Neurodegeneration in the centrally-projecting Edinger–Westphal nucleus contributes to the non-motor symptoms of Parkinson’s disease in the rat

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Abstract

Background: The neuropathological background of major depression and anxiety as non-motor symptoms of Parkinson's disease is much less understood than classical motor symptoms. Although, neurodegeneration of the Edinger–Westphal nucleus in human Parkinson’s disease is a known phenomenon, its possible significance in mood status has never been elucidated. In this work we aimed at investigating whether neuron loss and alpha-synuclein accumulation in the urocortin 1 containing (UCN1) cells of the centrally-projecting Edinger–Westphal (EWcp) nucleus is associated with anxiety and depression-like state in the rat.

Methods: Systemic chronic rotenone administration as well as targeted leptin–saporin-induced lesions of EWcp/UCN1 neurons were conducted. Rotarod, open field and sucrose preference tests were performed to assess motor performance and mood status. Multiple immunofluorescence combined with RNAscope were used to reveal the functional–morphological changes. Two-sample Student’s t test, Spearman’s rank correlation analysis and Mann–Whitney U tests were used for statistics.

Results: In the rotenone model, besides motor deficit, an anxious and depression-like phenotype was detected. Well-comparable neuron loss, cytoplasmic alpha-synuclein accumulation as well as astro- and microglial activation were observed both in the substantia nigra pars compacta and EWcp. Occasionally, UCN1-immunoreactive neuronal debris was observed in phagocytotic microglia. UCN1 peptide content of viable EWcp cells correlated with dopaminergic substantia nigra cell count. Importantly, other mood status-related dopaminergic (ventral tegmental area), serotonergic (dorsal and median raphe) and noradrenergic (locus ceruleus and A5 area) brainstem centers did not show remarkable morphological changes. Targeted partial selective EWcp/UCN1 neuron ablation induced similar mood status without motor symptoms.

Conclusions: Our findings collectively suggest that neurodegeneration of urocortinergic EWcp contributes to the mood-related non-motor symptoms in toxic models of Parkinson’s disease in the rat.

Keywords: Rotenone, Saporin, Urocortin 1, Depression, Anxiety, Rat, RNAscope, Immunofluorescence

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Background

Parkinson's disease (PD) is characterized by classic motor symptoms including rigor, tremor and bradykinesia attributed to neurodegeneration in the substantia nigra pars compacta (SNpc) and consequent loss of striatal dopaminergic afferentation [1]. According to the self-assessment of patients, non-motor symptoms deteriorate their quality-of-life more dramatically than motor symptoms themselves [2]. Therefore, the attention has been recently turned to PD-associated non-motor symptoms, such as anxiety and depression. While the main neuropathological hallmarks of motor symptoms such as Lewy body (LB) and Lewy neurite accumulation as well as nigral neurodegeneration are relatively well-investigated [1], much less knowledge has collected explaining the non-motor psychiatric symptoms. Anxiety disorders as well as major depression have been linked with altered noradrenergic, serotonergic and dopaminergic neurotransmission [3], but the underlying neuropathology of PD-associated mood disorders is poorly understood. Not surprisingly, the currently available pharmacotherapeutic strategies sharing the goal to increase serotonin (5-HT) and/or norepinephrine levels in the brain, provide limited efficacy [4].

Some neuropathological observations may explain the association between PD and mood disorders. For instance, LBs accumulate in the serotonergic raphe nuclei [3], dopaminergic ventral tegmental area (VTA) as well as in the noradrenergic locus ceruleus (LC) [5]. Besides these, neurodegeneration and LB accumulation have been observed in PD in the dorsal vagal nuclear complex and in the Edinger-Westphal nucleus (EW) [6, 7].

Classically, cholinergic parasympathetic preganglionic neurons of the EW project to the ciliary ganglion controlling the sphincter pupillae and ciliary muscles. Based on this, patients with PD would be expected to suffer from compromised pupillary light reflex and lens accommodation [8]. However, this is usually not the case, even though it is known that the “rostral part of the EW suffers 50% neural loss and 3% of the cells contain Lewy bodies” [6]. This paradox suggested us that the recently defined peptidergic centrally-projecting (EWcp) division [9] of the EW may be affected.

The EWcp expresses lepin receptors [10] and a number of neuropeptides [11–14] including urocrin 1 (UCN1) [15, 16]. UCN1 belongs to the corticotropin-releasing hormone (CRH) peptide family [15] and activates both CRH receptors. The significance of CRH receptor signaling has been assessed in numerous biological functions including stress adaptation response [17]. The involvement of UCN1 in stress and depression models has been demonstrated in mouse [18, 19], rat [20–23] and non-human primate [24] depression models. Importantly, EWcp samples of male depressed suicide victims contained decreased amount of Ucn1 mRNA [25].

In this work our goal was to investigate the putative underlying mechanisms of mood disorders in PD. Our main hypothesis was that the specific loss or damage of EWcp/UCN1 neurons directly contributes to anxiety and depression as non-motor symptoms of PD.

To test this, a systemic chronic rotenone treatment model for PD was applied in the rat [26]. As EWcp/UCN1 neurons carry lepin receptors [10], a lepin-conjugated saporin-induced selective ablation of UCN1 neurons was also performed to validate the depression-like phenotype in rats. Behavioral, functional–morphological, biochemical and histopathological tools were used to test the motor coordination, mood status as well as morphological changes in the brain.

Methods

Animals

In-house bred male Wistar rats (breeding pairs purchased from Animalab Kft., Vác, Hungary) were housed in three-per-cage (40 × 25 × 20 cm) groups in temperature and humidity-controlled environment. Animals were supplied with standard rodent chow and tap water ad libitum. Experiments were approved by the Animal Welfare Committee of Pécs University, the National Scientific Ethical Committee on Animal Experimentation in Hungary and the National Food Chain Safety Office in Hungary (license No: BA02/2000-49/2017).

Systemic rotenone treatment model of PD

Eleven-month-old animals were subjected to 6 weeks daily subcutaneous rotenone treatment ([R8875-1G, Sigma, Budapest, Hungary]) \(n=23\); 1.5 mg/kg/day rotenone dissolved in 20 µl/kg/day dimethyl-sulfoxide (Fisher Scientific, Loughborough, UK) and 1 ml/kg sunflower oil vehicle (8000-21-6, Molar Chemicals Kft., Halásztelek, Hungary) vs. vehicle injected controls \(n=12\). Five rats due to humane endpoint and six others showing less than 20% SNpc dopaminergic neuron loss upon rotenone treatment were removed from the study [27]. Finally, 12 rotenone-treated rats were included.

Sucrose preference test

The anhedonia level was determined by sucrose preference test (SPT). Seventy-two hours before testing 1 ml/ν% sucrose solution and tap water were offered. After 24 h, the place of the bottles was reversed. On the test day, water deprivation was applied between 6 a.m. and 4 p.m. Then, the two bottles were offered for individually caged animals for 3 h. Sucrose preference was calculated according to the widely used formula [28].
Open field test
The open field test (OFT) was performed in a brightly lit box [50 x 50 x 38 cm] with black background. Video recordings of 5 min were analyzed with SMART Junior Tracking software (PanLab, Barcelona, Spain) to assess the time spent in the periphery of the box, and the traveled distance [29].

Rotarod test
Rotarod performance test (RPT) was used to assess motor coordination. Animals were first pre-trained to run on the accelerating rotating axis three times. For testing, rats were placed on the axis of the device (47750, Ugo Basile, Gemonio, Italy) and the time interval rats spent on the rotating axis was registered [30].

Targeted toxin-induced lesion of EWcp/UCN1 neurons
The EWcp contains neuron populations that do not express UCN1 [14, 31]. Because in this project, our aim was to study the significance of UCN1 neuron loss, we applied a selective neuron ablation by saporin. This neurotoxin enters neurons only if it is conjugated to a substance that is internalized by receptor-mediated endocytosis and irreversibly inhibits the cells’ protein synthesis [32]. As EWcp/UCN1 neurons carry leptin receptors [10], leptin-conjugated saporin injection provides a reliable tool to perform selective UCN1 neuron ablation [33].

Thirty-two rats were intraperitoneally anesthetized by ketamine (78 mg/kg, Richter, Budapest, Hungary) and xylazine (13 mg/kg, Eurovet, Nagyatád, Hungary). Using a stereotaxic apparatus, 0.08 µl leptin-conjugated saporin (n = 16) or saporin (n = 16) solution (#KIT-47, ATS INC, Carlsbad, CA, USA) was injected into the EWcp area. From a point 1.5 mm left and 4.8 mm caudal to the Bregma [34] an oblique injection path closing 19° angle with the vertical axis was set and the Hamilton needle was introduced to a depth of 5.8 mm [33, 35]. Compounds were injected in 30 s. Thirty seconds later, the needle was retracted by 0.5 mm, and 1 min later, it was removed.

If the magnitude of UCN1 neuron loss was less than 20% upon leptin–saporin treatment, the animal was excluded (8 rats) from the experiment. Six rats were excluded, because the injection path deviated from the planned route, or the needle caused physical damage in the EWcp. Finally, nine saporin (control) and leptin–saporin-injected rats were included into the statistics. Behavioral testing was performed after 10 days recovery as described above. Behavioral data were converted into Z-scores using the formula $Z = (X - \mu) / \sigma$, where $(X)$ represents the raw data of the individual animal, $(\mu)$ and $(\sigma)$ the mean and standard deviation of the control group, respectively [36]. Tissue sampling was performed 2 weeks after surgery.

Tissue and sample preparation
Intraperitoneally anesthetized (urethane, 1.4 g/kg, Merck KGaA, Darmstadt, Germany) rats were perfused with 50 ml 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 250 ml 4% paraformaldehyde in Millonig’s buffer. Brains were dissected and post-fixed for 14 days. Thymus and adrenals were collected and weighed.

Thirty µm coronal vibratome (Leica Biosystems, Wetzlar, Germany) sections in six series were collected between the optic chiasm and middle cerebellar peduncle. Sections were stored in PBS containing 0.01% sodium azide at 4 °C [22].

Multiple immunofluorescence labeling
Sections of caudate–putamen (CPu) [Bregma −0.36 mm – (−0.84 mm)], EWcp [Bregma −5.88 mm – (−6.72 mm)], SNpc [Bregma −5.16 mm − (−6.24 mm)], VTA [Bregma −5.16 mm − (−6.24 mm)], dorsal- (DR) [Bregma −6.96 mm − (−7.32 mm)] and median (MNR) raphe nucleus [Bregma −7.20 mm − (−7.80 mm)] LC and A5 noradrenergic cells [Bregma −9.60 mm − (−10.08 mm)] were manually selected [34] for multiple immunofluorescence labelings on samples from the rotenone model. After epitope retrieval (10 min, 90 °C, sodium-citrate buffer; pH 6.0), permeabilization (0.5% Triton X-100; Sigma) and 2% normal donkey serum (60 min) treatment, sections were incubated in cocktails of primary antibodies for double (16 h, room temperature) or triple labelings (48 h, 4 °C). Ionized calcium-binding adapter molecule 1 (IBA1, rabbit)-tyrosine-hydroxylase (TH, mouse) double labeling was performed in the SN, VTA, LC and A5. 5-HT (mouse)-IBA1 labeling was conducted in the DR and MNR. CPu was assessed by TH (rabbit)-NeuN (mouse) labeling. UCN1 (goat)–IBA1–glial fibrillary acidic protein (GFAP, mouse); moreover, UCN1 (goat)–alpha-synuclein (mouse)–TH (rabbit) triple stainings were performed. Reactive microglia were identified by IBA1 (goat)–CD68 (rabbit) double labeling in the EWcp. Tumor necrosis factor alpha (TNF-alpha, rabbit)–GFAP (mouse)–IBA1 (goat), moreover, inducible nitric oxide-synthase–iNOS, rabbit)–GFAP (mouse)–IBA1 (goat) triple labelings were conducted in the SNpc. In the targeted toxin model, UCN1 (rabbit)–GFAP (mouse)–IBA1 (goat) and a UCN1 (goat)–TH (mouse)–caspase 3

$Z = \frac{X - \mu}{\sigma}$
(rabbit) triple labelings, moreover, a UCN1 (goat)–NeuN (mouse) double labeling was performed. The respective fluorophore-conjugated or biotinylated secondary sera were applied either for 3 h in double or overnight in triple stainings. Biotinylated donkey anti-rabbit serum was detected by Cy5-conjugated streptavidin (Jackson, 1:1500, 3 h). Finally, sections were mounted on gelatin-coated slides, air-dried and covered with glycerol-PBS.

Antisera were characterized earlier as referenced in Table 1. Omission and non-immune serum replacement

Table 1 Summary of antibody details

| Antibody name | Vendor | Cat# | References | RRID  | Antigen | Dilution |
|---------------|--------|------|------------|-------|---------|----------|
| Anti-tyrosine-hydroxylase (host: rabbit, polyclonal) | Abcam | ab112 | [65] | AB_297840 | Full length protein from rat pheochromocytoma | 1:2000 |
| Anti-tyrosine-hydroxylase (host: mouse, monoclonal) | Sigma | T2928 | [66] | AB_477569 | Rat tyrosine-hydroxylase | 1:2000 |
| Anti-UCN1 (host: goat, polyclonal) | Santa Cruz | SC1825 | [21] | AB_2304014 | C terminus peptide fragment of rat UCN1 | 1:200 |
| Anti-UCN1 (host: rabbit, polyclonal) | Salk Institute Prof. WW. Vale | PBL#5779 | [67] | AB_2315527 | Rat UCN1 (amino acids 1–40) | 1:20,000 |
| Anti-GFAP (host: rabbit, monoclonal) | Abcam | Ab33922 | [68] | AB_732571 | C terminus peptide (sequence commercially sensitive) | 1:1500 |
| Anti-GFAP (mouse monoclonal) | Novocastra | NCLLGFP-GAS | [69] | AB_563739 | Porcine spinal cord | 1:1000 |
| Anti-IBA1 (host: rabbit, polyclonal) | Wako Ltd | 019–19,741 | [70] | AB_839504 | C terminus synthetic peptide of IBA1 | 1:1000 |
| Anti-IBA1 (host: goat, polyclonal) | Wako Ltd | 011–27,991 | [71] | AB_302665 | Synthetic peptide corresponding to C-terminal of IBA1 | 1:500 |
| Anti-alpha-synuclein (host: mouse, monoclonal) | Abcam | Ab1903 | [72] | AB_3265 | Recombinant full length protein corresponding to human alpha-synuclein | 1:2000 |
| Anti-NeuN (host: mouse, monoclonal, clone A60) | Millipore | MAB377 | [73] | AB_2298772 | Purified cell nuclei from mouse brain | 1:1000 |
| Anti-5-HT (host: mouse, monoclonal) | Universite’ Claude Bernard, Lyon, France Dr. Luciene Leger | Custom made | [18] | BSA-conjugated 5HT | 1:20,000 |
| Anti-CD68 (Host: rabbit, polyclonal) | Abcam | ab125212 | [74] | AB_1097546 | Synthetic peptide AFCITRRRQSTYQPL aa167-175 within the p17 subunit (cleaved caspase) | 1:100 |
| Anti-caspase 3 (host: rabbit, polyclonal) | Abcam | ab49822 | [75] | AB_868673 | Recombinant full length protein corresponding to human TNFalpha | 1:4000 |
| Anti-TNF-alpha (host: mouse, monoclonal) | Abcam | ab220210 | [76] | AB_2892586 | Recombinant fragment (information is proprietary to the supplier) | 1:250 |
| Anti-iNOS (host: rabbit, monoclonal) | Abcam | ab178945 | [76] | AB_2861417 | Recombinant fragment (information is proprietary to the supplier) | 1:250 |
| Normal Donkey Serum | Jackson Immunoresearch | 017–000-121 | | AB_2337258 | - | 2% |
| Alexa Fluor 488 donkey anti-mouse IgG (H+L) | Jackson Immunoresearch | 706–545-148 | | AB_2340472 | - | 1:400 |
| Alexa Fluor 488 donkey anti-goat IgG (H+L) | Jackson Immunoresearch | 706–545-148 | | AB_2340472 | - | 1:600 |
| Cy3 donkey Anti-Rabbit IgG (H+L) | Jackson Immunoresearch | 711–165-152 | | AB_2307443 | - | 1:800 |
| Alexa Fluor 647 donkey Anti-Rabbit IgG (H+L) | Jackson Immunoresearch | 711–605-152 | | AB_2492288 | - | 1:500 |
| Alexa Fluor 647 donkey Anti-Goat IgG (H+L) | Jackson Immunoresearch | 705–605-003 | | AB_2340436 | - | 1:500 |
| Biotin-SP (long spacer) donkey anti-rabbit IgG (H+L) | Jackson Immunoresearch | 711–065-152 | | AB_2340593 | - | 1:200 |
of primary or secondary antibodies prevented all immunolabelings (images not shown).

**RNA prolongation in situ hybridization combined with immunofluorescence**

In the rotenone model, four EWcp sections per animal were pretreated for RNAscope according to our recently developed protocol [37] optimized for 30 µm-thick PFA fixed sections. Subsequent steps of RNAscope protocol was performed according to the supplier’s suggestions. Ucn1 mRNA was visualized by Cy5 (1:3000). A mouse Ucn1 mRNA probe (Cat No: 466261, Advanced Cell Diagnostics, Newark, CA, USA) was used as mouse and rat UCN1 show full peptide sequence identity [38]. Some randomly selected sections were hybridized with tripleplex positive control probes for the rat (320891) or with tripleplex negative control probes (320871). After channel development positive control gave well-recognizable signal, while no fluorescence was seen in negative controls (images not shown).

Subsequently, slides were further processed for double-immunofluorescence using goat anti-UCN1 (1:175) and rabbit IBA1 (1:1000) for 48 h at 4 °C. After washes, Alexa 488-conjugated donkey anti-goat and Cy3-conjugated rabbit IBA1 (1:1000) were counterstained with 4’,6-diamidino-2-phenylindole (DAPI) and covered with antifade medium.

**Microscopy, digitalization and morphometry**

Samples were digitalized using Olympus FluoView 1000 confocal microscope with × 20 (NA: 0.5), × 40 (NA: 0.8) and × 60 (NA: 1.49 oil) objectives. The excitation and emission of fluorophores were set according to the built-in settings of the FluoView software (Fv10-ASW; Version 0102). Blue (DAPI), red (Cy3), green (Alexa 488) and white (Cy5 and Alexa 647) virtual colors were used.

Morphometry including counting of cells, nerve terminals and signal dots of the RNA labeling, densitometry and cell size measurement was performed on non-edited digital images. Every quantitation was carried out on four to six images per animal. The intensity of fluorescent signal was semi-quantified by measurement of the cytoplasmic signal corrected for the background, yielding the specific signal density (SSD) [18]. The measurement and unbiased stereological cell counting [39] was performed by ImageJ software (version 1.52a, NIH). Glial cell morphology was conducted according to the scoring system by Harrison et al. [40]. For publication purposes, representative images were cropped, contrasted and edited using Adobe Photoshop software.

**Statistics**

Data were presented as mean of the group ± standard error of the mean. Statistics were performed by two-sample Student’s t test after assessment of data distribution. Outlier data beyond the 2-sigma range were excluded. Spearman’s rank test was also applied to search for correlations. Mann–Whitney U test was used to assess results of glial activity scores. Alpha was set to 5% in all cases.

**Results**

Partial loss of EWcp/UCN1 neurons in the rotenone model is associated with depression-like phenotype and increased anxiety

RPT revealed that rotenone-treated rats were practically unable to stay on the rotating rod (1.75 ± 1.25 s) in contrast to vehicle-treated animals which ran 68.83 ± 15.37 s (Fig. 1A, t(22) = 4.14; p = 0.002). In OFT, deterioration of motor skills was also reflected by the reduced distance traveled (Fig. 1B, t(19) = 2.68; p = 0.01).

Regarding mood status, OFT revealed that parkinsonian rats spent more time (Fig. 1C, t(21) = 2.36; p = 0.027) in the periphery suggesting increased level of anxiety. The anhedonia level increased also upon rotenone treatment as rats consumed less sweetened fluid in SPT (Fig. 1D, t(22) = 2.34; p = 0.029). Increased relative adrenal weight

**Fig. 1** Increased anxiety and depression-like behavior in the rotenone model of Parkinson’s disease. Rotenone-treated rats (red bars) were unable to run on the rotating rod in the rotated test (A) and they moved less in the open field test (OFT) (B). The increased anxiety level was proven by longer time spent in the periphery of the OFT arena (C). Roteneone-treated rats drank less sweetened water in the sucrose preference test (SPT) suggesting increased anhedonia (D). Relative adrenal (E) and thymus (F) weights as well as bodyweight (G) data suggest increased hypothalamus–pituitary–adrenal axis activity in the rotenone-treated group compared to vehicle (oil) injected rats (black bars). Tyrosine-hydroxylase (TH, green in H, I)—alpha-synuclein (aSYN, red in H, I) double labeling in the substantia nigra pars compacta (SNpc) revealed reduced SNpc/TH-immunoreactive (ir) cell count (J) in rotenone-treated rats. Cytoplasmic alpha-synuclein-ir inclusions (see arrowhead in insert of panel I) were observed upon rotenone treatment in SNpc/TH neurons (K). The assessment of microglia morphology in the SNpc by TH (green in L, M)—ionized calcium binding adaptor molecule 1 (IBA1), red in L, M) double labeling revealed increased activity score upon rotenone treatment (N). TH (green in P, Q)—gial fibrillary acidic protein (GFAP, red in P, Q) double labeling revealed also increased astrogliosis (O) in rotenone-treated rats. TH (red in R, S)—NeuN (green, R, S) labeling in the caudate-putamen (CPu) revealed reduced dopaminergic fiber density in rotenone-treated rats (T) occasionally with focal pattern (see the border of focal fiber loss area indicated by arrowheads in panel S). Rotenone treatment did not induce neuron loss in the striatum (U).

*p < 0.05, **p < 0.01, ***p < 0.001 in Student’s t test or in Mann–Whitney U test in N and O. n = 8–12. Bars: 50 µm
Fig. 1 (See legend on previous page.)

A Rotarod test (sec)

B Distance traveled in OFT (Z-score)

C Periphery time in OFT (Z-score)

D SPT (Z-score)

E Relative adrenal weight (×10⁻⁴ g/g)

F Relative thymus weight (×10⁻⁴ g/g)

G Bodyweight (g)

Oil

Rotenone

H TH-SYN

I TH-SYN

J N of TH-ir cells in the SNpc

K Alpha-synuclein-ir TH/SNpc neurons (%)

L TH-IBA1

M TH-IBA1

N Microglia activity score

O Astrocyte activity score

P TH-GFAP

Q TH-GFAP

R TH-NeuN

S TH-NeuN

T TH-ir fibers in the CPU

U N of NeuN-ir neurons in the CPU
IBA1 staining suggested elevated microglial (Fig. 1L–N, revealed increased astroglial activity (Fig. 1O–Q, found in 10.96 ± 0.00004) in rotenone-treated rats. Alpha-synuclein-ir inclusions were found in 10.96 ± 0.00004 of SNpc/TH neurons (Fig. 1H, I) in rotenone-treated rats, unlike (0.82 ± 0.42%) in controls (Fig. 1K, t (20) = − 6.18, p = 0.00005). In addition, IBA1 staining suggested elevated microglial (Fig. 1.1–N, U (22) = 14; p = 0.0008) activation scores, while GFAP revealed increased astrogial activity (Fig. 1O–Q, U (22) = 26.5; p = 0.0019) in rotenone-treated rats. The density of TH-ir nerve fibers was reduced by 69% (Fig. 1T, t (19) = 3.41; p = 0.003) in the CPu of rotenone-injected rats. Occasionally, also focal loss of striatal TH immunoreactivity was found (Fig. 1R, S). The counting of NeuN-ir cells revealed no neuron loss in the CPu (Fig. 1R, S, U, t(19) = 0.57; p = 0.57). Furthermore, in the SNpc of rotenone-exposed rats, increased TNF-alpha immunoreactivity in astrocytes (Fig. 2A–C, t (22) = − 4.94; p = 0.00006) and elevated microglial (Fig. 2D–F, t(10) = − 2.62; p = 0.025) and neuronal iNOS immunoreactivity (Fig. 2G, E, G, t(10) = − 4.25; p = 0.0008) was detected, compared to controls.

When testing our main hypothesis, we found that the number of SNpc neurons decreased upon rotenone treatment (Fig. 3A–C, t(22) = 2.52; p = 0.019) and they showed pyknotic morphology (Fig. 3A, B, D, t(18) = − 3.62; p = 0.0019). Occasionally, UCN1 neurons were found to disintegrate, where IBA1-ir microglial processes surrounded the UCN1-ir debris of the neurons (Fig. 3G, H; Additional file 1: video 1A, Additional file 2: video 1B). Microglia interacted about 10-times more frequently with UCN1 neurons in rotenone-treated rats (Fig. 3I, t (18) = 4.28; p = 0.0004) than in controls. Alpha-synuclein-ir cytoplasmic inclusions were observed in 11.94 ± 1.28% of the UCN1 neurons in rotenone-treated rats (Fig. 3B, E), unlike in controls (Fig. 3A, E; 0.62 ± 0.33%; p(20) = 8.52; p < 10−6). GFAP labeling in the EWcp (Fig. 3I, K) revealed that rotenone induced also astrocyte activation (Fig. 3L; Mann–Whitney test; U(22) = − 14.50; p = 0.0009). To test whether astrogliosis was restricted to EWcp within the periaqueductal gray matter (PAG), astrocyte morphology was assessed in the ventrolateral PAG (Fig. 3, K) and no remarkable astrogliosis was seen here (Fig. 3M, U (22) = 52.00; p = 0.24).

Correlation analyses revealed significant inverse relationship of the SNpc/TH immunoreactivity both with microglial (Fig. 2K; Spearman’s ρ: −0.48; p = 0.03) and with astrocyte activity scores (Fig. 2L; Spearman’s ρ: −0.57; p = 0.004) in the EWcp. In contrast, no correlation was found between SNpc/TH immunoreactivity and TNF-alpha (Spearman’s ρ: −0.26; p = 0.79) or iNOS (Spearman’s ρ: −0.82; p = 0.42) immunoreactivities (data not illustrated).

Rotenone treatment resulted in 126%-elevation of UCN1 peptide immunosignal (compare Fig. 3A with 3B or 3 C with 3D) (Fig. 3Q, t(22) = − 11.11; p < 10−6). We successfully combined immunofluorescence with RNAscope in situ hybridization for Ucn1 mRNA. The EWcp perikarya contained high amount of cytoplasmic Ucn1 mRNA that was often inhomogeneously distributed in clusters of confluent signal dots, especially in rotenone-treated animals. This uneven distribution made the quantitation of cytoplasmic mRNA signal difficult. In contrast, well-countable Ucn1 mRNA signal puncta were dispersed in the karyoplasms of neurons (Fig. 3N, O’). Quantitation of intranuclear Ucn1 mRNA transcripts revealed decreased Ucn1 mRNA expression by 57.8% in rotenone-treated rats (Fig. 3P; t(18) = − 4.08; p = 0.0008). A strong negative correlation was found between cellular

(See figure on next page.)
Fig. 2 (See legend on previous page.)
Selective EWcp/UCN1 neuron ablation replicates mood status-related symptoms observed in parkinsonian rats

EWCP/UCN1 neurons were selectively ablated by targeted leptin–saporin toxin injection to test whether the administration causes similar behavioral anomalies to those observed in the rotenone model.

SPT revealed that leptin–saporin caused increased anhedonia (Fig. 5A, t(16) = 2.13; p = 0.048). The UCN1 neuron-ablated rats spent longer time (Fig. 5B, t(16) = 2.70; p = 0.015) next to the walls in OFT, suggesting increased anxiety. Importantly, these animals traveled the same total distance as controls (Fig. 5C, t(16) = 0.45; p = 0.65). RPT also showed that leptin–saporin injection did not affect motor performance (Fig. 5D, t(16) = 0.20; p = 0.84).

UCN1–NeuN labeling proved that leptin–saporin injection reduced the number of EWcp/UCN1 cells by 24.5% (Fig. 5E, G, t(16) = 4.56; p = 0.0003). No significant reduction of UCN1 immunonegative but NeuN+ ir EWCP cells was seen (Fig. 5E, F, H, t(16) = 1.15; p = 0.26), suggesting selective neurodegeneration of EWCP/UCN1 neurons. In contrast to saporin-injected controls, a subset of UCN1 neurons in leptin–saporin treated rats were seen to contain nuclear caspase 3 immunoreactivity suggesting still ongoing apoptotic process (Fig. 5M–O, t(12) = −5.52; p = 0.0002). Both microglia (Fig. 5I–K, t(12) = 1.00; p = 0.006) and astrocyte (Fig. 5I, J, L, U(12) = 0.00; p = 0.004) activity scores were higher in the EWCP of leptin–saporin-injected rats than in saporin-treated controls. Microglia in UCN1-ablated rats showed a strong tendency (Fig. 5I, J, P, t(10) = −2.21; p = 0.051) to interact more frequently with UCN1 neurons.

In the SNpc, we did not observe dopaminergic neuron loss (Fig. 5Q, R, T, t(10) = 0.41; p = 0.68), considerable neuronal caspase 3 activity (Fig. 5Q–S, t(10) = 0.79; p = 0.43), micro- (Fig. 5U–W, U(12) = 13.00; p = 0.42)

(See figure on next page.)

**Fig. 3** UCN1-immunoreactive (ir) neurons of the centrally-projecting Edinger–Westphal nucleus (EWcp) undergo neurodegeneration and show altered functional neumromorphology in the rotenone model of Parkinson’s disease. In rotenone-treated rats (red bars) the number of EWCP/UCN1 neurons (green in B) decreased (G), compared with oil-injected controls (A, black bars). EWCP/UCN1 neurons also showed swollen morphology (D), and contained alpha-synuclein (αSYN, red) immunoreactive (ir) inclusions (see arrow in insets of panels B and histogram in E). Occasionally, ionized calcium adapter-membrane 1 (Iba1)–ir phagocytic microglial cells (red G, H) were observed around UCN1 immunonegative cell fragments (green) (inset in G, panels H and I) and see also Additional file 1 Video 1 and rotenone treatment increased the number of interactions between UCN1 neurons and microglia (I). Gliarial fibrillary acidic protein (GFAP) labeling (white in J, K) revealed astroglia (I) upon rotenone treatment in the EWcp, while astrocyte activity score in the adjacent ventrolateral periaqueductal gray (vlPAG) remained unchanged (M). RNAscope in situ hybridization for Ucn1 mRNA (white in N, No, O, O’) revealed reduced expression (P) in rotenone-treated rats. Immunolabeling in the same preparations revealed increased UCN1 peptide specific signal density (SSD) of the cells (green in N, No, O, O’) upon rotenone treatment (Q). Cells with very low Ucn1 mRNA and high UCN1 peptide content were occasionally approached by phagocytic microglial cells (IBA1, red in O, O’). DAPI (4’,6-diamidino-2-phenylindole dihydrochloride) labeling for nuclear staining is shown in blue (N’, No’, O’, O’). A negative correlation between UCN1 SSD and motor performance (R), SNpc/TH cell count and UCN1 SSD (S) as well as UCN1 SSD and Ucn1 mRNA expression (T) was detected. a.u.: arbitrary unit; *p < 0.05, **p < 0.01, ***p < 0.001 in Student’s t test, or in Mann–Whitney U test in L and M. ns not significant; n = 8–12. Bars: 50 μm.
Fig. 3 (See legend on previous page.)
and astroglisis (Fig. 5U–W, X, \( U(12) = 14.50; p = 0.57 \)) in the leptin–saporin model.

**Discussion**

In the systemic toxic model of Parkinson's disease, rotenone was chosen to assess whether the PD-like state can be associated with morphological changes also within the peptidergic EWcp and whether it is accompanied with depression-like phenotype and anxiety. Next, a local neuron ablation was performed to examine whether the selective EWcp-damage causes comparable deterioration in the mood status.

**Anxiety and depression as non-motor symptoms of PD are detected in the rotenone model**

Although not without limitations [41, 42], the rotenone model of PD reproduces many features of the human disease, including systemic mitochondrial impairment, selective dopaminergic nigrostriatal damage, microglia activation, alpha-synuclein accumulation with formation of LBs [43, 44]. The RPT, a widely used method [45, 46] unambiguously revealed severe motor coordination deficit in rotenone-treated rats. Reduced OFT locomotor activity further supported the deterioration of motor skills in full agreement with earlier studies [47, 48]. OFT also suggested increased anxiety in rotenone-treated rats [49]. In SPT, reduced sucrose consumption in parkinsonian rats suggested diminished reward-seeking behavior [50]. Although these in vivo results confirmed the efficacy of the PD model, one has to consider the limitations of these tests as motor skills of rats might directly affect their performance. Nevertheless, after pre-testing other mood-status assessing paradigms (e.g., forced swim test, light–dark box test) in our pilot studies, we decided for OFT and SPT as parkinsonian animals were also capable of performing these, and the within and between subject error was acceptable.

The number of SNpc/TH cells was reduced by 32.4% in rotenone-treated rats. Although this is considerably less than the 70% neuron loss in humans [1], rats with 20% loss of SNpc/TH neurons showed significantly impaired motor skills. Furthermore, in line with others, we found neurons with cytoplasmic alpha-synuclein inclusions in SNpc/TH [51] that correspond to LB-like structures. These two observations strongly suggest the validity of our model. Increased SNpc/astroglia TNF-alpha immunoreactivity and elevated microglial and neuronal iNOS signal in the SNpc strongly suggest that the rotenone treatment-related oxidative stress and consequent neuroinflammation contributed to the neuron loss [52, 53]. Furthermore, in the rotenone model a diffuse striatal axonal loss and also drastic focal TH-ir fiber loss may be observed [54]. Indeed, we also measured reduced CPu/TH-ir fiber content in rotenone-treated rats, and found some circumscribed striatal neuropil areas with almost undetectable TH-ir (Fig. 1S).

**EWcp/UCN1 neurons suffer neurodegeneration and surviving cells show functional damage in the rotenone model**

The magnitude of neuronal loss, the presence of cytoplasmic alpha-synuclein-inclusions, corresponding to LB-like structures and the increased microglial activity in the SNpc was in line and in statistical correlation with micro- and astroglial activity as well as with UCN1–neuron loss of the EWcp suggesting their similar susceptibility to the toxic agent. This is not surprising as EWcp neurons are developmentally also closely related with SNpc/TH neurons [55]. Although obvious cytoplasmic alpha-synuclein immunoreactive inclusions suggested the presence of LB-like structures in the SNpc and EWcp, we have to state an important limitation here is that our antibody, similarly to numerous other commercially available sera [56], was not specific for the high molecular weight isoforms found in LBs in the rat.

Beyond the obvious interaction between UCN1-ir cell fragments (Fig. 3H′) and CD68-ir reactive microglia (Fig. 2I) [57] that unambiguously proves the loss of EWcp/UCN1 cells, we also provide evidence that surviving UCN1 cells suffered functional damage. We assume that the mitochondrial complex 1 inhibition

(See figure on next page.)

**Fig. 4** Dopaminergic, serotonergic and noradrenergic centers do not show significant neurodegeneration or glial activation in the rotenone model in the rat. Dopaminergic neurons marked by tyrosine-hydroxylase (TH, green) labeling in the ventral tegmental area (VTA) (A, B) and dorsal raphe nucleus (DR) (E, F) do not show significant neurodegeneration as shown by histograms C and G, respectively. The number of ionized calcium binding adaptor molecule 1 (IBA1, red in A, B, E, F)-ir microglia-TH neuron interactions remained unchanged both in the VTA (D) and DR (H) upon rotenone treatment. The number of serotonin (5-HT)-producing neurons of the DR (green, I, J) and median raphe nuclei (MNR, green, M, N) were not affected significantly as shown in panel (K) and (O), respectively. The number of microglia (IBA1, red in L, M, N)—5-HT neuron interactions did not change in the DR (L) and MNR (P). Noradrenergic cells of the locus ceruleus (LC, Q, R) and A5 noradrenergic cells of the ventrolateral medulla (U, V) shown by TH immunolabeling (green) did not suffer significant neurodegeneration upon rotenone treatment as shown in histograms (S) and (W), respectively. The microglial activity score (IBA1, red in Q, R, U, V) did not change in the LC (T) and in the A5 (X). 4th: fourth brain ventricle. Black bars: vehicle (oil) injected rats, red columns: rotenone-treated group. \( n = 7-12 \). Bars: 50 µm

\[ R(W) \]
Fig. 4 (See legend on previous page.)
in UCN1 cells caused energy deficit [58] with consequent UCN1 peptide accumulation due to impaired axonal transport. The increased SSD of UCN1 suggests peptide accumulation in the perikarya of cells, which was also observed in stressed mice [19, 59]. The reduction of Ucn1 mRNA message is in line with findings in EWcp samples of chronically stressed rats [23] and depressed suicide victims [25]. Adrenal-, thymus- and bodyweight data suggest increased HPA axis activity that is commonly associated with depression-like states in animal models [19, 60] and human depression [17].

Although PD-related neurodegeneration was shown to affect the DR [3], VTA and LC [5], we did not observe remarkable damage of these serotonergic, dopaminergic and noradrenergic cell populations in our rats. We cannot exclude that a higher rotenone dose [30] or a longer treatment period would have also affected these or other centers [53], that were not examined in this project but may affect mood status. Nevertheless, our rat model ultimately supports further the significance of the EWcp/UCN1 in PD-associated mood disorder.

Local targeted toxic UCN1–neuron ablation results in altered mood status

To further corroborate that EWcp/UCN1 neuron loss contributes to deterioration in the mood status, a local targeted UCN1 cell ablation was conducted. The leptin-conjugated saporin is internalized into leptin receptor positive cells leading to disturbed protein synthesis [61] and apoptotic neuron loss [62]. As in this area the leptin receptor expression is restricted to a part of EWcp/UCN1 cells [10, 63], a selective partial UCN1 neuron death was expected. After pre-tests with multiple leptin–saporin doses we achieved a cell loss that was comparable with that observed in the rotenone model. Earlier works [33] and pre-tests suggested that the cell death requires 2 weeks in the leptin–saporin model. Nevertheless, the occasional caspase 3 immunoreactivity in UCN1 neurons suggested that in some cells the leptin–saporin-induced apoptotic process [64] was still in progress at the time of tissue sampling. Some activated micro- and astroglial cells were seen also in saporin-injected controls most likely due to the direct physical effect of the surgery. In leptin–saporin-treated rats both glial cell types were more reactive and microglia tended to interact more frequently with the UCN1 neurons. Nevertheless, the selectivity of ablation was confirmed by the unchanged number of UCN1 immunonegative, but NeuN positive EWcp cells upon leptin–saporin treatment. In leptin–saporin-injected animals, increased OFT-anxiety and SPT–anhedonia suggested that the loss of EWcp/UCN1 cells provoked mood changes but normal motor coordination and locomotor activity excluded parkinsonism. In line with this, dopaminergic neuron loss, apoptotic activity or reactive gliosis was not detected in the SNpc.

**Conclusion**

In this study we successfully applied the rotenone model of PD and detected the anxious and depression-like phenotype, considered as non-motor symptoms of PD in the rat. Selective local UCN1 neuron ablation evoked similar mood status without motor symptoms. With respect to the limitations, our findings collectively suggest that the impairment of the UCN1 neurons in the EWcp contribute to the non-motor symptoms of PD. Human studies have to determine the potential diagnostic and therapeutic significance of these observations.
Fig. 5 (See legend on previous page.)
Abbreviations
S-HT: Serotonin; CD68: Cluster of differentiation 68; CPU: Caudate–putamen; CRH: Corticotropin-releasing hormone; DAPI: 4’6-Diamino-2-phenylindole; DR: Dorsal raphe nucleus; EW: Edinger–Westphal nucleus; EWcp: Centrally-projecting Edinger–Westphal nucleus; GFAP: Glial fibrillary acidic protein; HPA: Hypothalamus–pituitary–adrenal axis; IBA1: Ionized calcium-binding adapter molecule 1; INOS: Inducible nitric oxide synthase; LB: Lewy body; LC: Locus ceruleus; MNR: Median raphe nucleus; OCT: Open field test; PAG: Periaqueductal gray matter; PD: Parkinson’s disease; RPT: Rotarod performance test; SNpc: Substantia nigra, pars compacta; SPT: Sucrose preference test; SSD: Specific signal density; TH: Tyrosine-hydroxylase; TNF-alpha: Tumor necrosis factor alpha; UCN1: Urocortin 1 peptide; UCN1 mRNA; VTA: Ventral tegmental area.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12974-022-02399-w.

Additional file 1: Video 1A shows a 3D reconstruction of confocal Z-stack images demonstrating a disintegrating UCN1 neuron (green) surrounded by phagocytic IBA1 immunoreactive microglia (red) cells in the EWcp. (See the same cell in Fig. 3 also.)

Additional file 2: Video 1B shows a 3D reconstruction of confocal Z-stack images demonstrating a disintegrating UCN1 neuron (green) surrounded by phagocytic IBA1 immunoreactive microglia (red) cells in the EWcp. (See the same cell in Fig. 3 also.)

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Authors’ contributions
Animal experiments were performed by BU, BP, ZsM, MB, LÁK, JF, TF, NF, and BG. Behavioral tests and their assessments were conducted by JF, BU, BP, ZsM, BF, NF, and BG. Tissue and sample preparation was done by BU, MB, LÁK, TF, and BG. Immunolabelings were performed by BU, NF, and BG. RNAscope was carried out by BU, AK, VF, and BG. Microscopy and digital imaging, morphometry was done by GB, BU, and BG. Data analysis and statistics were performed by BG, JF, NF, and BU. First manuscript draft was written by BU, supervised by BG. The study design, preliminary tests were planned and assessed by BU and BG. Figures were prepared by BU, GB, and BG. All authors contributed to the final version of the manuscript.

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Availability of data and materials
The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations
Ethics approval and consent to participate
Experiments were approved by the Animal Welfare Committee of Pécs University, the National Scientific Ethical Committee on Animal Experimentation in Hungary and the National Food Chain Safety Office in Hungary (license No. BA02/2000–49/2017).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References
1. Poewe W, Seppi K, Tanner CM, Halliday GM, Brundin P, Volkmann J, et al. Parkinson disease. Nat Rev Dis Primers. 2017;3(3):17013. https://doi.org/10.1038/ndrpd.2017.13.
2. KumaRes M, Khan S. Spectrum of non-motor symptoms in Parkinson's disease. Cureus. 2021;13(2):e13275. https://doi.org/10.7759/cureus.13275.
3. Boronovo J, Allende-Castro C, Laliena A, Guerrero N, Silva H, Concha ML. Changes in neural circuitry associated with depression at pre-clinical, pre-motor and early motor phases of Parkinson's disease. Parkinsonism Relat Disord. 2017;35:17–24. https://doi.org/10.1016/j.parkreldis.2016.11.009.
4. Starkstein SE, Brockman S. Management of depression in Parkinson's disease: a systematic review. Mov Disord Clin Pract. 2017;4(4):470–7. https://doi.org/10.1002/mdc3.12503.
5. Goodert M, Jakes R, Anthony Crowther F, Grazià SM. Parkinson's disease, dementia with lewy bodies, and multiple system atrophy as α-synucleinopathies. Methods Mol Med. 2001;62:33–59. https://doi.org/10.1385/1-59259-142-6.33.
6. Hunter S. The rostral mesencephalon in Parkinson's disease and Alzheimer's disease. Acta Neuropathol. 1985;68(1):53–8. https://doi.org/10.1007/BF00388956.
7. Wakabayashi K, Tani J, Mori F, Takahashi H. The Lewy body in Parkinson's disease: molecules implicated in the formation and degradation of alpha-synuclein aggregates. Neuropathology. 2007;27(5):494–506. https://doi.org/10.1111/j.1440-1789.2007.00803.x.
8. Stankovic I, Petrovič T, Pekmezović T, Marković V, Stojković T, Dražašević-Miljković N, et al. Longitudinal assessment of autonomic dysfunction in early Parkinson's disease. Parkinsonism Relat Disord. 2019;66:74–9. https://doi.org/10.1016/j.parkreldis.2019.07.008.
9. Kozic T, Bittencourt JC, May PJ, Reiner A, Gamin PD, Palkovits M, et al. The Edinger–Westphal nucleus: a historical, structural, and functional perspective on a dichotomous terminology. J Comp Neurol. 2011;519(8):1413–34. https://doi.org/10.1002/cne.22580.
10. Xu L, Scheinen WJ, Leshan RL, Patterson CM, Elias CF, BouwHUw S, et al. Leptin signaling modulates the activity of urocortin 1 neurons in the mouse nonpreganglionic Edinger–Westphal nucleus. Endocrinology. 2011;152(3):979–88. https://doi.org/10.1210/en.2010-1143.
11. Innis RB, Aghajanian GK. Cholecystokinin-containing and nociceptive neurons in rat Edinger–Westphal nucleus. Brain Res. 1986;363(2):230–8. https://doi.org/10.1016/0006-8993(86)91008-5.
12. Xu L, Bloem B, Gaszner B, Roubos EW, Kozicz T. Stress-related changes in the activity of cocaine- and amphetamine-regulated transcript and nesfatin neurons in the midbrain non-pregnanglionic Edinger–Westphal nucleus in the rat. Neuroscience. 2010;170(2):478–88. https://doi.org/10.1016/j.neuroscience.2010.07.001.

13. Xu L, Janssen D, van der Knaap N, Roubos EW, Leshan RL, Myers MG Jr, et al. Integration of stress and leptin signaling by CART producing neurons in the rodent midbrain centrally projecting Edinger–Westphal nucleus. Front Neuroanat. 2014;8(8). https://doi.org/10.3389/fnana.2014.00008.

14. Zuniga A, Ryabinin AE. Involvement of centrally projecting Edinger–Westphal nucleus neuropeptides in actions of addictive drugs. Brain Sci. 2020;10(2):67. https://doi.org/10.3390/brainsci10020067.

15. Vaughan J, Donaldson C, Bittencourt J, Perrin MH, Lewis K, Sutton S, et al. urocortinergic neurons respond in a differentiated manner to various acute stressors in the Edinger–Westphal nucleus. Nature. 1995;378(6554):287–92. https://doi.org/10.1038/378287a0.

16. Kozicz T, Yanaihara H, Arimura A. Distribution of urocortin-like immunoreactivity in the central nervous system of the rat. J Comp Neurol. 1998;391(1):1–10. https://doi.org/10.1002/(SICI)1096-9861(19980202)391:1<1::AID-CNE3>3.0.CO;2-6.

17. Deussing JM, Chen A. The corticotropin-releasing factor family: physiology of the stress response. Physiol Rev. 2018;98(4):2225–86. https://doi.org/10.1152/physrev.00042.2017.

18. Kormos V, Gáspár L, Kovács LA, Farkas J, Gaszner T, Csernus V, et al. Reduced response to chronic mild stress in PACAP mutant mice is associated with blunted FosB expression in limbic forebrain and brainstem centers. Neuroscience. 2016;325(330):335–58. https://doi.org/10.1016/j.neuroscience.2016.06.004.

19. Farkas J, Kovács LA, Gászár L, Nafiz A, Gaszner T, Ujvári B, et al. Construct and face validity of a new model for the three-hit theory of depression using PACAP mutant mice on CD1 background. Neuroscience. 2017;23(354):11–29. https://doi.org/10.1016/j.neuroscience.2017.04.019.

20. Gaszner B, Csernus V, Kozicz T. Urocortinergic neurons respond in a differentiated manner to various acute stressors in the Edinger–Westphal nucleus in the rat. J Comp Neurol. 2004;480(2):170–9. https://doi.org/10.1002/cne.20343.

21. Gaszner B, Korosi A, Palkovits M, Roubos EW, Kozicz T. Neuropeptide Y activates urocortin 1 neurons in the nonpreganglionic Edinger–Westphal nucleus. J Comp Neurol. 2007;500(4):708–19. https://doi.org/10.1002/cne.21177.

22. Gaszner B, Jensen KO, Farkas J, Reglodi D, Csernus V, Roubos EW, et al. Effects of maternal separation on dynamics of urocortin 1 and brain-derived neurotrophic factor in the rat non-preganglionic Edinger–Westphal nucleus. Int J Dev Neurosci. 2009;27(5):439–51. https://doi.org/10.1016/j.ijdevneu.2009.05.006.

23. Aschrafi A, Verheijen JM, Gordebeke PM, Olde Loohuis NF, Menting K, Jager A, et al. MicroRNA-326 acts as a molecular switch in the regulation of somatostatin SST4 receptor transgenic mice: construction and brain expression pattern characterization. Int J Mol Sci. 2021;22(7):3758. https://doi.org/10.3390/ijms22073758.

24. Quellacci C, Pelagalli A, Liguori G, Mirabella N. Urocortins in the mammalian endocrine system. Acta Vet Scand. 2019;61(1):46. https://doi.org/10.1186/s13028-019-0480-2.

25. Ip CW, Cheong D, Vollmann J. Stereological estimation of dopaminergic neuron number in the mouse substantia nigra using the optical-fractonomator and standard microscopy equipment. J Vis Exp. 2017;127:56103. https://doi.org/10.3791/56103.

26. Harrison L, Pfuhlmann K, Schriever SC, Pfluger PT. Profound weight loss induces reactive astroglia in the accumbens of obese mice. Mol Metab. 2019;40:149–55. https://doi.org/10.1016/j.molmet.2019.03.009.

27. Blesa J, Przedborski S. Parkinson’s disease: animal models and dopaminergic cell vulnerability. Front Neuropathol. 2014;15(8):155. https://doi.org/10.3389/fneph.2014.00155.

28. Huang YL, Zeng NX, Chen J, Ni J, Luo WJ, Liu P, et al. Dynamic changes of behaviors, dentate gyrus neurogenesis and hippocampal miR-124 expression in rats with depression induced by chronic unpredictable mild stress. Neural Regen Res. 2020;15(6):1150–9. https://doi.org/10.4103/1673-5374.270414.

29. Feyissa DD, Aher YD, Engidawork E, Höger H, Lubeck G, Kozicz T. Individual differences in male rats in a behavioral test battery: a multivariate statistical approach. Front Behav Neurosci. 2017;11(11):26. https://doi.org/10.3389/fnhbeh.2017.00026.

30. Zhang ZN, Zhang JS, Xiang J, Yu ZH, Zhang W, Cai M, et al. Subcutaneous rotenone rat model of Parkinson’s disease: dose exploration study. Brain Res. 2017;15(1655):104–13. https://doi.org/10.1016/j.brainres.2016.11.020.

31. Zuniga A, Smith ML, Canuso M, Ryabinin AE. Vesicular glutamate transporter 2-containing neurons of the centrally-projecting Edinger–Westphal nucleus regulate alcohol drinking and body temperature. Neuropharmacology. 2021;21(200):108795. https://doi.org/10.1016/j.neuropharm.2021.108795.

32. Riley RG, Kline RH IV. Neuronal lesioning with axonally transported toxins. J Neuropathol Methods. 2000;103(1):73–82. https://doi.org/10.1016/s1096-3011(00)00029-7.

33. Xu L, Furedi N, Lutter C, Geenen B, Pětěřával V, Baláškov A, et al. Leptin coordinates efferent sympathetic outflow to the white adipose tissue through the midbrain centrally-projecting Edinger–Westphal nucleus in male rats. Neuropharmacology. 2021;1205:108898. https://doi.org/10.1016/j.neuropharm.2021.108898.

34. Paxinos G, Watson C. The rat brain in stereotaxic coordinates. 6th ed. New York: Elsevier Academic Press; 2007.

35. Furedi N, Nagy Á, Mikoł A, Berta G, Kozicz T, Pětěřával V, et al. Melanocortin 4 receptor ligands modulate energy homeostasis through urocortin 1 neurons of the centrally projecting Edinger–Westphal nucleus. Neuropearmacology. 2017;115(18):26–37. https://doi.org/10.1016/j.neuropharm.2017.03.002.

36. Guilloux JP, Seney M, Edgari N, Bilibelle E. Integrated behavioral z-scoring increases the sensitivity and reliability of behavioral phenotyping in mice: relevance to emotionality and sex. J Neuropsychosci. 2011;197(1):21–31. https://doi.org/10.1177/1473287911007001001.

37. Nemes B, Bölcsei K, Kecskés A, Kormos V, Gaszner B, Acszell T, et al. Human somatostatin SST4 receptor transgenic mice: construction and brain expression pattern characterization. Int J Mol Sci. 2021;22(7):3758. https://doi.org/10.3390/ijms22073758.

38. Betarbet R, MacKenzie G, Garcia-Okuna M, Panov AV, Greenamyre JT. Chronic systemic pesticide exposure reproduces features of Parkinson’s disease. Nat Neurosci. 2000;3(12):1301–6. https://doi.org/10.1038/71834.

39. Betarbet R, Canet-Aviles RM, Sherer TB, Mastroberardino PG, McLendon C, Myre JT. Chronic systemic pesticide exposure reproduces features of Parkinson’s disease. Nat Neurosci. 2005;8(11):1496–502. https://doi.org/10.1038/nneurosci.2005.12003.

40. Campos FL, Carvalho MM, Cristovão AC, Je G, Baltazar G, Salgado AJ, et al. Rodent models of Parkinson’s disease: beyond the motor symptomatology. Front Behav Neurosci. 2013;26:7(175). https://doi.org/10.3389/fnbeh.2013.00175.
rotenone-induced Parkinson’s disease in rats: Possible role of endocan-nabinoxid system modulation. Int Immunopharmacol. 2021;94: 107431. https://doi.org/10.1016/j.intimp.2021.107431.

47. Zaitone SA, Abo-Elmatty DM, Elshazy SM. Piracetam and vincopetine ameliorate rotenone-induced Parkinsonism in rats. Indian J Pharmacol. 2012;44(6):774–9. https://doi.org/10.4103/0253-7613.103300.

48. Sun C, Wang Y, Mo M, Song C, Wang X, Chen S, et al. Minocycline protects against rotenone-induced neurotoxicity correlating with upregulation of Nurr1 in a parkinson’s disease rat model. Biomed Res Int. 2019;5(2019):6843265. https://doi.org/10.1155/2019/6843265.

49. Valvasori S, Samita, Roger B, Varela and Joao Quevedo (2017): Animal Models of Mood Disorders: Focus on Bipolar Disorder and Depression, Pages 991–1001, Open field test

50. Scheggi S, De Montis MG, Garbanara C. Making sense of rodent models of anhedonia. Int J Neuropsychopharmacol. 2018;21(11):1049–65. https://doi.org/10.1093/ijnp/pyx083.

51. Prasad EM, Hung SY. Behavioral tests in neurotoxin-induced animal models of Parkinson’s disease. Antioxidants (Basel). 2020;9(10):1007. https://doi.org/10.3390/antioxidants1010007.

52. Clausen BH, Wierenfeldt M, Høgedal SS, Frich LH, Nielsen HH, Schrøder HD, Clausen BH, Wirenfeldt M, Høgedal SS, Frich LH, Nielsen HH, Schrøder HD, Prasad EM, Hung SY. Behavioral tests in neurotoxin-induced animal models of Parkinson’s disease. J Neuroinflammation. 2020;17(1):1. https://doi.org/10.1186/s12974-019-01055-1.

53. Zhang D, Li S, Hou L, Jing L, Ruan Z, Peng B, Zhang X, Hong JS, Zhao J, Zhang W. Microglial activation contributes to cognitive impairments in rotenone-induced mouse Parkinson’s disease model. J Neuroinflammation. 2018;15(1):1. https://doi.org/10.1186/s12974-017-0808-3.

54. Höglinger GU, Féger J, Prigent A, Michel PP, Parain K, Champy et al. Rozenkevich SA, Abo-Elmatty DM, Elshazy SM. Piracetam and vinpocetine disease patients. Acta Neuropathol Commun. 2014;2:90. https://doi.org/10.1186/2051-9355-2-90.

55. Joksimovic M, Anderegg A, Roy A, Campochiaro L, Yun B, Kittappa R, Zaitone SA, Abo-Elmatty DM, Elshazy SM. Piracetam and vinpocetine disease patients. Acta Neuropathol Commun. 2014;2:90. https://doi.org/10.1186/2051-9355-2-90.

56. Kumar ST, Jagannath S, Francois C, Vanderstichele H, Stoops E, Lashuel AM. Microglial phenotypes and toll-like receptor 2 in the substantia nigra degeneration in rats. J Neurochem. 2003;84(4):491–502. https://doi.org/10.1046/j.1471-4159.2003.01533.x.

57. Kojismovic M, Andregas M, Roy A, Campochiaro L, Yun B, Kittappa R, et al. Spatiotemporally separable Shh domains in the midbrain define distinct dopaminergic progenitor pools. Proc Natl Acad Sci USA. 2009;106(45):19185–90. https://doi.org/10.1073/pnas.0904283106.

58. Kumar ST, Jagannath S, Francois C, Vanderstichele H, Stoops E, Lashuel AM. Microglial phenotypes and toll-like receptor 2 in the substantia nigra degeneration in rats. J Neurochem. 2003;84(4):491–502. https://doi.org/10.1046/j.1471-4159.2003.01533.x.

59. Heinz S, Freyberger A, Lawrenz B, Schladt L, Schmuck G, Ellinger-

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