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NEURONAL EXCITABILITY IN THE PERIAQUEDUCTAL GREY MATTER DURING THE ESTROUS CYCLE IN FEMALE WISTAR RATS

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Abstract—Extracellular recordings were made from output neurons in the dorsal half of the periaqueductal gray matter (dPAG) in urethane-anesthetized female Wistar rats. All the neurons were quiescent. A basal level of firing was therefore induced by continuous iontophoretic application of D,L-homocysteic acid (DLH). In the presence of the $GABA_A$ receptor antagonist bicuculline methiodide (BIC 0–30 nA) the DLH-induced firing increased further, revealing the presence of ongoing GABAergic inhibitory tone on the recorded neurons. The BIC-induced increase in firing rate was significantly greater in neurons recorded during estrus (Est) and late diestrus (LD) compared with proestrus (Pro) and early diestrus (ED) suggesting that GABAergic tone was lower in Est and LD.

I.v. injection of the panicogenic $(CCK)_B$ receptor agonist pentagastrin (PG, 40 $\mu g\ kg^{-1}$) produced an increase in firing rate in 12/17 (70%) of neurons tested in the dPAG. Iontophoretic application of PG (10–30 nA) also produced a current-related increase in firing rate in 73.6% of the neurons tested. The excitatory response was reduced during application of the selective CCK_B receptor antagonist β-[2-([2-(8-azaspiro[4.5]dec-8-ylcarbonyl)-4,6-dimethylphenyl]amino)-2-oxoethyl]-(R)-napthalenepropanoic acid (CR2945) (60 nA, $n=6$). The PG-evoked increase in firing rate was significantly greater in neurons recorded during Est and LD compared with during Pro and ED. Juxtacellular labeling with neurobiotin in eight neurons revealed multipolar cells 12–44 μm diameter with up to six primary dendrites. In three of eight neurons, a filled axon was present and coursed without branching toward the perimeter of the periaqueductal gray matter (PAG).

The estrous cycle-related change in responsiveness to BIC and PG suggests that the panic circuitry in the PAG may become more responsive to panicogenic agents during estrus and late diestrus as a consequence of a decrease in the intrinsic level of inhibitory GABAergic tone. The findings may have implications for understanding the neural processes that underlie the development of premenstrual dysphorias in women.

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Abbreviations: BIC, bicuculline methiodide; CCK, cholecystokinin; CR2945, β-[2-([2-(8-azaspiro[4.5]dec-8-ylcarbonyl)-4,6-dimethylphenyl]amino)-2-oxoethyl]-(R)-napthalenepropanoic acid; DLH, D,L-homocysteic acid; dPAG, dorsal half of the midbrain periaqueductal gray matter; ED, early diestrus; Est, estrus; HR, heart rate; LD, late diestrus; MAP, mean arterial pressure; PAG, periaqueductal gray matter; PB, phosphate buffer; PG, pentagastrin; Pro, proestrus; PSB, Pontamine Sky Blue; RR, rate of respiration.

Key words: estrous cycle, GABA, panic, periaqueductal grey, CCK_B receptors, rat.

In women, the premenstrual (late luteal) phase of the menstrual cycle is commonly associated with psychological changes that include irritability, anxiety and mood swings (Halbreich, 2003). Women afflicted by other disease states, e.g. catamenial epilepsy, irritable bowel syndrome and panic disorder, also experience exacerbation of symptoms during the premenstrual period (Ensom, 2000). Cyclical changes in female behavior are not confined to humans. During diestrus, female Wistar rats also display increases in aggressive behaviors and heightened responsiveness to panic-inducing drugs (Olsson et al., 2003).

The premenstrual phase of the menstrual cycle in women and late diestrus (LD) in rats are both associated with a rapid fall in plasma progesterone (McLaughlan et al., 1987; Watanabe et al., 1990). Several studies have shown that a fall in progesterone precipitated by withdrawal from prolonged dosing with the steroid, leads to plasticity of $GABA_A$ receptor subunit expression in several brain regions. Upregulation of $\alpha 4$ and δ $GABA_A$ receptor subunit mRNA and protein levels has been reported in hippocampus, amygdala and periaqueductal gray matter (PAG) (Smith et al., 1998a,b; Gulinello et al., 2003; Griffiths and Lovick, 2005a). In the PAG a parallel increase in $\alpha 4$, δ and $\beta 1$ $GABA_A$ receptor subunit expression was also seen in rats during the LD phase of the estrous cycle (Lovick et al., 2005). Thus the natural fall in endogenous progesterone levels that occurs during LD appears to be a sufficient stimulus to trigger plasticity of $GABA_A$ receptor subunit expression in the PAG (Lovick, 2006).

The dorsal half of the periaqueductal gray matter (dPAG) contains neural circuitry that can initiate panic behavior. Stimulation in the dPAG in humans and in experimental animals evokes autonomic and behavioral signs of panic-like anxiety (Lovick, 2000). In humans panic can also be triggered by systemic injection of agents that are agonists at cholecystokinin $(CCK)_B$ receptors (e.g. van Megen et al., 1994). CCK_B receptor agonists are also panicogenic in rats and a major site of action appears to be the dPAG (Bertoglio and Zangrossi, 2005; Zanovelli et al., 2004). A recent study in anesthetized female rats has shown that the panic-like pattern of cardio-respiratory response evoked by i.v. injection of the CCK_B agonist pentagastrin (PG) was enhanced during LD (Brack et al., 2006). This suggests that the responsiveness of the panic circuitry to PG may change according to the stage of the estrous cycle. The central actions of CCK are thought to be linked to activity in GABAergic systems (Acosta, 2001;

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Ferraro et al., 2000; Ranovska, 1995; Perez de la Mora et al., 1993; Siniscalchi et al., 2003). Within the PAG, estrous cycle-related upregulation of α4, β1 and δ $GABA_A$ receptor subunit expression was confined to GABAergic interneurons (Griffiths and Lovick, 2005b). Thus it is possible that changes in the functional properties of the GABAergic cell population in the PAG during the estrous cycle are associated with changes in responsiveness of the panic circuitry to CCK_B agonists. In order to test this hypothesis, we investigated the functional excitability of output neurons in the PAG at different stages of the estrous cycle in anesthetized female rats.

Some of the results have been published in abstract form (Brack et al., 2004; Brack and Lovick, 2004; Jeffery et al., 2005).

EXPERIMENTAL PROCEDURES

All experiments were carried out on female Wistar rats (244.6±3.1 g body weight, n=50), approximate age 10–12 weeks. Animals were housed in pairs at 21±1 °C under a 12-h light/dark cycle (lights on at 7 a.m.) and were given free access to food and water throughout their maintenance. All procedures were undertaken in accordance with the University of Birmingham local guidelines on the ethical use of animals, the UK Animals (Scientific Procedures) Act 1986 and conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1985). All attempts were made to minimize the number and suffering of animals used.

Determination of the stage of the estrous cycle

Prior to starting any surgical procedures, a vaginal smear was taken from the unanesthetized animal and stained using a Quick-Diff FIX staining set (Reagena, Takojantie, Toivala, Finland) to determine the stage of the estrous cycle (Brack et al., 2006). Additional smears were taken from the anesthetized animal throughout the day to ensure that each animal remained in the same stage of the estrous cycle for the duration of the experiment.

Surgical preparation

Rats were anesthetized with urethane (0.5 ml 100 g, 20% solution i.p., Sigma, Poole, Dorset, UK) and instrumented to record femoral arterial pressure. Heart rate (HR) was derived from the ECG signal recorded from a chest electrode positioned at approximately V4. Respiratory rate was recorded using a spirometer (AD Instruments, Chalgrove, Oxfordshire, UK) attached to a tracheal cannula. Rectal temperature was maintained at 37 °C throughout the experiment via a heating blanket. A femoral vein was cannulated for administration of drugs and supplementary fluids: saline or the plasma substitute gelofusine (B Braun Medical, Sheffield, South Yorkshire, UK). Rats were held in a stereotaxic frame in the attitude described by Paxinos and Watson (1986). A craniotomy was performed and the dura was cut and reflected to expose the cortical surface overlying the PAG.

Once the surgical preparation was finished a stabilization period of at least 1 h was allowed before experimentation began. The depth of anesthesia was monitored throughout the experiment by testing corneal and pedal reflexes. Supplementary doses of sodium pentobarbitone (Sagatal, 1–3 mg i.v., Rhone Merieux, Harlow, Essex, UK) were given as needed to maintain a stable and consistent level of anesthesia.

Neuronal recording

Extracellular recordings of single unit activity in the PAG were made using five-barreled glass microelectrodes, overall tip diameter 5 μm. The barrels were filled respectively with 4 M NaCl for recording, 1% Pontamine Sky Blue (PSB) in 0.5 M sodium acetate (pH 7.7) for current balancing and marking recording sites, D,L-homocysteic acid (DLH, 0.1 M, pH 8.4) for activating neurons and two of the following: GABA (0.25 M, pH 4), the $GABA_A$ receptor antagonist bicuculline methiodide (BIC, 0.2 M, pH 4), the CCK_B receptor agonist PG (13 mM, pH 9) or the CCK_B receptor antagonist β-[2-([2-(8-azaspiro[4.5]dec-8-ylcarbonyl)-4,6-dimethylphenyl]amino)-2-oxoethyl]-(R)-napthalenepropanoic acid (CR2945) (Revel et al., 1998) (10 mM, pH 9). For barrels filled with GABA and BIC, a retaining current of −15 nA was applied in between ejection periods to prevent diffusion of drugs from the pipette tip. For barrels filled with DLH, PG and CR2945 the retaining current was +15 nA.

The electrode tip was positioned within the dorsal half of the PAG at sites between 5.6 mm and 8.7 mm caudal to bregma and between 3.5 mm to 5.5 mm below the cortical surface (Paxinos and Watson, 1986). Spike activity was amplified using a Neurolog amplifier (NL104, Digitimer, Welwyn Garden City, Hertfordshire, UK) and fed through a window discriminator and a histogram of firing rate was computed on-line. A four-channel iontophoretic device (Grayden Electronics, Birmingham, West Midlands, UK) was used to apply drugs directly in the vicinity of each recorded neuron. Recording sites were marked by iontophoretic deposition of PSB (5 μA for 10 min negative current). At the end of each experiment the brain was removed and fixed in 10% formol saline. Frozen sections 60 μm thick were stained with Neutral Red. The anatomical location of blue spots in the tissue was made with reference to the atlas of Paxinos and Watson (1986).

In conscious rats the excitability of the circuitry in the dorsal half of the PAG is normally subject to an ongoing inhibitory GABAergic influence (Schenberg et al., 1983; Brandao et al., 2005). Previous studies in anesthetized animals have reported that the majority of the neurons that could be recorded using multibarrel micropipettes, were quiescent (Lovick, 2001). In the present study searching for neurons was therefore carried out during continuous ejection of DLH (<5 nA) in order to induce neuronal firing. Once a single unit recording had been established the level of ejecting current for DLH was adjusted to produce an average firing rate of around 5 Hz. No neurons that were spontaneously active, i.e. firing in the absence of DLH, were investigated.

Juxtacellular labeling

In a separate group of animals the neurons from which recordings had been made were identified by juxtacellular labeling with neurobiotin (Pinault 1996). Initial attempts to entrain neuronal activity using traditional five-barreled micropipettes proved unsuccessful. We therefore constructed 'piggyback' electrodes as described by Jones et al. (2002) by gluing a single barrel pipette filled with 1.5% neurobiotin in 0.5 M sodium acetate (pH unadjusted, resistance 7–35 mΩ, mean 22.6±4.0 mΩ) to a five-barrel micropipette so that the single barrel protruded approximately 10 μm beyond the tip of the multibarrel micropipette. The barrels were filled respectively with DLH, PSB and a selection of the solutions described above. Searching for neuronal activity was carried out during continuous ejection of DLH (see above). Recordings were made using an intracellular bridge mode amplifier (model BA-1S, npi Electronic GmbH, Hauptstrasse, Tamm, Germany). Once a spike had been isolated, a qualitative assessment of the responsiveness to pharmacological agents was made. Neurons were then entrained by applying 200 ms on 200 ms off positive current pulses through the recording pipette (1–10 nA for periods between 40 and 360 s), gated by an isolated square pulse voltage stimulator (model SD9, Astro-Med, West Warwick, RI, USA). After