Circadian and ultradian rhythms of free glucocorticoid hormone are highly synchronized between the blood, the subcutaneous tissue and the brain

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Supplemental Information

A. Full description of surgical procedures, experimental designs and corticosterone assay

Animals

Male Wistar rats (body weight approximately 175-200 g) were purchased from Harlan (Loughborough, UK). Rats were housed three per cage under standard lighting (14/10-hours light/dark cycle, lights on at 05:00 h) and temperature (21-22 °C) conditions. Food and water were available *ad libitum*. Rats were handled once per day (about 5 min per rat) starting one week before surgery. At the time of surgery, the body weight of the rats was approximately 250 g. All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986. The experimental protocols were designed to minimize animal suffering and numbers of animals used and were approved by the Ethical Review Group of the University of Bristol and the Home Office, United Kingdom.

Protocol-1: Single-probe microdialysis in the blood or the subcutaneous tissue

Surgical and microdialysis procedures

To investigate the biological rhythms of free corticosterone levels in different peripheral compartments on two consecutive days, we performed single-probe *in vivo* microdialysis in freely

behaving rats and collected dialysate samples from either the blood (jugular vein) or the subcutaneous tissue. In a first group of rats, a microdialysis probe (CMA20 Elite: membrane polyarylethersulfone; 20 kDa cut-off; length 10 mm; membrane diameter 0.5 mm; *in vitro* recovery of free corticosterone 51.5 ± 2.2% (n=6) at 37°C; CMA Microdialysis, Stockholm, Sweden) was inserted into the jugular vein under isoflurane (Merial Animal Health Ltd., Harlow, UK) anesthesia. The inlet and outlet tubing of the probe were tunneled under the skin to the neck region and secured. In a second group of rats, a microdialysis probe (with the same specifications as above) was inserted under the skin of the neck region and secured. Dental cement and anchor screws were used to fix a small metal peg (for later connection to a liquid swivel) to the skull. The skin was closed with surgical silk (4.0, Ethicon, Somervill, NJ, USA) and carprofen (Rimadyl, 4 mg/kg, s.c., Pfizer, Sandwich, UK) was given for post-operative pain relief. After surgery, animals were housed individually in Plexiglas cages (length × width × height = 27 × 27 × 35 cm) with food and water *ad libitum* and connected to a dual-channel liquid swivel and counterbalance arm system (Microbiotech AB, Stockholm, Sweden) via the peg on the head. This system allows the animals to move freely in all three dimensions, including full rearing and sleeping in a curled-up body posture. Probes were perfused with sterile, pyrogen-free Ringer solution (147 mM NaCl, 4 mM KCl, 2.25 mM $CaCl_2$; Delta Pharma, Pfüllingen, Germany) at 2.05 µl/min using a microinfusion pump (KD Scientific, Holliston, MA, USA). The syringes with the perfusion fluid were filled each day between 08:00-09:00 h. Fluorethylenepolymer tubing (Microbiotech AB, Stockholm, Sweden) with a dead volume of 1.2 µl/100 mm length was used for all connections. Dead volumes were taken into account during sample collection.

Experimental design

Two days after insertion of the microdialysis probe in the peripheral compartment, sampling started at 09:00 h and continued for 47 hours. Microdialysis samples were collected in intervals of 10 min (between 09:00 – 21:00 h) or 30 min (between 21:00 – 08:00 h). During the whole experiment animals were left undisturbed in their home cages. Microdialysis samples were collected in pre-cooled vials using automated refrigerated sample collectors (CMA470, CMA Microdialysis). The samples were stored at –80 °C for later determination of the concentrations of corticosterone. Experiments were performed on six animals simultaneously.

Protocol-2: Simultaneous dual-probe microdialysis in the blood and the subcutaneous tissue

Surgical and microdialysis procedures

To directly compare the biological rhythms of free corticosterone in the blood and in a peripheral target compartment, we performed dual-probe *in vivo* microdialysis in freely behaving rats and collected samples from the blood (jugular vein) and the subcutaneous tissue simultaneously. Rats were prepared for microdialysis as described under Protocol-1 with the exception that each animal received microdialysis probes in both the jugular vein and the subcutaneous tissue during the same surgery. After surgery, animals were housed individually in Plexiglas cages (as above) with food and water *ad libitum* and were connected to a motorized 5-channel swivel and counterbalance arm system (MCS/5A; Instech Laboratories Inc., Plymouth Meeting, PA, USA) via the peg on the head. Similar to the dual-channel swivel system, this 5-channel system allows the animals to move freely in all three dimensions. Importantly, the power-assisted swivel has a low torque despite its 5-channel design and follows the movements of the rats accurately. Probes were connected and perfused as described under Protocol-1.

Experimental design

Two days after insertion of the microdialysis probes, sampling started at 09:00 h and continued for 20 h. Microdialysis samples were collected in intervals of 10 min (between 09:00 – 21:00 h) or 30 min (between 21:00 – 05:00 h). During the whole experiment animals were left undisturbed in their home cages. Samples were collected and stored as described under Protocol-1.

Protocol-3: Simultaneous dual-probe microdialysis in the blood and the hippocampus

Surgical and microdialysis procedures

To directly compare the biological rhythms of free corticosterone in the blood and the brain, we performed dual-probe *in vivo* microdialysis in freely behaving rats and collected samples from the blood (jugular vein) and the hippocampus simultaneously. Nine days before the start of the experiment, under isoflurane anesthesia, a guide cannula (CMA12 Guide Cannula, CMA

Microdialysis) was implanted just entering the hippocampus at the dorsal site. Hippocampal coordinates based on the rat atlas of Paxinos and Watson (1) with the tooth bar set at -3.3 mm and bregma as overall zero were posterior 5.2 mm, lateral 5.0 mm, and ventral 4.0 mm. Dental cement and anchor screws were used to fix a peg to the skull as described under Protocol-1. Carprofen was given for post-operative pain relief. After surgery, animals were housed individually in Plexiglas cages (as above) with food and water *ad libitum*. After seven days of recovery, a microdialysis probe (CMA12 Elite Microdialysis Probe: polyarylethersulfone membrane, 20 kDa cut-off, length 4 mm; membrane diameter 0.5 mm; *in vitro* recovery of free corticosterone 27.0 ± 1.7 % (n=6) at 37°C; CMA Microdialysis) was inserted through the guide cannula into the hippocampus and a peripheral microdialysis probe (CMA20 Elite Microdialysis Probe: see above; length 4 mm; recovery rate 27.0 ± 2.8 % (n=6) at 37°C; CMA Microdialysis) was inserted into the jugular vein under isoflurane anesthesia. Carprofen was given for post-operative pain relief. The rats were connected to a 5-channel swivel and counterbalance arm system as described under Protocol-2. Probes were connected and perfused as described under Protocol-1.

Experimental design

Two days after insertion of the microdialysis probes, sampling started at 09:00 h and continued for 20 h. Microdialysis samples were collected in intervals of 10 min (between 09:00 – 21:00 h) or 30 min (between 21:00 – 05:00 h). During the whole experiment animals were left undisturbed in their home cages. Samples were collected and stored as described under Protocol-1.

Histology

After the completion of the experiments described under Protocol 3, animals were killed using an overdose of pentobarbital (Euthatal, 200 mg/kg body weight, i.p., Merial). The brains were collected and stored in a 4% paraformaldehyde fixative solution. Brains were cut into 50 µm sections using a cryostat and stained with Cresyl Violet to verify the localization of the microdialysis probe in the hippocampus as described previously (2). No misplacements were found.

Measurement of corticosterone

Dialysate corticosterone concentrations were measured in undiluted samples using a ^{125}I-corticosterone radioimmunoassay (MP Biomedicals, Solon, OH, USA)(2) and expressed as µg/dl dialysate. In addition to running the standard curve supplied by the manufacturer for quality control purposes, a separate standard curve of freshly prepared corticosterone solutions in Ringer solution (i.e. the physiological solution used in the microdialysis experiments), spanning the free corticosterone dialysate concentration range, was used. Because of the small sample volumes, the volumes of the assay reagents were adjusted (25% of the volumes stated in the manufacturer's instruction) but their ratio remained the same. For the standards 25 µl was used; for 10-min samples, 15 µl dialysate was used and 10 µl Ringer solution was added (concentrations were corrected for the added Ringer); for 30-min samples, 25 µl dialysate was used. The detection limit was 0.00125 µg/dl; the inter- and intra-assay variations were 16 and 14%, respectively (3). Dialysate corticosterone concentrations were not corrected for probe recovery. There were only rare occasions that sample values were "off-curve". Off curve values were not replaced. To calculate pulse characteristics, a "-1" was placed for each missing value in the Excel datasheet, which was subsequently recognized and treated by the PULSAR program as a missing value.

References

1. **Paxinos G, Watson C** 1982 The rat brain in stereotactic coordinates. Sydney: Academic Press

2. **Linthorst ACE, Flachskamm C, Holsboer F, Reul JMHM** 1994 Local administration of recombinant human interleukin-1 beta in the rat hippocampus increases serotonergic neurotransmission, hypothalamic-pituitary-adrenocortical axis activity, and body temperature. Endocrinology 135:520-532

3. **Droste SK, De Groote L, Atkinson HC, Lightman SL, Reul JMHM, Linthorst ACE** 2008 Corticosterone levels in the brain show a distinct ultradian rhythm but a delayed response to forced swim stress. Endocrinology 149:3244-3253

B. Table 1: Results of ANOVA Analyses

PROTOCOL-1: single-probe microdialysis					
Compartment	**Pulse Parameter**	**Effect (main or interaction)**	**Degrees of freedom**	**F value**	**P value**
Blood	Pulse frequency	day	1,11	8.80	<0.02
		time period	1,11	0.98	n.s.
		day x time period	1,11	0.0005	n.s.
	Mean pulse amplitude	day	1,11	0.84	n.s.
		time period	1,11	29.12	≤0.0005
		day x time period	1,11	0.284	n.s.
	Mean pulse height	day	1,11	1.59	n.s.
		time period	1,11	68.13	≤0.0005
		day x time period	1,11	0.48	n.s.
	Mean free corticosterone	day	1,11	4.39	n.s.
		time period	1,11	90.04	≤0.0005
		day x time period	1,11	2.37	n.s.
	AUC	day	1,11	4.46	n.s.
		time period	1,11	84.42	≤0.0005
		day x time period	1,11	2.67	n.s.
Subcutaneous tissue	Pulse frequency	day	1,9	3.47	n.s.
		time period	1,9	3.47	n.s.
		day x time period	1,9	0.05	n.s.
	Mean pulse amplitude	day	1,9	1.52	n.s.
		time period	1,9	19.46	<0.01
		day x time period	1,9	0.53	n.s.
	Mean pulse height	day	1,9	1.56	n.s.
		time period	1,9	52.09	≤0.0005
		day x time period	1,9	2.10	n.s.
	Mean free corticosterone	day	1,9	2.52	n.s.
		time period	1,9	145.44	≤0.0005
		day x time period	1,9	4.05	n.s.
	AUC	day	1,9	2.43	n.s.
		time period	1,9	115.86	≤0.0005
		day x time period	1,9	3.92	n.s.
PROTOCOL-2: dual-probe microdialysis in periphery					
Compartment	**Pulse Parameter**	**Effect (main or interaction)**	**Degrees of freedom**	**F value**	**P value**
Blood and subcutaneous tissue	Pulse frequency	compartment	1,5	0.003	n.s.
		time period	1,5	0.19	n.s.
		compartment x time period	1,5	2.77	n.s.
	Mean pulse amplitude	compartment	1,5	0.89	n.s.
		time period	1,5	18.05	<0.01
		compartment x time period	1,5	0.34	n.s.
	Mean pulse height	compartment	1,5	3.18	n.s.
		time period	1,5	18.58	<0.01
		compartment x time period	1,5	7.35	<0.05
	Mean free corticosterone	compartment	1,5	4.57	n.s.
		time period	1,5	25.79	<0.01
		compartment x time period	1,5	13.33	<0.02
	AUC	compartment	1,5	4.62	n.s.
		time period	1,5	20.95	<0.01
		compartment x time period	1,5	7.40	<0.05

PROTOCOL-3: dual-probe microdialysis in periphery and brain					
Compartment	**Pulse Parameter**	**Effect (main or interaction)**	**Degrees of freedom**	**F value**	**P value**
Blood and hippocampus	Pulse frequency	compartment	1,16	0.19	n.s.
		time period	1,16	6.14	=0.03
		compartment x time period	1,16	0.12	n.s.
	Mean pulse amplitude	compartment	1,16	0.39	n.s.
		time period	1,16	36.33	≤0.0005
		compartment x time period	1,16	2.89	n.s.
	Mean pulse height	compartment	1,16	2.78	n.s.
		time period	1,16	77.89	≤0.0005
		compartment x time period	1,16	7.88	<0.02
	Mean free corticosterone	compartment	1,16	8.38	<0.02
		time period	1,16	125.76	≤0.0005
		compartment x time period	1,16	15.62	=0.001
	AUC	compartment	1,16	4.93	<0.05
		time period	1,16	142.80	≤0.0005
		compartment x time period	1,16	16.13	=0.001

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C: Table 2: Direct comparison of the circadian and ultradian rhythms of free corticosterone levels in the blood and the subcutaneous tissue as calculated using the PULSAR algorithm (Protocol-2, dual-probe microdialysis)

Time period (time of day, h)	Compartment	Pulse frequency (pulse per hour)	Mean pulse amplitude (µg/dl)	Mean pulse height (µg/dl)	Mean free corticosterone (µg/dl)	AUC (arbitrary units)
09:00-15:00	Blood	1.15 ± 0.16	0.06 ± 0.02	0.15 ± 0.04	0.10 ± 0.03	0.65 ± 0.15
	Subcutaneous tissue	1.04 ± 0.15	0.07 ± 0.02	0.14 ± 0.03	0.09 ± 0.02	0.57 ± 0.08
15:00-21:00	Blood	1.11 ± 0.11	0.23 ± 0.05**	0.46 ± 0.10**	0.33 ± 0.07**	1.93 ± 0.41**
	Subcutaneous tissue	1.23 ± 0.08	0.23 ± 0.06$^{\#}$	0.40 ± 0.09$^{\#\#}$	0.28 ± 0.05$^{\#\#}$	1.64 ± 0.31$^{\#\#}$

Free corticosterone levels were simultaneously monitored in the blood and the subcutaneous tissue by dual-probe microdialysis in the same animals. Pulse characteristics were calculated using the PULSAR algorithm on data depicted in Figure 2. All rats (n=6) showed significant circadian and ultradian rhythms in both compartments during the analyzed 12-hour sampling period between 09:00 – 21:00 h (10 min interval samples). Post-hoc statistical analysis of the morning/early afternoon period (09:00-15:00 h) versus the late afternoon/early night period (15:00-21:00 h) revealed significant circadian variations in the pulse amplitude, pulse height, mean free corticosterone level, and AUC with higher values attained during the later time period in both compartments. No significant circadian variations were found for the pulse frequency in either compartment. Importantly, post-hoc statistical analysis revealed no significant differences between the parameters calculated for the blood and the subcutaneous tissue, demonstrating the high similarity of the free corticosterone rhythms in these peripheral compartments.

**, blood: time period 15:00-21:00 h vs. time period 09:00-15:00 h ($P<0.0125$). $^{\#}$ and $^{\#\#}$, subcutaneous tissue: time period 15:00-21:00 h vs. time period 09:00-15:00 h ($P=0.015$ and $P<0.0125$, respectively). Paired Student's t-tests with Bonferroni correction. See Table S1 for ANOVA results. Values shown are mean ± SEM.

D: Table 3: Direct comparison of the circadian and ultradian rhythms of free corticosterone levels in the blood and the hippocampus as calculated using the PULSAR algorithm (Protocol-3, dual-probe microdialysis)

Time period (time of day, h)	Compartment	Pulse frequency (pulse per hour)	Mean pulse amplitude (µg/dl)	Mean pulse height (µg/dl)	Mean free corticosterone (µg/dl)	AUC (arbitrary units)
09:00-15:00	Blood	1.16 ± 0.10	0.03 ± 0.002	0.07 ± 0.01	0.04 ± 0.004	0.27 ± 0.03
	Hippocampus	1.17 ± 0.08	0.04 ± 0.003	0.07 ± 0.01	0.04 ± 0.004	0.27 ± 0.03
15:00-21:00	Blood	1.32 ± 0.08	0.09 ± 0.01**	0.18 ± 0.01**	0.13 ± 0.01**	0.80 ± 0.04**
	Hippocampus	1.38 ± 0.07	0.07 ± 0.01$^{\#\#}$	0.15 ± 0.01$^{\#\#}$	0.11 ± 0.01$^{\#\#,\$}$	0.67 ± 0.04$^{\#\#,\$}$

Free corticosterone levels were simultaneously monitored in the blood and the hippocampus by dual-probe microdialysis in the same animals. Pulse characteristics were calculated using the PULSAR algorithm on data depicted in Figure 3. All rats (n=17) showed significant circadian and ultradian rhythms in both compartments during the analyzed 12-hour sampling period between 09:00-21:00 h (10 min interval samples). Post-hoc statistical analysis of the morning/early afternoon period (09:00-15:00 h) versus the late afternoon/early night period (15:00-21:00 h) revealed significant circadian variations in the pulse amplitude, pulse height, mean free corticosterone level, and AUC, but not in pulse frequency, with higher values attained during the later time period in both compartments. Importantly, post-hoc statistical analysis revealed no significant differences in pulse frequency, pulse height and pulse amplitude between the blood and the hippocampus demonstrating the high similarity of the ultradian free corticosterone rhythms in these two compartments. However, the mean free corticosterone level and the AUC were slightly lower in the hippocampus as compared to the blood during the later time period (15:00-21:00 h) only.

**, blood: time period 15:00-21:00 h vs. time period 09:00-15:00 h ($P<0.0125$); $^{\#\#}$, hippocampus: time period 15:00-21:00 h vs. time period 09:00-15:00 h ($P<0.0125$); $^{\$}$, time period 15:00-21:00 h: hippocampus vs. blood ($P<0.0125$)). Paired Student's t-tests with Bonferroni correction. See Table S1 for ANOVA results. Values shown are mean ± SEM.