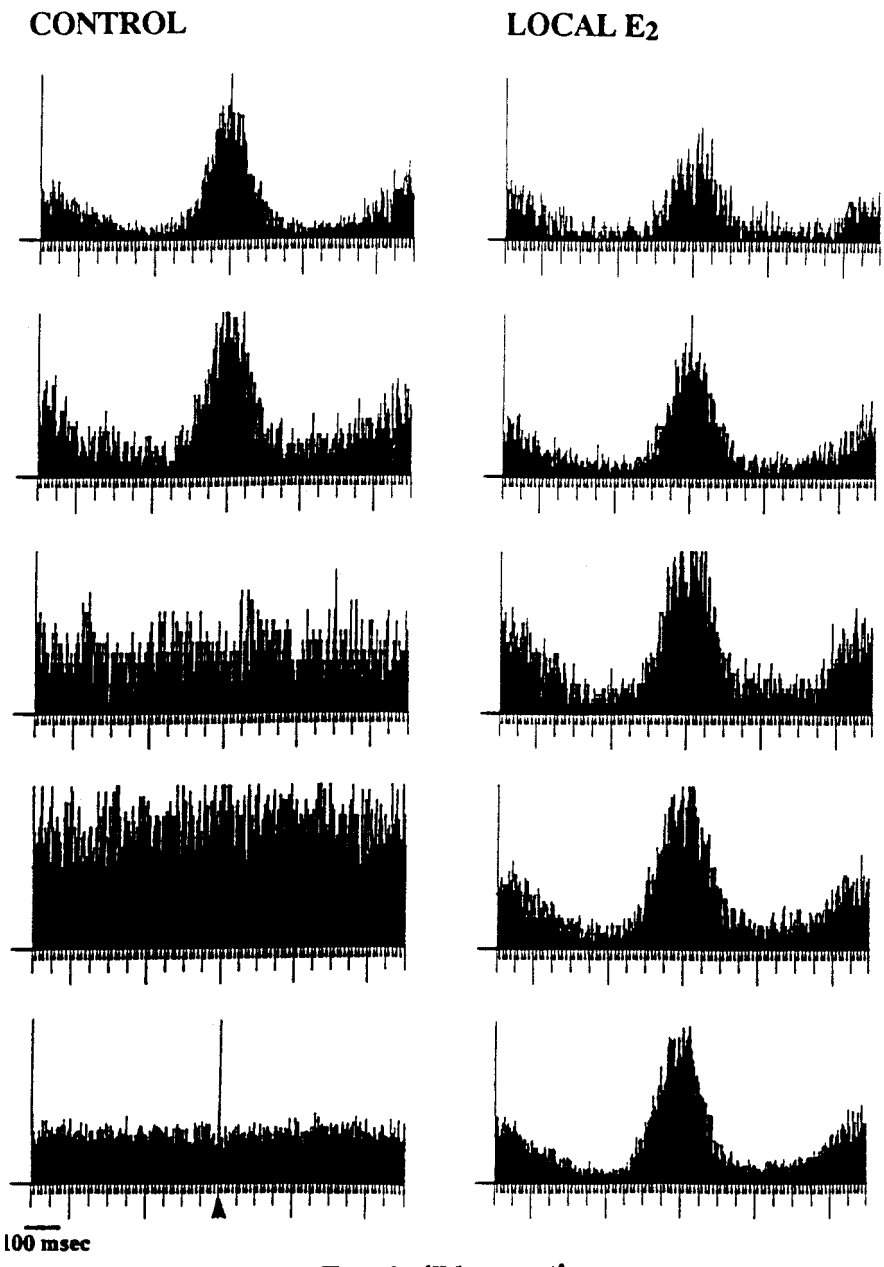
For the first study, whisker-responsive DAO neurons were recorded during whisking behaviour and rhythmicity assessed across hormone state. The spatial extent of coupling was also determined by examining the diameter occupied by neurons firing synchronously. [Synchronization here is defined by the presence of a 1 ms peak at the cross-correlation node, with an $r > 0.1$, $P < 0.0005$, where $n = 10^3$–10^5 spikes, using correlogram analysis.] Characteristically, whisker-responsive DAO neurons discharge with a rhythm time-locked to whisker movement (7–9 Hz). Following treatment with E2, whisking frequency was consistent at 8.5 Hz, with no deviations (SEM, 0; variance, 0). In contrast, on dioestrus, a highly variable whisking frequency was observed (5–10 Hz), with a lower average frequency than observed on oestrus (6.9 ± 0.60) and a greater variance (3.08, dioestrus; 0, oestrus). E2 treatment produced similar

effects on DAO discharge patterns, resulting in a faster, more consistent rhythmic DAO discharge frequency (mean, 8.5; variance, 1.91) versus dioestrus values (mean, 7.4; variance, 8.82; $P<0.05$). In addition, the amplitude of the oscillation was increased 86% by E2, while trough amplitude was depressed by 80%, also suggesting that DAO rhythmicity is more consistent at this time, a result similar to that observed for the forepaw area of the DAO. Further analysis with interspike interval histograms and autocorrelation raster analysis demonstrates that the interspike interval exhibits more peaks (3.5 vs. 1.5, $P<0.05$) of shorter duration (98 vs. 265 ms, $P<0.05$) following treatment with E2 than observed on dioestrus.

Two days of E2 treatment also produced maximal effects on synchronization of DAO oscillations, increasing the number of synchronized neurons to 90%, which effectively increased the coupling diameter by $150\pm45\,\mu$m ($P<0.001$). The correlation coefficient of coupling was also increased from 0.1 to 0.148 following E2 treatment ($n=4.5\times10^5$, 2 ms bins/cell, $P<0.05$).

These results suggest that the effect of E2 in increasing the extent of rhythmic, synchronized DAO discharge is associated with facilitation of rhythmic whisker movement. To discern more completely the relationship between DAO discharge patterns and rhythmic whisking behaviour, we also compared cross-correlation analysis of DAO discharge during periods of active whisking (rhythmic movement) versus non-whisking and across hormone state. E2 treatment resulted in rhythmic DAO discharge (8.5 Hz) during periods of both whisking and non-whisking, while dioestrus conditions were associated with rhythmic discharge dependent upon rhythmic whisker movement. These results suggest that E2 effects on whisking, *per se*, are not producing the enhanced, synchronized discharge observed from DAO recordings.

Rhythmic discharge of olivary neurons was also tightly coupled to step cycle rhythmicity during treadmill locomotion under control conditions. Increases in the number of synchronized, rhythmically discharging neurons following oestrus hormone treatment produced an increase in the spatial extent of the oscillating neuronal cluster recorded during locomotion. When recorded during treadmill locomotion at speeds of 11 cm/s under control dioestrus conditions, the average diameter of a coupled cluster was $50\pm10\,\mu$m. Local administration of E2 increased the coupling diameter by $150\pm20\,\mu$m compared to dioestrus values ($P<0.05$, see Fig. 3), suggesting that E2 produces direct effects on the DAO. In a similar manner, cyclic increases in circulating oestrus hormones on oestrus increased the diameter of the oscillating cluster by $100\pm11\,\mu$m (light phase, $P<0.01$) and $151\pm12.5\,\mu$m (dark phase, $P<0.01$); administration of E2 + progesterone to an E2-primed rat replicated oestrus conditions in that the spatial extent of rhythmic DAO discharge was increased by $135\pm5.4\%$ ($P<0.01$).


CONTROL
LOCAL E2
100 msec
Treadmill locomotion
(variable acceleration)

Gap junction proteins

Coupling between olivary neurons is accomplished via dendrodendritic gap junctions (Lang et al 1996). Gap junction conductance is modulated by both NMDA receptors (Pereda & Faber 1996) and GABAergic input (Lang et al 1996), systems which are in turn altered by chronic E2 exposure (Smith 1989, Murphy et al 1998, Weiland 1992b). However, more compelling evidence for E2-modulation of gap junctions is found in a number of reports demonstrating that E2 can increase formation of gap junction proteins in uterus and liver via genomic as well as non-genomic mechanisms (Grümmer et al 1999). In addition, the Cx43 gene contains an E2 receptor response element (Yu et al 1994), suggesting a transcriptional effect of E2 on this gap junction protein. In order to determine a possible mechanism for the observed E2 facilitation of rhythmic DAO discharge, we tested the hypothesis that connexin 32 (Cx32), a marker for neuronal gap junction proteins, and the dendritic lamellar body (DLB), a specific marker for dendrodendritic gap junctions, are increased by E2. As before, female rats were treated with E2 (2 μg, i.p.) or vehicle for 2 d before testing, in some cases in conjunction with sustained periods of treadmill running. Western blot analysis of connexin band density revealed a profound effect of both hormone and locomotor activity on connexin levels. Cx32 band staining was evident as a single band at 38 kDa, as previously described. Cx32 levels were highest in rat both exposed to E2 and rhythmic treadmill running (300% increase above control; $P < 0.001$). Cx32 levels in rats only treated with E2 were threefold higher than control ($P < 0.05$). Treadmill locomotion alone produced no significant effect on Cx32 levels. Staining with the DLB antibody revealed a single band at 110 kDa. Levels of the DLB were increased 175% by E2 + treadmill running ($P < 0.05$); E2 alone increased DLB levels by 120% ($P < 0.05$). Treadmill locomotion alone increased DLB levels by 52%. In contrast, Cx43 levels were increased only by E2 treatment (200% increase above control levels, $P < 0.01$) but not by treadmill locomotion + E2. Cx26 levels were not altered by either hormonal or behavioural state. All changes in Cx band density observed were unaccompanied by corresponding changes in levels of the control protein, GAPDH.

Although less well established, gap junctions are known to exist in the adult hippocampus, where they may play a role in mediating high frequency (200 Hz)

FIG. 3. Locally applied E2 enhances olivary oscillations. Correlograms of olivary discharge recorded during a variable acceleration paradigm before (left) and 20–40 min after (right) local infusion of 600 pM E2 to an E2-primed progesterone-treated rat. Locally applied E2 increased from two to five the number of synchronized, oscillating neurons compared to control values. Control records were obtained 20–40 min after infusion of vehicle alone. These results are representative of 15 neurons recorded in three rats. (Reprinted with permission from Smith 1998, © Elsevier Science.)

oscillations (Draguhn et al 1998). Two-day treatment with E2 also significantly increased band density for both DLB and Cx32 (by twofold and fivefold, respectively), suggesting an additional role for this hormone on electrotonic coupling and oscillation states.

In the inferior olive, E2-induced increases in levels of both Cx32 and DLB suggests an increase in dendrodendritic gap junctions. Oscillatory discharge of the DAO is an ensemble-like property which requires both an intrinsic pacemaker potential at the cellular level as well as electrotonic coupling of neuronal populations by gap junctions (Lang et al 1996). Thus, these results suggest that stimulatory effects of E2 on rhythmic DAO discharge and facilitation of rhythmic movement may be mediated by an increase in gap junction density. The results from this study not only have implications for hormonal control over the DAO circuit, but suggest that E2-induced increases in high frequency oscillations may be more of a global CNS phenomenon.

$GABA_A$ receptor subunit composition

In addition to its established activating effects via EAA receptors and gap junction formation, another possible activating effect of this steroid is via decreases in GABA inhibition. There is evidence that E2 treatment can alter levels of GAD, the GABA synthesizing enzyme in areas including CA1 hippocampus (McCarthy et al 1995, Murphy et al 1998, Weiland 1992b). Our recent results suggest that, in CA1 hippocampus, 48 h exposure to E2 decreases GABA inhibition via up-regulation of the α4 subunit of the $GABA_A$ receptor (GABA-R). Our previous findings suggest that abrupt discontinuation after chronic exposure to the GABA-modulatory metabolite 3α,5α-THP increases CNS excitability via α4 GABA-R subunit up-regulation (Smith et al 1998). This increase in excitability was evidenced by increases in seizure susceptibility and can be explained mechanistically by decreases in the decay time for GABA-gated current which would decrease total charge transfer. Both endpoints were prevented after α4 expression was suppressed using antisense technology (Smith et al 1998). Because our preliminary data suggest that 48 h exposure to 3α,5α-THP also increases GABA-R subunit levels, we tested 48 h E2 treatment on this system because this steroid is also known to enhance conversion of progesterone to 3α,5α-THP due to its facilitating effect on the 5α-reductase enzyme. E2 is able to increase levels of the α4 subunit by two- to threefold after injection of 0.4–20 μg/kg for two days (i.p.) to female adult rats, with maximal effects at 4–8 μg/kg of the steroid (Fig. 4). A dose of 8 μg/kg E2 significantly increased hippocampal levels of 3α,5α-THP (2.1 ± 0.07 ng/g) above dioestrus, control values (1.59 ± 0.19 ng/g; $P < 0.04$), assessed using radioimmunoassay procedures. These results suggest

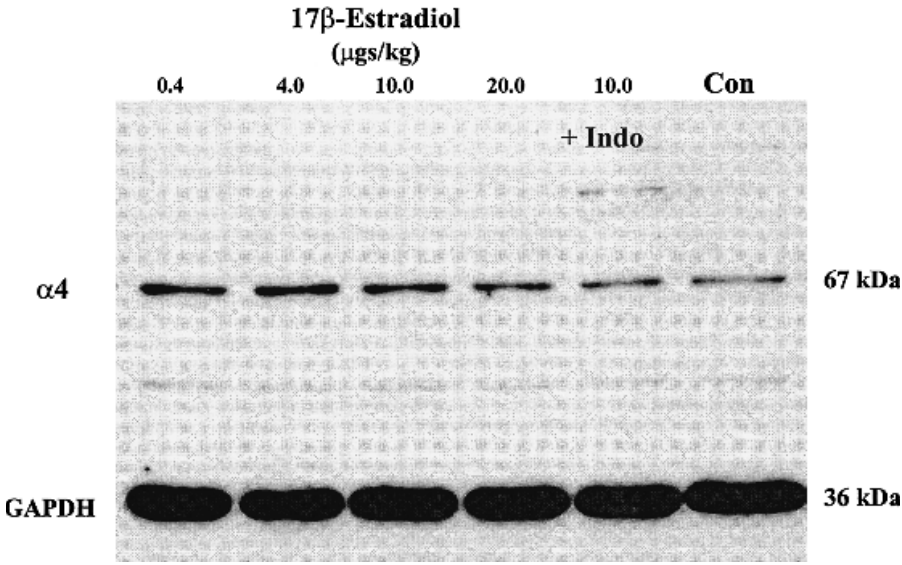


FIG. 4. E2 increases levels of the α4 subunit of the $GABA_A$ receptor. Two-day treatment with E2 (2 μg, i.p.) increased significantly levels of the α4 subunit in hippocampus compared to control, dioestrus values. In contrast, no changes in GAPDH protein were noted. These results suggest that chronic E2 treatment alters GABA-R subunit composition ($n=3$ rats/group, performed in triplicate).

that up-regulation of the α4 subunit in the CA1 hippocampus can be accomplished by E2.

Synaptic current

In order to assess the impact of chronic E2 exposure on GABA physiology, we recorded GABAergic synaptic current from pyramidal cells in CA1 hippocampus following 48 h E2 treatment (8 μg/kg, i.p.). Tetrodotoxin (TTX)-resistant miniature inhibitory postsynaptic currents (mIPSCs) were recorded using the whole cell approach at −70 mV at 34–35 °C and the data were analysed using software developed in the laboratory of H. Korn (Ankri et al 1994). mIPSCs represent the minimal (quantal) postsynaptic response to transmitter released from a single vesicle. Therefore, mIPSC characteristics are not complicated by factors which would influence evoked responses, such as the number or pattern of vesicle release, and would not unphysiologically activate extrasynaptic GABA-R. Frequency, peak amplitude and kinetics of mIPSCs were determined

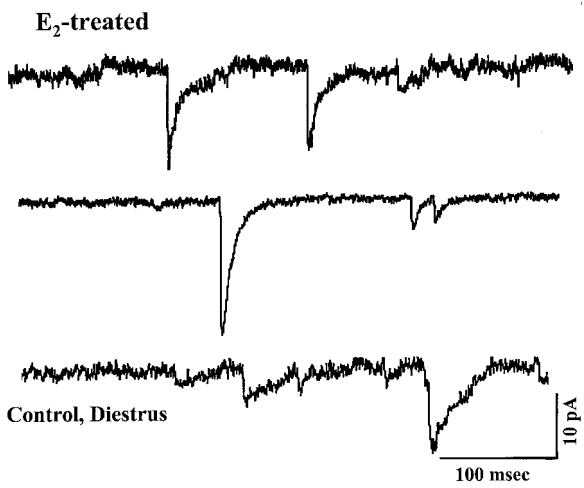


FIG. 5. E2 decreases the decay time constant of mIPSCs recorded from CA1 hippocampus. Individual traces of mIPSCs recorded from CA1 hippocampus at −60 mV at room temperature (27 °C) using whole cell recording techniques in the hippocampal slice preparation. Following two days of E2 treatment (2 μg, i.p.) mIPSCs are of shorter duration, but reflect a similar amplitude distribution as observed on the day of dioestrus. mIPSC frequency was slightly decreased after E2 treatment.

in cells from E2-treated animals and compared to those from control, vehicle-treated dioestrus rats. Decay time constants for mIPSCs were analysed as either mono- or biexponential decay time constants using non-linear curve fitting routines and least squares approximations (Fig. 5). Under control conditions, spontaneous mIPSCs were low amplitude (5–40 pA), infrequent events (6–8 Hz). A kinetic analysis revealed a bimodal population with a larger population (90%) decaying with a monoexponential decay time constant (τ) = 3.88 ± 0.56 ms. A smaller population (10%) decayed with a biexponential decay; $\tau f = 0.8 \pm 0.33$, $\tau s = 5.2 \pm 1.2$ ms. Following treatment with E2, there was a striking increase in the percentage of cells exhibiting the biexponential decay time (30–40%), and a significant decrease in the slow τ compared with control; $\tau f = 0.9 \pm 0.43$ ms, $\tau s = 3.7 \pm 0.43$ ms. A significant decrease in τ was also noted for the monoexponentially decaying time constant ($\tau = 2.77 \pm 0.32$ ms). However, mIPCs amplitude distribution was not altered by E2 treatment. The significant decrease in decay time overall would tend to decrease total GABA inhibition. Such a change is consistent overall with the concept that increases in neuronal excitability follow elevations in circulating levels of E2.

Global activation of CNS circuits by E2, via increased glutamate excitation and reduced GABA inhibition, may result in significant facilitation of processing, both

for sensorimotor systems, as well as for higher cognitive events. Theoretical analysis of oscillating, excitatory systems such as would predominate under high E2 conditions suggests that faster processing of input may result. However, under conditions where both E2 and progesterone are present, contrast enhancement of excitatory versus inhibitory input may lead to finer resolution of problem-solving processing.

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DISCUSSION

Simpkins: Have studies been done in either female athletes or musicians, looking at the stage of menstrual cycle and performance?

Smith: That's an interesting question. I have heard on one study that was fairly anecdotal, which was a study of gymnasts that showed improved performance with higher oestrogen levels. I don't know if this was ever published. The other studies I know about have to do with fine movements such as typing.

Simpkins: And do they change over the menstrual cycle?

Smith: Rapid movements such as finger tapping, walking, typing and repeating vowels are facilitated during the mid-cycle peak in oestradiol.

Herbison: In models of neural networks, the most efficient way of increasing the output of network is to increase both the excitatory and the inhibitory inputs (see Nelson & Turrigiano 1998). To alter one without the other is highly destabilizing. What you have shown here is a nice biological demonstration of this hypothesis.

Smith: That is a good point. Enhancing the contrast of both excitatory and inhibitory input as we have shown may be especially relevant for on-beam versus off-beam activity in cerebellum or centre–surround activity in the visual system. However, achieving a balance between excitatory and inhibitory events, as you suggest, would also ensure stability.

Murphy: A quick comment on your structural evidence for gap junctions with connexins, which I think is very interesting. I did some studies on hippocampal slice cultures with Lucas Pozzo-Miller. Although unpublished, we found an increase in the structural proteins N-cadherin and β-catenin in hippocampal synapses from slice cultures after oestrogen treatment. From a perspective of oestrogenic effects on synaptogenesis, it's very interesting that structural modifications occur that may enhance the function of the synapses electrically.

Smith: That is an interesting finding and certainly suggests that oestrogen produces structural changes in addition to its modulating effects.

Gustafsson: Benita Katzenellenbogen (Urbana, Illinois) has studied effects of oestrogens on MCF-7 cells. Using differential display she has picked up a couple of interesting target genes. One of them is what she calls EBP50 (ERM-binding phosphoprotein), which is a protein that seems to bind to a family of structural membrane proteins. Interestingly, she thinks that this sort of potential morphological effect of oestrogen is perhaps mechanistically related to one of the phenotypes of the ERKO mice recently described in *Nature* by Rex Hess, where it was found that there is less reabsorption of fluid in the epididymal ducts, owing to morphological changes (Hess et al 1997). This leads to a back-pressure in the testes which ultimately destroys sperm production. Those are yet other examples of interesting effects of oestrogens on structural proteins in the membrane.

I would like to point to an interesting finding that we made when we studied expression of the various nuclear receptors in the brain. Take for example PPARα, which is activated by fatty acids and prostaglandin, and LXRα and β, which are activated by oxysterols. These nuclear receptors and many others that we have studied seem invariably to be more highly expressed in two structures

in the brain, namely the hippocampus and the cerebellum. I wonder about the specificity of these phenomena. If you gave ligands like WY14643, which is a PPARα agonist, do you think these parameters of locomotion would be affected?

Smith: I guess you're asking about the specificity of our effect. Initially we did see specificity in some of our initial studies investigating oestrogen effects on glutamate responses of cerebellar Purkinje cells. In this case, oestrogen exerted rapid effects (<1 hour) in potentiating glutamate responses. This effect was specific for 17β-oestradiol (not 17α), but was not blocked by tamoxifen or protein synthesis inhibitors, suggesting that it is *not* acting through a classic steroid mechanism. We are just beginning to look at our effects on gap junctions. We have started to use oestrogen blockers to see if this is really a classic effect.

Baulieu: Have you tested progesterone specifically on the gap junctions?

Smith: If you give progesterone with oestrogen it doesn't really seem to change our effect. We haven't looked at progesterone alone on the gap junctions, but we have looked at progesterone alone just on the olivary oscillations. I don't know whether it makes it worse, but it certainly doesn't do anything.

Baulieu: What mechanism do you postulate for the effect on α4?

Smith: Our initial finding was that it was an effect of chronic exposure and withdrawal. Interestingly, other GABA modulators, such as benzodiazepines and alcohol given chronically, also up-regulate α4, so it may be a kind of homeostatic mechanism to allow the $GABA_A$ receptor to be less inhibitory. It appears that the primary effect of an increase in the α4 subunit is to decrease the decay time of GABA-gated current, which would mean less Cl^- getting through the channel and less inhibition. If $GABA_A$ receptors are stimulated for a long time by one of these steroids, then a compensatory decrease in the inhibition would protect CNS circuits from becoming overly depressed.

I don't think it's going to be mediated by classic steroid receptor, because alcohol and benzodiazepines do the same thing. This makes me think that the mechanism is via the $GABA_A$ receptor.

Herbison: With regard to the effects of steroids on the hippocampus, have you looked at the effects of oestrogen and progesterone on $GABA_A$ subunits other than the α4? There was a paper a few years ago by Nancy Weiland looking at the effects of several steroids, including allopregnanolone, on several $GABA_A$ receptor subunits (Weiland & Orchinik 1995). Also, in our own experience, working on hypothalamic oxytocin neurons, we know that progesterone up-regulates the α1 subunit, and this is probably what makes that receptor sensitive to allopregnanolone. Is anything going on other than just the α4 subunit?

Smith: We have done most of this work with either progesterone or allopregnanolone; we've only recently tried oestrogen. We were actually surprised that oestrogen increased α4: we thought it would have no effect. Although we

haven't looked at alterations in other GABA-R subunits using oestrogen, we have with allopregnanolone. The most striking change is an increase in the δ subunit, which is a subunit that may alter sensitivity of the GABA-R to allopregnanolone. We see other small changes. We think we see a change in $\beta 2$, $\beta 3$; it looks like $\alpha 2$ and $\gamma 2$ are not changing, and I'm not sure about $\alpha 1$. We are getting conflicting results.

Gibbs: Do you know of any work that has looked at effects of oestrogen on saccadic eye movements? These are heavily influenced by GABA.

Smith: I know that Sundström et al (1997) are using this technique to look at GABAergic function, but I don't believe they have looked at oestrogen. They look at women with premenstrual syndrome (PMS), and the results are consistent with what I found. $\alpha 4$ is benzodiazepine insensitive, and they find that women with PMS have saccades which are relatively insensitive to benzodiazepine, whereas in normal women benzodiazepine treatment slows the saccades.

Gibbs: Could you comment on whether your results would lead to any predictions about interactions between hormone replacement and benzodiazepine action in women?

Smith: This is hard to say, because we only give oestrogen for 48 h and then we see this change. We haven't really got into all the possible mechanisms and what their repercussions may be. It is hard to say what longer treatment with oestrogen might do. It could be that longer treatment makes this effect go away, because this is what happens with progesterone. Progesterone has an odd multiphasic effect. After 2 d there is an increase in $\alpha 4$, which then gradually returns to normal. The oestrogen effect might just be a transient increase in response to a perturbation.

Murphy: We have been trying to look at the effects of progesterone on dissociated hippocampal cultures, with very heterogeneous results. Can you speculate a bit about whether you think that *in vivo* there exist subpopulations of GABAergic cells in the hippocampus that may diverge in their response to progesterone?

Smith: Absolutely. The populations of GABA receptors in the hippocampus are heterogeneous; we see different subunits. Although the subunit which is mainly responsible for altered allopregnanolone sensitivity, the δ subunit, is in low concentration, it's definitely there. This means that if you hit a cell that has that subunit, it would not respond to the same degree. I believe it is much more common in the granule cells than in the dentate gyrus, so if you're looking at everything including the dentate gyrus you should be hitting some populations which are not as sensitive.

Toran-Allerand: However, if you are making cultures of dissociated hippocampal cells, the cells used are very immature (prenatal), and many neurons

have not yet been born, so one may be missing the very neurons that may be important and should be responding.

Murphy: What I meant was that for every cell that we can record a significant response from, we find one or two cells from which we get no response.

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