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Effects of prolonged treatment with corticosterone on brain transcriptome in laboratory mice

Summary of the doctoral dissertation

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The research performed was financed by OPUS grant 2017/27/B/NZ2/02796

Jastrzębiec 2023

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List of publications constituting a doctoral dissertation

1. Aneta Jaszczyk, Grzegorz R. Juszczak. Glucocorticoids, metabolism, and brain activity. Neuroscience & Biobehavioral Reviews, 2021

DOI: https://doi.org/10.1016/j.neubiorev.2021.03.007

Impact Factor = 9.052

Points of Ministry of Education and Science = 200p

2. Aneta Jaszczyk, Adrian M. Stankiewicz, Grzegorz R. Juszczak. Dissection of mouse hippocampus with its dorsal, intermediate, and ventral subdivisions combined with molecular validation. Brain Sciences, 2022

DOI: https://doi.org/10.3390/ brainsci12060799

Impact Factor = 3.333

Points of Ministry of Education and Science = 100p

3. Aneta Jaszczyk, Adrian M. Stankiewicz, Joanna Goscik, Alicja Majewska, Tadeusz Jezierski, Grzegorz R. Juszczak. Overnight corticosterone and gene expression in mouse hippocampus. International Journal of Molecule Sciences, 2023

DOI: https:// doi.org/10.3390/ijms24032828

Impact Factor = 6.208

Points of Ministry of Education and Science = 140p

Summary

Glucocorticoids are natural steroid hormones synthesized by the adrenal cortex. In humans, the dominant substance of this group is cortisol while in other mammals such as mice, it is corticosterone. These hormones have a critical role for the organism. They take part in the metabolism of carbohydrates, proteins, fats, and perform several other functions. The process of glucocorticoid secretion is mediated by the hypothalamic-pituitary-adrenal (HPA) axis that is controlled by the circadian cycle and brain areas orchestrating responses to the external and internal threats. Released glucocorticoids bind to the mineralocorticoid and glucocorticoid receptors located in the target cells. Glucocorticoids are important in medicine because of their anti-inflammatory and immunosuppressive effects. Their major role is to prepare an organism to activity both in basal and stress-induced conditions and to curb excessive responses of the immune system that could pose a threat to the organism.

The aim of the doctoral dissertation was to examine the effects of prolonged treatment with glucocorticoid hormone (corticosterone) on transcriptomic changes in the hippocampus of laboratory mice and to relate them to biological processes in the brain. The doctoral dissertation includes three publications in indexed scientific journals.

The first publication aimed at reviewing the current state of knowledge on the biological effects induced by glucocorticoids in the brain and to identify the most important gaps in the available literature. A detailed analysis of the literature has revealed that the most important limitation of existing knowledge results from the fact that the research was focused mainly on the effects observed within the first 3-4 hours after glucocorticoid hormone administration while longer periods especially within the range of 6 to 12 hours were very rarely studied. Importantly, the few available data indicate that longer time periods are crucial to understand the effects of glucocorticoid hormones on the brain. Moreover, corticosterone research conducted to date is dominated by a few topics such as memory-related brain plasticity and inflammation while the metabolic effects of glucocorticoids in the brain are still studied fragmentarily. Additionally, many brain studies conducted so far have methodological limitations related, for example, to the use of synthetic glucocorticoids, which show limited ability to penetrate the blood-brain barrier. Therefore, many aspects of glucocorticoid action in the brain are still poorly understood indicating a need for new experimental studies.

The goal of the second publication was to develop and validate a dissection method allowing for collection of a high-quality samples for transcriptomic experiments. We performed

this methodological experiment because our previous studies have shown that contamination with the choroid plexus may lead to the occurrence of both false-positive and false-negative results in transcriptomic studies performed on brain samples. The experiment confirmed that the method allows for collection of well-preserved hippocampi with negligible amount of choroid plexus. Therefore, the method became a methodological basis for further experiments testing the effect of corticosterone on the transcriptome in the hippocampus.

Considering the identified gaps in existing knowledge, we conducted a pharmacological experiment using the hippocampal dissection method described in publication number 2. The obtained results are described in the third publication, which aimed at assessing the effect of elevated glucocorticoids on transcriptomic changes in the mouse hippocampus after 12h of corticosterone treatment and during subsequent resting period when the level of corticosterone returns to the baseline. Obtained results show that transcriptomic responses to glucocorticoids are heterogeneous in terms of the decay time and that changes in the expression of glucocorticoid-responsive genes depend on the duration of the resting period. Analysis of the results indicates that glucocorticoids affect the expression of genes related to lipid, glycogen and iron metabolism, immune response and the cardiovascular system. Transcriptomic effects induced by 12-hour corticosterone treatment consist of acute effects (genes described in previous studies after about 3 to 4 hours) and delayed effects (genes specific for prolonged glucocorticoid action). The delayed effects include also changes in gene expression that achieve significance at the time when the blood level of the hormone returns to the baseline. Finally, we have found a considerable overlap between genes regulated by corticosterone and genes implicated previously in stress response.

Streszczenie

Glikokortykoidy to naturalne hormony sterydowe syntezowane przez korę nadnerczy. U człowieka dominującą substancją z tej grupy jest kortyzol, u innych ssaków takich jak myszy jest to kortykosteron. Hormony te pełnią kluczową rolę dla organizmu. Biorą udział w metabolizmie węglowodanów, białek i tłuszczy oraz spełniają szereg innych funkcji. W procesie wydzielania glikokortykoidów pośredniczy oś podwzgórze-przysadka-nadnercza (HPA), która jest kontrolowana przez cykl okołodobowy oraz obszary mózgu regulujące reakcje na zagrożenia zewnętrzne i wewnętrzne. Uwolnione glukokortykoidy wiążą się z receptorami mineralokortykoidowymi i glikokortykoidowymi zlokalizowanymi w komórkach docelowych. Glikokortykoidy są ważne w medycynie między innymi ze względu na swoje właściwości przeciwzapalne i immunosupresyjne. Główną rolą glikokortykoidów jest przygotowanie organizmu do aktywności zarówno w warunkach podstawowych, jak i wywołanych stresem oraz hamowanie nadmiernych reakcji układu odpornościowego, które mogłyby stanowić zagrożenie dla organizmu.

Celem doktoratu było zbadanie wpływu długotrwałego działania hormonu glikokortykoidowego (kortykosteronu) na zmiany transkryptomiczne w hipokampie myszy laboratoryjnych oraz powiązanie ich z procesami biologicznymi zachodzącymi w mózgu. Rozprawę doktorską stanowi zbiór trzech spójnych tematycznie artykułów opublikowanych w czasopismach naukowych.

Celem pierwszej publikacji było dokonanie przeglądu dotychczasowego stanu wiedzy na temat efektów biologicznych wywoływanych przez glikokortykoidy w mózgu oraz identyfikacja najważniejszych nie zbadanych dotąd aspektów w dostępnej literaturze. Szczegółowa analiza literatury wykazała, że najważniejszym ograniczeniem dotychczasowej wiedzy jest skupienie się na efektach obserwowanych w ciągu pierwszych 3-4 godzin po podaniu hormonów glikokortykoidowych podczas gdy dłuższe okresy czasowe w zakresie od 6 do 12 godzin są powszechnie pomijane. Nieliczne dostępne dane wskazują jednak, że dłuższe okresy czasowe są kluczowe dla zrozumienia oddziaływania hormonów glikokortykoidówych na mózg. Co więcej realizowane do tej pory badania nad kortykosteronem są zdominowane przez niektóre tematy takie jak plastyczność mózgu związana z pamięcią oraz stany zapalne podczas gdy efekty metaboliczne glikokortykoidów w mózgu są nadal zbadane jedynie fragmentarycznie. Dodatkowo, wiele badań mózgu realizowanych w przeszłości zawiera niedoskonałości metodologiczne związane

ze stosowaniem syntetycznych glikokortykoidów, które wykazują ograniczoną zdolność przenikania przez barierę krew-mózg. Dlatego, pomimo kilku dekad badań wiele aspektów działania glikokortykoidów w mózgu jest nadal słabo poznanych co uzasadnia podjęcie tej tematyki w badaniach eksperymentalnych.

Celem drugiej publikacji było opracowanie i walidacja metody pobierania hipokampu pozwalającej na zebranie wysokiej jakości próbek do eksperymentów transkryptomicznych. Wcześniejsze badania wykazały, że zanieczyszczenie splotem naczyniówkowym może prowadzić do wystąpienia fałszywie dodatnich oraz fałszywie ujemnych wyników w badaniach transkryptomicznych mózgu. Przeprowadzony eksperyment potwierdził, że zastosowana metoda pozwala na pobranie dobrze zachowanego hipokampu ze znikomą ilością splotu naczyniówkowego. Metoda ta została następnie wykorzystana w badaniach wpływu kortykosteronu na transkryptom w hiopokampie.

Biorąc pod uwagę zidentyfikowane braki w dotychczasowej wiedzy zaplanowano eksperyment farmakologiczny wykorzystujący metodę pobierania tkanki mózgowej opisaną w publikacji numer 2. Uzyskane wyniki zostały opisane w trzeciej publikacji, której celem było zbadanie wpływu podwyższonego poziomu glikokortykoidów na zmiany transkryptomiczne w hipokamie myszy po 12 godzinnym podawaniu kortykosteronu i podczas późniejszego okresu spoczynku, kiedy poziom kortykosteronu wraca do poziomu wyjściowego. Otrzymane wyniki wskazują, iż transkryptomiczna odpowiedź na glikokortykoidy jest heterogenna pod względem czasu zaniku, a zmiany w ekspresji genów reagujących na glikokortykoidy zależą od czasu trwania okresu spoczynku. Analiza uzyskanych wyników wskazuje, że glikokortykoidy wpływają na ekspresje genów związanych z metabolizmem lipidów, glikogenu i żelaza, odpowiedzią immunologiczną oraz układem sercowo-naczyniowym. Efekty transkryptomiczne wywołane 12 godzinnym podawaniem kortykosteronu składają się z efektów ostrych (geny opisane wcześniej po około 3 - 4 godzinach) i efektów opóźnionych (geny specyficzne dla przedłużonego działania glikokortykoidów). Dwunasto-godzinne podawanie kortykosteronu wywołuje również efekty transkryptomiczne, które osiągają istotność statystyczną w momencie, gdy poziom hormonu we krwi wraca do wartości wyjściowej. Uzyskane w badaniu dane dotyczące genów regulowanych przez kortykosteron w znaczonym stopniu pokrywają się z genami związanymi z odpowiedzią na stres.

1. Introduction

Glucocorticoids (GCs) are the steroid hormones synthesized by the adrenal glands (Kalafatakis et al., 2019). GCs regulate many physiological functions, such as glucose and lipid metabolism, cell growth and proliferation, immune responses, brain plasticity and memory processes (Juszczak and Stankiewicz, 2018; Vitellius et al., 2018; Timmermans et al., 2019; Jaszczyk and Juszczak, 2021). The process of glucocorticoid secretion is mediated by the hypothalamic-pituitary-adrenal (HPA) axis that is controlled by the circadian cycle and brain areas orchestrating responses to the external and internal threats (de Kloet, 2013; Nicolaides et al., 2015). The mechanism of GCs action is mediated by two ligand-activated transcription factors: mineralocorticoid (MR) and glucocorticoid (GR) receptors. The response to GCs differs between individuals, tissues, and cells (Weikum et al., 2017). Due to their effects on the immune system, synthetic GCs are often used to treat many inflammatory (Adcock and Mumby, 2016) and autoimmune diseases (Spies et al., 2011).

The stress response involves the organism's series of complex reactions to restore homeostasis disturbed by a real or perceived threat (Joseph and Whirledge, 2017). The response includes activation of sympathetic nervous system leading to rapid release of adrenaline from adrenal medulla and activation of the HPA axis triggering delayed release of glucocorticoids from the adrenal cortex (Nicolaides et al., 2015; Joëls et al., 2018). Adrenal glands release both corticosterone and cortisol although the proportion between these two glucocorticoids varies greatly between species. For example, the main glucocorticoid in mice is corticosterone while in humans cortisol is the dominant hormone (Joëls et al., 2018). The released hormones activate receptors both in the brain and peripheral organs allowing for adaption to changes in the environment (Joëls et al., 2018).

1.1. Mechanisms of GCs action

1.1.1. Glucocorticoid receptors

The effective action of glucocorticoids is mediated by the mineralocorticoid (MRs) and glucocorticoid (GRs) receptors in the brain (Mifsud and Reul, 2018). Both receptors have different ligand affinity and distribution (de Kloet, 2013). This results in a precise hormonal response that depends on the balance between mineralocorticoid and glucocorticoid receptors as well as hormone concentrations (Datson et al., 2008). Mineralocorticoid receptors have a higher affinity for GCs than glucocorticoid receptors (Reul et al., 1987). Mineralocorticoid receptors are activated at basal levels of circulating GCs, whereas glucocorticoid receptors are involved when GCs levels increase, for example during stress (Reul et al., 1987; Datson et

al., 2008). Mineralocorticoid receptors are strongly expressed in limbic structures such as the hippocampus, amygdala, septum, and prefrontal cortex. Glucocorticoid receptors are common in neurons and glial cells. Their highest expression occurs in structures involved in stress responses such as the paraventricular nucleus, hippocampus, amygdala, and ascending aminergic neurons (de Kloet, 2013). The two types of receptors play different roles in regulating hypothalamic-pituitary-adrenal axis activity. Mineralocorticoid receptors maintain the basal activity of the axis, while glucocorticoid receptors are responsible for negative feedback activated by increased hormone concentrations (Datson et al., 2008). Both types of receptors belong to the nuclear hormone receptor superfamily and are encoded by the *NR3C1* and *NR3C2* genes (Plieger et al., 2018).

1.1.2. Regulation of glucocorticoid signaling

The production and secretion of glucocorticoid hormones is controlled by the hypothalamic-pituitary-adrenal axis (Spencer and Deak, 2017). Under non-stressful conditions, GCs are released into the blood in a circadian rhythm. Peak levels are obtained during the active phase (in humans in the morning, in mice at night). During stress, HPA axis activity is increased (Mifsud and Reul, 2018). Activation of the HPA axis causes the release of corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) from the hypothalamic paraventricular nucleus (PVN). CRH and AVP stimulate release of adrenocorticotropic hormone (ACTH) (Timmermans et al., 2019). ACTH in turn acts on the adrenal cortex and stimulates the release of GCs that not only affect various processes such as cell growth metabolism and immunity (Juszczak and Stankiewicz, 2018) but also triggers negative feedback controlling the activity of the HPA axis at the level of the anterior pituitary, the hypothalamus, and higher brain centers (McCarty, 2016). Negative feedback is important for terminating the HPA axis response under physiological conditions and during stress (Timmermans et al., 2019).

1.1.3. Genomic and non-genomic effects

Glucocorticoids use both genomic and non-genomic mechanisms to trigger their action in the organism (Makara, 2001). Genomic mechanisms of action are mediated by the cytosolic glucocorticoid and mineralocorticoid receptors (Gray et al., 2017). These are the most well-studied mechanisms of action (Panettieri et al., 2019). After GCs attach to GR, the receptor moves from the cytosol to the cell nucleus, where it binds to GC response elements (GREs) and then acts as a transcription factor affecting gene expression (Mir et al., 2021). The non-genomic action of GCs is rapid and does not involve transcription or protein synthesis. GCs non-genomic

effects involve non-specific interactions with the cell membrane, or specific interactions with cytosolic GRs (cGR) or membrane-bound GRs (mGR) (Panettieri et al., 2019). Some of the non-genomic actions of GCs result from the interaction between GCs and membrane lipids which alters their physicochemical properties and modulates of the MAPK (mitogen activated protein kinases) signaling cascade (Timmermans et al., 2019).

1.2. Glucocorticoids in rodent brain

The most precise information on the timing of GCs entry into the brain has been collected in rodents subjected to stress procedures or treated with corticosterone. Available data indicate that brain corticosterone levels are typically elevated 10 -15 min after peripheral injection or stress exposure, peak after 20-60 min, and return to baseline after 60-120 min in most cases (Jaszczyk and Juszczak, 2021). Based on experimental data, it can be observed that many factors affect the timing of elevated GCs and the resulting brain response. These include genetic background, initial exposure to mild stress (Thoeringer et al., 2007), the age of the animals (Yau et al., 2015) and the amount of hormone injected or released because of the stress exposure. A physiological mechanism contributing to the delay of brain entrance of corticosterone is a concomitant release of Corticosteroid-Binding Globulin from the liver that is most pronounced during moderate and severe stress (Qian et al., 2011). A confounding factor that can also contribute to the variability of results is stress associated with preparation of animals for experiments, for example transport of animals between different rooms. Such inadvertent stress can initiate release of GCs before the start of the procedure intended for inducing the stress response. Finally, significant changes in the brain level of corticosterone are easier for detection in adrenalectomized animals (Conway-Campbell et al., 2007; Venero and Borrell, 1999) because of a negligible basal level of GCs and smaller between-subject variability due to the absence of changes in the level of endogenously released hormone.

1.2.1. Metabolic effects of glucocorticoids

One of the most well-known effects of glucocorticoids is an elevated blood glucose level, which is due to decreased uptake in some tissues such as muscles and body fat (Sakoda et al., 2000; Su et al., 2014) and increased gluconeogenesis in the liver (Khani and Tayek, 2001; Kuo et al., 2015; Mcmahon et al., 1988). Although GCs were primordially associated with the glucose metabolism, they also have a profound effect on lipid metabolism and influence the availability of numerous energy substrates (Jaszczyk and Juszczak, 2021). Acute metabolic responses to GCs can be divided into early (first 2 h) and delayed ($\geq$ 4 h) effects based on the time-course of concomitant changes in blood glucose (Jaszczyk and Juszczak, 2021).

1.2.2. Brain effects of glucocorticoids

As indicated by the available data GCs affect the dynamics of neuronal activity. Depending on their interaction with other factors, GCs can lead to variable neuronal responses involving both excitation and inhibition. This, in turn, is expected to promote task-related activity in response to environmental challenges (Jaszczyk and Juszczak, 2021). The effects of GCs on the brain are pleiotropic and elevated GCs levels affect changes in synapse formation, dendritic arborization and hippocampal volume (Fukumoto et al., 2009). Long-term exposure to stress affects the function of many brain regions including the prefrontal cortex and amygdala (Fukumoto et al., 2009). Increased levels of GCs are detrimental to brain development and function (Fukumoto et al., 2009).

1.3. Significance of glucocorticoid research

Glucocorticoids are important in medicine for a multitude of reasons. First, they are crucial to maintain homeostasis in basal conditions (Dunlop, 1963) and participate in the mechanism of the stress response (Nicolaides et al., 2015). Second, they are widely used in medicine for their potent anti-inflammatory properties (Reichardt et al., 2021). Finally, prolonged changes in the level of glucocorticoids lead to serious diseases because insufficient release leads to Addison's disease (Dunlop, 1963) while excessive levels of endogenous or exogenous glucocorticoids cause Cushing's syndrome (Bista and Beck, 2014). However, despite their medical importance, there are significant gaps in understanding the mechanism mediating the effects of glucocorticoids on brain metabolism and physiology (Jaszczyk and Juszczak, 2021). To fill the existing gaps in knowledge, an in vivo experiment was performed (the results are described in Publication 3). It was designed to study the effects of corticosterone administered for 12 h during the period of natural activity associated with the light-dark cycle to provide a better understanding of the brain processes following one-day stress or glucocorticoid treatment and during the subsequent resting period associated with the light-dark cycle.

2. Hypotheses

- Transcriptomic effects triggered by overnight treatment with corticosterone (12 hours) will differ from acute effects reported previously after about 3 - 4 hours.
- Changes in expression of genes responsive to glucocorticoids will depend on the duration of the resting period.
- Treatment with corticosterone can induce delayed transcriptomic effects that will achieve significance at the time when the blood level of the hormone returns to the baseline.

3. Objective of the study

- Explaining processes taking place in the brain in response to prolonged (12h) elevated levels of corticosterone

4. Material and methods

4.1. Animals

Seventy Swiss-Webster male mice were used in both experiments. Five were used in experiment 1 (Publication 2), and sixty in experiment 2 (Publication 3). The mice were bred at the Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences in Jastrzębiec. Animals were housed in cages with fine sawdust bedding (4-5 mice per cage) at standard conditions (12/12 h light cycle, 22±2°C, and 55±5 % humidity). The animals had an enriched environment and free access to dry food (Labofeed H, Kcynia, Poland) and tap water. Both experiments were conducted as part of project 2017/27/B/NZ2/02796, which has been approved by the local ethics committee (permit no. WAW2/090/2018) in accordance with polish Act of 15 January 2015 on the protection of animals used for scientific and educational purposes.

4.2. Experimental procedure

4.2.1. Experiment 1 - validation of dissection method

Assessment of dissection precision was performed on tissues obtained from 5 mice, which were 3.5 months old and weighed 35.7 ± 1.3 g. From each mouse, two hippocampi were collected as separate samples, as well as a portion of brain tissue that was a positive control for the expression of the choroid plexus marker (*Ttr*) gene. Dissection was performed on ice using an illuminated table magnifier (3X) and a Petri dish painted black to increase the contrast between the background and the dissected tissue. Each step was preceded by a thorough rinsing of the brain with ice-cold, sterile water stored in a laboratory wash bottle. The first step was to

remove the olfactory bulb and the anterior portion of the brain located 3-4 mm from the frontal pole. Then, using a needle and spatula, the cerebral cortex covering the hippocampi was removed, starting from the interhemispheric fissure. The hippocampi were separated from the white matter along their anterior and posterior edges. The white matter along the anterior edge, together with the associated tissues and cortex removed at an earlier stage, was frozen in liquid nitrogen, and served as a positive control for the expression of the choroid plexus marker (*Ttr* gene). The dorsal part of the hippocampus was separated from the tissues around the third ventricle using an oblique cut that was perpendicular to the longitudinal axis of the hippocampus. Finally, we released the ventral part of the hippocampus using a jet of ice-cold sterile water and a dissecting needle. All visible connections between the ventral hippocampus and the white matter were removed with a scalpel. The stream of ice-cold sterile water was used not only to separate the hippocampus from the rest of the brain, but also to remove and make visible the remnants of other tissues from the dorsal and ventral surfaces of the hippocampus. All pieces of tissue attached to the hippocampus were cut off with a scalpel. Each hippocampus was divided into 3 equal parts corresponding to the dorsal, intermediate and ventral parts.

4.2.2. Experiment 2 - testing an effect of corticosterone on hippocampal transcriptome

Three months old mice (weight 39g ± 3.8g,) were relocated from family cages to individual cages. After the separation, the mice were divided into the control and corticosterone groups. The mice assigned to the corticosterone group were divided randomly into 3 subgroups (n = 10). For each corticosterone group there was assigned a separate control group (n = 10) of siblings so that obtained results could be compared between brothers from both groups. Each group contained animals from 5 different litters. As a result of the salivary gland tumor observed at a later stage of the experiment, the number of animals in one group was reduced to 9. In the other groups, the number of mice was unchanged. According to the procedure used in our laboratory (Stankiewicz et al., 2015, 2014) the mice were habituated to the new conditions for 21 days. This time allows for the normalization of corticosterone levels and responses of animals to environmental stimuli (Stankiewicz et al., 2014; Hunt and Hambly, 2006). On the 22 day at the beginning of the dark phase, the main part of the experiment began. Half of the mice received corticosterone (100 μg/ml) dissolved in drinking water with hydroxypropyl-β-cyclodextrin (0.45%) which is a cyclic oligosaccharide used to dissolve steroid hormones in water. Corticosterone was pre-dissolved in a 30% hydroxypropyl-β-cyclodextrin solution using a vortex/magnetic stirrer and diluted to final concentration. The dose of corticosterone was selected based on previous studies and additional

pilot experiment. Animals in the control group received water with the addition of hydroxypropyl-β-cyclodextrin. Mice were given new bottles of corticosterone solution or water at the beginning of the 12-hour activity phase. On the next day, the animals were sacrificed at a three time points to collect samples for analysis. The first group of corticosterone-treated mice and assigned control subjects were sacrificed during the first hour of the light phase when animals are still awake although their activity is decreases, while the remaining corticosterone-treated and control mice were sacrificed during the 5th and 9th hours when the animals are asleep. Animals from control and corticosterone groups were sacrificed in alternating order. Blood was collected in Eppendorf tubes containing 20 µl of 0.4mM Na_2EDTA. Brains were removed for hippocampal dissection performed according to protocol described in Publication 2. Dissected whole hippocampi were placed in freezing vials, then frozen in liquid nitrogen and stored at a temperature of -80°C.

4.3. Analysis of blood samples

After the blood was collected, the glucose level was tested using a glucometer (Microdot) and dedicated test strips. For this purpose, 1 µl of blood was used. Remaining blood was centrifuged (10 min / 5000 RPM at +4 ºC) to collect plasma that was stored in -20°C. From the collected plasma, corticosterone levels were tested. The plasma corticosterone level was checked by enzyme-linked immunosorbent assay (Demeditec Corticosterone rat/mouse ELISA kit). One sample was replicated twice on the plate. The test was performed according to the protocol provided by the manufacturer and the absorbance for each well was read at 450 nm.

4.4. RNA isolation

Total RNA from both experiments was extracted from individual samples using the GeneMATRIX universal RNA purification kit (EURx Ltd. Poland), according to the protocol provided by the manufacturer. The quantity and quality of all RNA samples were assessed by spectrophotometry (ND-1000, Nanodrop) and microcapillary electrophoresis (Bioanalyzer 2000). For experiment 2, high quality samples (260/280 ~ 2.1, RIN >9) were selected for microarray analysis (n = 8 in each group).

4.5. Microarray analysis

The analysis of gene-expression profile was performed using SurePrint G3 Rat Gene Expression v2 8x60K Microarray, 8x60K (Agilent Technologies, USA) and Agilent Technologies Reagent Set according to the manufacturer's procedure. RNA Spike In Kit (Agilent Technologies, USA) was used as an internal control, the Low Input Quick Amp Labeling Kit was applied to amplify and label (Cy3 or Cy5) target RNA to generate complementary RNA (cRNA) for oligo-microarrays. 300 ng of cRNA from control (Cy3-labelled) and corticosterone-treated (Cy5-labelled) mice were hybridized together on two-color microarrays without pooling of samples from the same groups. In total we used 24 microarrays printed on 3 slides with 8 microarrays applied for each time point. Both control and corticosterone-treated animals from all analyzed time points were assigned to each slide in a pseudorandom way. Gene Expression Hybridization Kit was used for fragmentation and hybridization and Gene Expression Wash Buffer Kit was used for washing slides after hybridization. Acquisition and analysis of hybridization intensities were performed using Agilent DNA Microarray Scanner G2505C. Data were extracted, and background subtracted using the standard procedures included in the Agilent Feature Extraction Software version 10.7.3.1. Data extraction included Lowess normalization. The data were deposited in GEO database (accession number GSE218508)

4.6. Annotation of microarray data

Due to the variability between different genomic databases (Allen et al., 2012; Stankiewicz et al., 2019), a consensus annotation combining two different annotation approaches was used (Allen et al., 2012). Each probe was annotated with a gene symbol list using biomaRt R package with "agilent sureprint g3 ge 8x60k" attribute (Durinck et al., 2009), GPL21163-3202.txt annotation file from GEO database (Barrett et al., 2012) and GPL21163_noParents.an.txt annotation file from gemma database (Lim et al., 2021). If none gene symbol existed, the probe sequence was annotated with Ensembl identifiers using rBLAST R package [Basic local alignment search tool, https://github.com/mhahsler/rBLAST] followed by translation of these identifiers to gene symbols using biomaRt. The second annotation was based on Biomart/Enseble database and combined data from mouse and mouse strain databases (version 107) since some probes are included only in the mouse strain databases. Biomart/Ensembl does not contain the most recent version of the Agilent mouse microarrays that were used in our experiments (v2 8x60K). Therefore, we combined data retrieved for 8x60K and WholeGenome agilent microarrays. In the retrieved annotation dataset,

we included information about the gene name and type, transcript name and type and assignment to the Ensembl canonical category of transcripts having the highest coverage of conserved exons, highest expression, longest coding sequence and represented in other key resources (https://www.ensembl.org/info/genome/genebuild/canonical.html). Finally, the first and second annotation was compared with each other to identify consistently annotated probes, including gene synonyms retrieved from Biomart/Ensembl with the term "gene name" selected in filter panel and the terms "gene name" and "synonyms" selected in attributes panel.

4.7. Microarray data analysis

The raw data files were analyzed with the Limma package from the Bioconductor project using the same criteria for all files (Smyth, 2005). The 'normexp' background correction method (Ritchie et al., 2007) has been applied. The background correction was followed by within-array normalization carried out with the loess procedure and between-array normalization conducted with the quantile method (Bolstad et al., 2003; Smyth and Speed, 2003). Normalized data without offset were used for the calculation of fold changes and retrieval of separate channel intensities obtained with the following formula:

$$Red\ channel = \sqrt{2^{2 \cdot A\ value} \cdot 2^{M\ value}}$$

$$Green\ channel = \frac{Red\ channel}{2^{M\ value}}$$

M value means binary logarithm of red/green intensity ratio while A value means average log2 intensity of the microarray spot. Data with offset 50 (variance stabilizing transformation) were used for the calculation of p values following previous guidelines (Ritchie et al., 2007; Silver et al., 2009). The statistical analysis was performed with separate channel tests which take under consideration the intra-spot correlation (Smyth and Altman, 2013). P-values were corrected using the Benjamini and Hochberg procedure controlling False Discovery Rate (Benjamini and Hochberg, 1995). Genes showing adjusted p-values < 0.05 were considered as differentially expressed.

4.8. PCR validation

In both experiments, gene expressions were analyzed by SYBR Green-based qPCR performed in 96-well plates on a Roche LightCycler® 96 thermocycler. Primers were designed using the Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The designed primers were located on two different exons and contained all the mRNA

transcripts of each of the specified genes. The annealing temperature for each primer was determined by performing PCR with a set temperature gradient (55º-65º) during a 3-step amplification. The specifications of the primers are shown in Publication 2 Table 1 and Publication 3 Table 2. In the first experiment we have analyzed the expression of marker genes *Trhr*, *Lct* and *Ttr* to verify the precision of dissection (Publication 2) with *Hmbs* gene selected as the reference gene. In the second experiment we have analyzed the expression of *Sult1a1*, *Lao1*, *Etnppl*, *Apoc3* to validate the microarray results (Publication 3) with *Tbp* gene used as the reference gene. In both experiments, the reference gene was selected using NormFinder software (https://moma.dk/normfinder-software) from among 4 different genes (*Hmbs*, *Ywhz*, *Tbp*, and *Gapdh*). The reverse transcription in both experiments was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche) and either 500 ng (Publication 2) or 1µg (Publication 3) of total RNA was used.

All genes were tested in triplicate, and each replicate was on a separate plate. In addition, each plate contained a series of 5-fold dilutions of the cDNA sample to determine the efficiency of the reaction. The plate also contained negative controls without cDNA. The final reaction volume for each gene was 20 µl. The exception was the *Trhr* gene where the final reaction volume was 40 µl. PCR products were subjected to melting curve analysis to confirm amplification specificity using Light Cycler 96 software (https://sequencing.roche.com/us/en/products/group/lightcycler-96.html). The relative expression of genes was calculated using the Pfaffl method.

4.9. Statistical analysis

In both experiments data were first tested for variance homogeneity with dedicated tests (C Cochran, Hartley and Bartlett / Levene's tests). Data that did not meet the requirement of variance homogeneity were first subjected to the square root transformation and next were tested again. Data with homogenous variance were analyzed with ANOVA followed by the Fisher's least significance difference (LSD) test. The data that did not meet the requirement of variance homogeneity even after SQRT transformation were analyzed with the nonparametric Mann–Whitney U test. Pearson's coefficient was used to assess the correlation between microarray and PCR results. The data analysis was performed with Statistica software, release 7.1 (StatSoft Inc., Tulsa, USA). Values are presented as mean ± SEM (column bar graphs) and scatter plots.

5. Results

5.1. Experiment 1 - validation of dissection method

5.1.1. Expression of Choroid Marker Gene *Ttr*

The analysis revealed that the *Ttr* gene had very low expression in all parts of the hippocampus (dorsal, intermediate and ventral; n = 9) in contrast to control tissue (n = 5) (Publication 2, Figure 5). The average expression was about 400 to 700 times higher in the control tissue than in the hippocampal samples. The differences were significant for all parts of the hippocampus compared to the control (p = 0.003).

5.1.2. Expression of Marker Genes Differentiating between Dorsal and Ventral Hippocampus

Real-time PCR analysis showed that *Lct* expression was highest in the dorsal hippocampus (n = 9) and lowest in the ventral hippocampus (n = 9), while the intermediate part (n = 9) showed intermediate levels of expression (Publication 2, Figure 6). Differences between neighboring parts of the hippocampus (dorsal vs. intermediate and intermediate vs. ventral) were significant at p = 0.0003. The *Trhr* gene showed an opposite expression pattern characterized by the highest level of expression in the ventral hippocampus (n = 9) and the lowest in the dorsal hippocampus (n = 7), as indicated by real-time PCR analysis (Publication 2 Figure 7). For two samples from the dorsal hippocampus, we obtained negative results for all replicates of the PCR analysis (triplicate). Due to uncertainty about the reasons for the negative results (technical error vs. lack of expression), we omitted these two samples from the statistical analysis, reducing the total number of samples to seven. The intermediate portion (n = 9) showed intermediate levels of Trhr expression. The differences were significant, with p = 0.0009 (dorsal part vs. intermediate part) and p = 0.0003 (intermediate part vs. ventral part).

5.2. Experiment 2 - testing an effect of corticosterone on hippocampal transcriptome

5.2.1. Blood Corticosterone and Glucose

Animals that received corticosterone at the beginning of the dark phase showed increased hormone levels in the first hour of the bright phase (Publication 3 Figure 1A [1 hour]). In this group of mice, corticosterone returned to baseline during the 5-hour rest period and remained at this level after 9 hours of rest (Publication 3 Figure 1A [5 and 9 hours]). In the control animals, corticosterone levels were at low levels at the first two time points and increased in the last time point (Publication 3 Figure 1A,B). Statistical analysis showed significant differences between corticosterone-treated and control animals during the 1st and 9th hours, and no differences at the 5th hour (Publication 3 Figure 1A). For the blood glucose level

(Publication 3 Figure 1C), they were similar in both groups at the first time point and showed a decrease in the corticosterone-treated group at 5 and 9 hours. ANOVA revealed a significant effect of treatment ($p < 0.0001$) and a significant interaction between treatment and time of sampling ($p = 0.007$) with significant differences between the corticosterone and control groups during the 5th ($p = 0.002$) and 9th hour ($p < 0.0001$) of the resting period.

5.2.2. Effect of Corticosterone - General Characteristics of Microarray Results

Analysis of the microarray results showed significant changes between corticosterone-treated and control animals at all three time points tested. A total of 17 444 unique probes showed significant differences between groups during at least one time point studied. The remaining probes (39 161) showed non-significant results. The microarray results were divided into primary effects (10 969 probes), which were already significant when corticosterone levels were elevated (Publication 3, Figure 8), and secondary effects (6475 probes), which became significant at 5 and 7 hours, when corticosterone levels returned to baseline in treated animals (Publication 3, Figure 9). The primary effects were classified into long-term (persisting throughout the study period), intermediate (showing a difference at 1 and 5 hours), short-term (showing differences in the first hours of the resting period) and primary effects that reversed the direction of altered expression during the resting period.

5.2.3. Primary Effects

A comparison of corticosterone-treated and control animals sacrificed over the first hour (Publication 3 Figure 8A) revealed 10 969 unique probes differing between groups. Half (51,2%) of these showed small changes of no larger than 25% (absolute value of log2 fold change $\leq$ 0,32). In contrast, there were 3764 probes differing between 25% and 50% (absolute value of log2 fold change > 0,32 and $\leq$0,58), 1376 probes differing between 50% and 100% (absolute value of log2 fold change > 0,58 and $\leq$1) and only 210 probes showing differences greater than 100% (absolute value of log2 fold change > 1). The primary effects reduced with time. After 9 h of rest, the number of probes showing a major effect of corticosterone decreased to 43% of all significant effects observed during the first hour (Publication 3 Figure 8C).

5.2.3.1. Long-Lasting Primary Effects

Analysis of microarray probes revealed 3451 probes specifically annotated to 3144 genes, which showed significant differences in all three testing periods with the same direction of change between groups. Most of these probes code proteins, but there were also lncRNAs (154), miRNAs (1) lincRNAs (2), rRNAs (1), TEC (To be Experimentally Confirmed) genes

(14), 169 pseudogenes (4,9%) and 96 dysfunctional transcripts (2,8%) classified in the Ensembl database as processed transcripts, transcripts retaining introns, nonsense mediated decay and antisense transcripts.

Most probes (2991) indicated small (<25%) and medium (<50%) differences between groups. Differences greater than 100% (log2 fold change > 1) after 9 h of rest were indicated by only 77 probes annotated to 71 genes. After rejecting probes with low signal intensity, the group was limited to seven protein-coding genes (*Etnppl, Sult1a1, Heph, Pygm, Pla2g3, Clcnka and Lao1*). For these genes, prolonged expression was detected by probes binding canonical transcript variants. Smaller differences (range from 50 to 100% after 9 h of rest) were indicated by 383 probes annotated to 327 genes. After discarding probes with low signal intensity, the group was reduced to 22 genes (*Mt1*, *Ptgds*, *Apod*, *Fam107a*, *Timp4*, *Phyhd1*, *Aqp4*, *Pxmp2*, *Hmgcs2*, *Agt*, *Pygm*, *Plin4*, *Vmn1r48*, *Kansl3*, *Rgs12*, *Opalin*, *Smim4*, *Col5a3*, *Apoc3*, *Ugt1a6b*, *Olfr145*, *Gm10447*). In the case of *Opalin*, this effect was detected by a transcript variant binding to a probe with conserved intron sequences. Examples of genes showing the most persistent changes in expression during all time points tested are shown in publication 3 in figure 10.

5.2.3.2. Intermediate Primary Effects

A number of 1956 probes specifically annotated to 1781 genes showed significant differences during the first and fifth hours with the same direction of change during both time points tested but returned to baseline during the ninth hour of the resting period. Examples of this expression model can be seen in Publication 3 Figure 11. Most of them code proteins, although there are also lncRNAs (77), miRNAs (1) lincRNAs (1), 12 TEC (To be Experimentally Confirmed) genes, 2 unknown but probable coding genes, 41 (2.1%) pseudogenes and 52 (2. 7%) dysfunctional transcripts, classified in the Ensembl database as processed transcripts, intron-preserving transcripts, antisense transcripts, nonsense decay transcripts and LoF transcripts.

Most of probes (1737) indicated small (<25%) and medium (<50%) differences between groups. Differences larger than 100% (log2 fold change > 1) after 5 h of rest were indicated by only 11 probes annotated to 11 genes but most of them displayed very low signal intensity (mean < 50). After the rejection of these probes, the group was restricted to two protein-coding genes (*Cdkn1a* and *Maff*). In the case of *Cdkn1a*, the effect was specific for some variants of the transcripts. Importantly, highly significant changes occurring during the first and fifth hour were detected by a probe binding canonical variant of *Cdkn1a* transcripts. Smaller but still

considerable differences (the range between 50 and 100% after 5 h of rest) were indicated by 208 probes annotated to 181 genes. This group was also dominated by probes with small signal intensity (mean < 50). After their rejection, the group was restricted to only 26 genes (*8430426J06Rik, Alpl, Atp2c2, Ccl12, Cmtm2a, Cntfr, Ecscr, Fmo2, Gbp2, Gbp3, Gjb6, Glp2r, Gm12022, Gm4285, Lrg1, Map3k6, Mt2, Ninj2, P2ry12, Pdpn, Sdc4, Slc38a5, Stab2, Tekt4, Tmem52 and Tmprss6*).

5.2.3.3. Short-Lasting Primary Effects

2893 probes specifically annotated to 2490 genes indicated differences between groups only during the first hour of rest, which was associated with increased glucocorticoid levels in animals receiving exogenous corticosterone. Examples of this expression model can be seen in Publication 3 Figure 12. Most of these genes code proteins, although there are also lncRNAs (198), miRNAs (2) lincRNAs (2), snRNAs (1), 26 TEC (To be Experimentally Confirmed) genes, 71 (2.5%) pseudogenes and 74 (2.6%) dysfunctional transcripts classified in the Ensembl database as processed transcripts, intron-preserving transcripts, antisense transcripts, nonsense decay transcripts and LoF transcripts.

The majority of probes (2490) indicated small (<25%) and medium (<50%) differences between groups. Differences larger than 100% (log2 fold change > 1) were indicated by only 33 probes but most of them displayed very low signal intensity (mean < 50). After the rejection of these probes, the group was restricted to six genes (*Kcnq2, Depp1, Galnt15, Plekhf1, Cxcl10* and *Phactr3*). In the case of *Kcnq2*, *Cxcl10* and *Phactr3* the effect was specific for some variants of the transcripts but only in the case of *Cxcl10* the significant effect was detected by a probe binding canonical variants of the transcripts. The most perplexing case is the *Kcnq2* gene because a significant effect was detected by a probe binding only dysfunctional variants of the *Kcnq2* transcripts (retained intron and processed transcript lacking an open reading frame) while five other probes indicated a lack of differences between groups. Smaller but still considerable differences (the range between 50 and 100% after 5 h of rest) were indicated by 370 probes. This group was also dominated by probes with small signal intensity (mean < 50). After their rejection, the group was restricted to only 20 genes (*Hes5, Sgk1, Mgp, Fzd2, Arrdc2, Pdk4, Vgll3, Thbs4, Rtp4, Gata2, Ifit3b, Tnfsf10, Cytl1, Tcim, BC018473, A330032P22Rik, Phf11d, Lhx3, BC053393 and Acss3*).

5.2.3.4. Time-Dependent Reversal of Primary Effects

A relatively small number of corticosterone-responsive genes significantly reversed the direction of expression during the resting period. Examples of this expression pattern are shown

in publication 3 Figure 13. There were 75 probes indicating a reversal in expression and most of them detect protein-coding genes with exception of one lncRNA, two processed pseudogenes and one transcript containing intron. Additionally, some probes are inconsistently annotated in different databases (two probes) or are annotated to more than one gene in the Ensembl/BioMart database (two probes). After the removal of probes that are lacking specificity, 71 genes were found that had reversed expression, including 39 genes that were unique for the category of primary transcriptomic responses with the time-dependent reversal. The remaining 32 genes also displayed other expression patterns detected by additional probes. All unique 39 genes displayed signal intensity larger than 80 and the largest differences between groups (of more than 50%) were found in the case of seven protein-coding genes (*Paqr5, Fas, Nfkbia, Fkbp5, Fgfrl1, Mc4r, Smim3*).

5.2.4. Secondary Effects

One of our assumptions was that the transcriptomic changes induced during an elevated level of corticosterone (first hour) will trigger an additional wave of transcriptomic effects that will develop when corticosterone returns to the baseline. However, the secondary effects were smaller in terms of the number of probes, indicating significant differences only during the fifth and ninth hour of the resting period (Publication 3 Figure 9B,C) compared with the first hour (Publication 3 Figure 9A). Although there were 60 probes indicating changes larger than 100% after 9 h of rest, all of them were characterized by small signal intensity (mean < 50). Very low signal intensity was also found in the case of probes indicating differences in the range between 50 and 100% after 9 h of rest because most of them (99%) were characterized by very low signal intensity (mean < 50). After the rejection of probes with the lowest signal intensity, the group was restricted to only five genes (*Zbtb16, Sh3pxd2b, Rhcg, Asb4, LOC102635912, Gjb3 and Gipc2*).

5.2.5. PCR Validation of Microarray Results

Validation of microarray results was performed for *Sult1a1*, *Lao1*, *Etnppl* and *Apoc3*. Genes were selected based on the significant effects observed for specific probes A 55 P2117155 (*Apoc3*), A 51 P391616 (*Etnppl*), A 55 P2101021 (*Lao1*), A 55 P2005475 (*Sult1a1*) and A 51 P321341 (*Sult1a1*). The results of the PCR analysis were compared with all microarray probes annotated to the selected genes (Publication 3, Figure 2). Calculated correlations show high concordance between PCR results and preselected microarray probes (Publication 3, Figure 2A-D, G), indicating that microarrays reliably detect levels of validated genes. Similar conclusions emerged from comparisons between groups. PCR analysis showed

increased expression of *Sult1a1* and *Lao1* at all time points tested (Publication 3 Figure 3) with a p-value < 0.001, as indicated by the Mann-Whitney U test (U = 1, n1 = n2 = 8) and the same expression pattern was found in the microarray data. In addition, the analysis shows variability among the different probes in their ability to detect the expression of annotated genes. These differences were most striking for the *Apoc3* gene (Publication 3 Figure 2D-F). Between-group comparisons showed that the two best probes for detecting *Apoc3* detected similar changes between control and corticosterone-treated animals (Publication 3 Figure 4B, C). This finding is consistent with the PCR results (Publication 3 Figure 4A), which revealed a significant treatment effect [F(1,42) = 129.78.49, p < 0.0001] with significant differences between the corticosterone and treatment groups during all time points tested (p < 0.0001, Fisher's LSD test). In contrast, a third probe, which was not correlated with PCR results (Publication 3 Figure 2F), did not detect a treatment effect (Publication 3 Figure 4D). In the case of the *Etnppl* gene, detected by multiple probes, more divergent results in terms of correlation with PCR were evident (Publication 3 Figure 2G-I). Intergroup comparisons of PCR data (Mann-Whitney U test) showed significant differences during the first (U = 1, n1 = n2 = 8, p = 0.001), fifth (U = 9, n1 = n2 = 8, p = 0.016) and ninth hours of the resting period (U = 1, n1 = n2 = 8, p = 0.001) (Publication 3 Figure 5A). Intergroup comparisons of the microarray data showed that one probe detected increased expression at all three time points (Publication 3 Figure 5B), consistent with the PCR data. A second probe detected an increase at two time points (Publication 3 Figure 5C). The last detected increased expression at the first time point and the opposite effect at the last time point (Publication 3 Figure 5D). In addition, these probes showed significant differences in signal intensity (Publication 3 Figure 5B-D). Data downloaded from the Ensembl/BioMart database showed that a probe detecting the canonical Ensembl transcript (Publication 3 Figure 5B) provided results showing the highest correlation with PCR, while the lowest correlation was obtained for a probe detecting only alternatively spliced transcripts thought to contain intron sequences (Publication 3 Figure 5D).

6. Discussion

6.1. Experiment 1 - validation of dissection method

The goal of the first experiment was to develop and validate a dissection method allowing for collection of a high-quality samples for transcriptomic experiments. Our dissection method is different from most of the other gross dissection protocols that require initial separation of hemispheres before exposition of the hippocampus (Publication 2 Table 2). Most of these protocols also require complete removal or displacement of the brainstem and diencephalon (thalamus and hypothalamus) to access the hippocampus from inside the mouse or rat brain. In general principle, our protocol resembles the approach used previously by Spijker (Spijker, 2011), although the details of these protocols are different in many respects. The unique features of our protocol are angled cuts used to separate the right and left hippocampus without collection of tissues located between them in the third ventricle (including choroid plexus) (Publication 2 Figure 4), and usage of a stream of water to minimize damage or distortion of hippocampi during the dissection and to remove potential tissue contaminations. Furthermore, we put a special emphasis on the removal of all remnants of white matter to avoid tissue contamination that can be caused by inclusion of fimbria with the adjacent choroid plexus.

Performed experiment confirmed that our method allows for collection of well-preserved hippocampi with negligible amount of choroid plexus. Brain tissue is soft and malleable and therefore can be easily damaged leading to omission of larger parts of dissected samples. Dissection of the hippocampi with preserved major parts was confirmed by the pattern of expression of molecular markers of dorsal and ventral hippocampi. Consistently with expectations based on previous studies (Fanselow and Dong, 2010; Cembrowski et al., 2016) the *Lct* gene had high expression in dorsal portion and negligible in ventral part (Publication 2 Figure 6) while *Trhr* gene displayed an opposite pattern of expression (Publication 2 Figure 7). Dissection of all major parts of the hippocampus is important due to the functional and transcriptomic differences between these subdivisions (Fanselow and Dong, 2010; Cembrowski et al., 2016; Lee et al., 2017; Floriou-Servou et al., 2018).

The second important issue is the risk of contamination of brain samples with choroid plexus. To verify the efficacy of our protocol we used the transcriptomic marker (*Ttr*) of the choroid plexus (Sousa et al., 2007; Stankiewicz et al., 2015). The PCR analysis showed that the transthyretin gene (*Ttr*) is expressed at a residual level in hippocampal samples that display an mRNA level several hundred lower than adjacent control tissue colocalized with the choroid

plexus (Publication 2 Figure 5) consistently with our previous assessment based on analysis of brain slices (Stankiewicz et al., 2015). This indicates that the applied method for dissecting hippocampus from fresh brain allows for replicable removal of majority of choroid plexus from hippocampal samples. Therefore, the presented dissection method is especially suitable for molecular studies performed on homogenized tissues that are sensitive to contamination (Stankiewicz et al., 2015; Mathew et al., 2016; Stankiewicz et al., 2022). Such tissue contamination can be responsible not only for false positive findings that are present in many published datasets but may also obscure genuine changes in expression of some genes shared between tissues (Stankiewicz et al., 2022). Importantly, no other gross dissection protocol for mice or rats (Publication 2 Table 2) has tested the presence of contaminations in collected samples, and most of these papers (Chiu et al., 2007; Hagihara et al., 2009; Mathis et al., 2011; Spijker, 2011; Sultan, 2013; Villers and Ris, 2013) do not even mention the fact that the choroid plexus is in dissected brain tissue. This issue is also neglected in protocols describing free-hand dissection of rat and mouse hippocampi from brain slices (Heffner et al., 1980; Wager-Miller et al., 2020). The only available alternative that was proved to be effective in the removal of the choroid plexus is laser microdissection (Sousa et al., 2007) but this method requires expensive equipment and is time consuming, which severely limits its application. A special emphasis on the removal of tissue contamination and avoidance of tissue distortions makes our protocol especially suitable for molecular experiments. Therefore, the method became a methodological basis for further experiments testing the effect of corticosterone on the transcriptome in the hippocampus.

6.2. Experiment 2 - testing an effect of corticosterone on hippocampal transcriptome

The aim of the experiment was to uncover the effect of elevated level of corticosterone on brain transcriptome immediately after overnight treatment (12h) and during subsequent resting period when the level of corticosterone returns to the baseline. To enable noninvasive prolonged treatment, the corticosterone was administered in drinking water. This rote of treatment administration is not disturbing animals but requires confirmation that intestinal absorption is sufficient to increase the blood level of the drug. This is especially important in case of corticosterone that is difficult to dissolve in water. Therefore, we performed analysis of blood level of corticosterone that confirmed significantly elevated level of the hormone shortly after overnight treatment (Publication 3 Figure 1A,B). These data show also that the level of corticosterone returned to the baseline after 5 hours of rest providing the basis for the assesment of the decay time of transcriptomic effects. The low level of blood corticosterone

was maintained after 9 hours of rest in corticosterone-treated mice in contrast to control animals that displayed small but significant increase consistently with typical circadian rhythm of corticosterone release (Qian et al., 2012). These differences show that exogenous glucocorticoids inhibited the release of endogenous corticosterone according to known regulatory mechanisms (Dallman and Jones, 1973; Gjerstad et al., 2018). The corticosterone data confirm the effectiveness of the applied treatment and provide an important background for interpretation of the transcriptomic effects and verification of the proposed hypotheses.

First, we assumed that transcriptomic effects triggered by overnight treatment with corticosterone (12 hours) will differ from acute effects reported previously after about 3 - 4 hours. Our experiment revealed considerable number of genes affected by the treatment but comparison with previous data requires solving some problems that are inherent in transcriptomic data. First, comparability between different datasets retrieved from literature is hindered by frequent changes in gene nomenclature and inconsistencies between different databases used to annotate microarray probes (Stankiewicz et al., 2019). As a result, the same gene may be denoted in different studies with alphanumeric codes attributed to newly identified transcripts or different names based on its predicted or confirmed function depending on the progress in gene identification and nomenclature standardization between species. Furthermore, some genes may not be listed in older studies in case when microarray probes were not successfully annotated at the time of paper publication. Therefore, reliable comparison between different datasets requires reannotation and standardization of gene nomenclature. Second, a common problem with transcriptomic studies performed in the past is low statistical power resulting from small experimental groups and a frequently applied practice of sample pooling decreasing the number of analyzed microarrays per group (typically 3-4) (Stankiewicz et al., 2022). As a result, such data suffer from large number of poorly replicated results both in terms of frequency of detection and direction of observed changes in expression that are consistent with random effects leading to appearance of false positive findings (Juszczak and Stankiewicz, 2018; Stankiewicz et al., 2022). Therefore, the results obtained in our experiment were compared with a list of replicable findings identified by previous analysis of standardized data taken from 17 studies testing effect of glucocorticoids on brain transcriptome (Juszczak and Stankiewicz, 2018) after the most recent update of gene nomenclature (Stankiewicz et al., 2022). The reference dataset (Juszczak and Stankiewicz, 2018) is based on both in vivo (Datson et al., 2001; Alfonso et al., 2004; Sato et al., 2008; Datson et al., 2011; Polman et al., 2012; Chen et al., 2013; Datson et al., 2013; Gray et al., 2014) and in vitro experiments (Morsink et al., 2006a, 2006b; Salaria et al., 2006; Fukumoto et al., 2009; Anacker et al., 2013; Carter et

al., 2013; Slezak et al., 2013; Jenkins et al., 2014; Peffer et al., 2014) and is dominated by acute data obtained after about 3-4 hours after glucocorticoid administration. The list of already established GC-responsive genes is additionally divided into a core list that contains 88 genes displaying the same direction of change in at least 4 previous papers and the extended list containing 251 genes that displayed the same direction of change in three independent studies in response to glucocorticoids (Juszczak and Stankiewicz, 2018; Stankiewicz et al., 2022). Importantly, both these lists contains almost exclusively genes that change the expression up to 4 hours after treatment with glucocorticoids with exception of 1 core gene (*Ndrg2*) and 11 genes from the extended list (*Spp1*, *Cdk1*, *Lpl*, *Dab2*, *Psmb8*, *Ctsh*, *Ctsc*, *Atp1a2*, *Npy*, *Igfbp3* and *Sparc*). The comparison between our current results and the reference list of genes identified by previous studies confirmed the hypothesis that the transcriptomic effects triggered by the overnight treatment with corticosterone (12 hours) are different from acute effects reported previously after about 3 - 4 hours. In fact, the transcriptomic effects observed after 12 hours are composed of both acute effects observed up to 4 hours of treatment and a new set of genes specific for prolonged effects of glucocorticoids. The acute effects included most of the previously identified genes because we detected significant changes in expression of 78% of core genes and 75% of GC-responsive genes from the extended list. Presence of these acute effects is not surprising because the analysis included period of elevated level of corticosterone immediately after the overnight treatment. However, the identified acute effects constitute minority of all detected changes because they contribute only about 2% of all unique microarray probes displaying significant differences in at least one time point. Presence of delayed effects can be easily explained by the fact that glucocorticoids regulate wide range of transcription factors that can trigger a second wave of transcriptomic events (Juszczak and Stankiewicz, 2018). Especially striking findings are genes displaying a replicable pattern of expression during two and three independent time points with a high magnitude of detected changes (for example *Pip5k1a*, *Pmaip1*, *Gbp3*, *Tekt4*, *Gm11627*, *Maff*, *Ddc*, *Pnpla2*, *Pglyrp1*, *Alpl*, *Slc38a5*, *Lao1*, *Etnppl*, *Clank*, *Heph*, *Phyhd1*, *Timp4*, *Agt*, *Timp4*, *Vmn1r48*, *Pdzd2*, *Pygm*, *Apod*, *Serpinb1a*, *Crybb1*, and *Tfcp2l1*) that were not previously implicated in the glucocorticoid response (Juszczak and Stankiewicz, 2018). Importantly, our microarray study is based on large number of samples that were collected from 48 animals to increase the statistical power. This indicates that the previous list of glucocorticoid-responsive genes (Juszczak and Stankiewicz, 2018) should be extended with a special emphasis on genes that are regulated at longer intervals such as 12 h.

The second hypothesis was that changes in expression of genes responsive to glucocorticoids will depend on the duration of the resting period. Consistently with this assumption we have found that transcriptomic responses to glucocorticoids are highly heterogeneous in terms of fading time (Publication 3 Figure 9-12). The number of transcriptomic responses that show a short duration or even time-dependent reversal at rest (*Errfi1, Cdkn1a/p21, Ddit4/Redd1, Ndrg2, Sesn1, Wnt7a*) are involved in the negative control of cell growth and proliferation (Ball, 1997; Takeichi et al., 2011; Qu et al., 2013; Sun et al., 2014; Dungan et al., 2019; Guo et al., 2019). Acute stress is known to trigger extensive activation involving 96% of the brain (Bonapersona et al., 2022). Therefore, stress-induced inhibition of cell growth and proliferation is considered an adaptive mechanism that protects the brain from the adverse effects of overstimulation, including the genotoxic effects of reactive oxygen species and the redundant tropic effect of glutamate (Stankiewicz et al., 2022). However, long-term trophic inhibition may adversely affect cognitive processes dependent on neurogenesis and neuronal plasticity. Our results suggest that GC-induced impairment of cell growth and proliferation is susceptible to regeneration during resting periods associated with low glucocorticoid levels. In contrast, some of the GC-responsive genes showed persistent changes in expression throughout the resting period, despite rapidly normalizing blood corticosterone levels. The persistent transcriptomic responses occurring during the resting period indicate long-term processes affected by glucocorticoids. Inspection of the most affected genes, which varied by more than 50% after 9 hours of rest, indicates that GCs can induce long-term effects involving metabolism of lipids (*Etnppl*, *Apod* and *Pla2g3* (Sato et al., 2008; Rassart et al., 2020; White et al., 2021)), ketones (*Hmgcs2* (Puchalska and Crawford, 2017)) and glycogen (*Pygm* (Migocka-Patrzałek and Elias, 2021)), homeostasis of iron (*Heph* (Xu et al., 2018)), water and potassium (*Aqp4* (Vandebroek and Yasui, 2020)), blood pressure (*Agt* (Takeda et al., 2021)), peroxisomal transport (*Pxmp2* (Rokka et al., 2009)), actin dynamics (*Fam107a* (Kretzschmar et al., 2018)), inhibition of tissue remodeling (*Timp4* (Brew et al., 2000)), epigenetic regulation (*Kansl3* (Sheikh et al., 2020)), voltage-sensitive chloride channels (*Clcnka* (Rivera et al., 2021)) and finally removal of toxins and signaling molecules (*Ugt1a6b*, *Sult1a1* and *Mt1* (Coyle et al., 2002; Ouzzine et al., 2003; Wu et al., 2005; Riches et al., 2007; Vašák and Meloni, 2011)). Some of these genes also exert pleiotropic effects. For example, *Ptgds* (L-PGDS) is responsible for the synthesis of postaglandin D2, which regulates a wide range of processes such as vasodilation, immune responses and sleep homeostasis (Urade and Hayaishi, 2000). The functions of affected genes are consistent with a wide range of glucocorticoid-induced effects, including lipid, glycogen and iron metabolism (Jaszczyk and

Juszczak, 2021), immune response (Cain and Cidlowski, 2017) and cardiovascular system (Walker, 2007; Goodwin, 2015).

Finally, we assumed that the treatment with corticosterone can induce delayed transcriptomic effects that will achieve significance at the time when the blood level of the hormone returns to the baseline. Such secondary effects were detected during the 5th and 9th hour of the resting period confirming our hypothesis. However, the secondary effects (Publication 3 Figure 9B,C) were much smaller than the primary effects (Publication 3 Figure 9A) observed already at the time of elevated level of corticosterone (1st hour of rest). These differences between primary and secondary effects of corticosterone are visible in terms of the number of affected genes, their fold changes and the signal intensity. Therefore, we assume that the secondary effects play rather a minor role in response to glucocorticoids during the studied time period.

An important question is to what extent the pharmacological effects induced by corticosterone recapitulate effects observed during the stress response involving not only increased level of glucocorticoids but also other hormones and neurotransmitters. There are also some data indicating that there are interactions between glucocorticoids and other mediators of the stress response leading to unique effects that are not observed at the time when only GCs are elevated (Jaszczyk and Juszczak, 2021). A comparison of the results obtained in publication 3 with a reference list of stress-responsive genes (Stankiewicz et al., 2022) showed that 1702 GC-responsive genes are also reliably detected in experiments examining the effects of stress on the brain transcriptome. This indicates that GCs may contribute to 63.7% of the transcriptomic responses observed during a stress response, and this is a much higher estimate than the previous one based mainly on acute responses obtained between 1 and 6 hours after glucocorticoid administration (Stankiewicz et al., 2022). The group of transcriptomic responses common to GC and stress responses also includes genes showing the most sustained changes during the resting period, such as *Etnppl, Heph, Fam107a, Apod, Aqp4, Agt, Ptgds, Mt1, Plin4, Sult1a1* and *Pla2g3*. Importantly, we also found several genes that have not previously been implicated in the glucocorticoid response (Juszczak and Stankiewicz, 2018) but have been identified as top genes in the stress response (Stankiewicz et al., 2022) such as *Depp1, Galnt15, Mgp, Hes5, Txnip, Il1r1* and *Elovl7* for short-term primary effects, *Slc2a1, Acer2, Fabp7, Pglyrp1, Lrg1, Htra1, Fmo2, Htra1, Gjc2, Lfng, Thbd, Jdp2, Slco1c1, Fjx1, Pllp* for intermediate primary effects and *Opalin, Mobp, Slc4a4, Tmem88b, Trf, Ptn, Actb, Qk, Homer1, Junb, Ptn, Creb5* and *Kif5a* (long-term primary effects). This indicates that the

overnight corticosterone treatment model is a useful tool for studying the mechanisms underlying the stress response.

Despite our efforts to improve methodology and to increase the sample size, our data have also some limitations resulting from problems associated with the microarray technology. Despite considerable progress there is still a challenge associated with interpretation of results provided by alternative probes annotated to the same gene but, in fact, detecting different variants of transcripts including some that are not functional. The comparison with PCR results showed that different probes annotated to the same gene may provide a highly discrepant results because some of them provide highly correlated results while other provide results that are inconsistent with PCR validation. Some remedy constitutes retrieval of detailed information about the type of transcripts detected by individual probes but, unfortunately, there are still gaps in databases such as Ensembl. As a result, we were not able to collect complete information about all microarray probes. The second problem is the large number of probes indicating significant but small differences between groups (Publication 3 Figure 7 and 8). Such small effects in transcriptomic data may represent both true changes in gene expression and artefacts. It should be noted that transcriptomic changes restricted to a small population of highly specialized cells are diluted in the total pool of transcripts isolated from homogenized tissue (Stankiewicz et al., 2022) and such a scenario is particularly likely in the brain, which is composed of highly heterogeneous population of cells (Zeisel et al., 2018). Moreover, sampling may occur at an early or late stage of gene regulation, when the observed changes are small. Our results support such a hypothesis, as some known GC-responsive genes, such as *Errfi1*, *Klf9*, *Bcl6*, which respond to acute glucocorticoid administration (Juszczak and Stankiewicz, 2018), show significant but small differences (< 30%) after overnight corticosterone administration. On the other hand, small changes in detected expression may represent systematic errors generated by background correction and matrix normalization. Therefore, it should be assumed that the smaller the magnitude of the detected effects, the higher the probability of false-positive results in transcriptomic data. Nevertheless, there is no perfect method to separate true effects from technical errors in a single study. However, this problem can be overcome by using a meta-analytic approach that identifies replicable results and separates them from random effects in pooled data sets from different studies (Stankiewicz et al., 2022). Therefore, we plan a meta-analisis of transcriptomic effects of glucocorticoids that will include present data and will be based on methods of data curation and analysis developed previously (Juszczak and Stankiewicz, 2018; Stankiewicz et al., 2022).

7. Conclusion

- The transcriptomic effects induced by overnight corticosterone treatment (12h) are partially different from the acute effects previously described after about 3 - 4 hours. They consist of acute effects (genes previously described after about 3 - 4 hours) and delayed effects (genes specific for prolonged glucocorticoid action).
- The transcriptomic responses to glucocorticoids are heterogeneous in terms of the decay time and changes in the expression of glucocorticoid-responsive genes depend on the duration of the resting period.
- Treatment with corticosterone induce also changes in gene expression that achieve significance at the time when the blood level of the hormone returns to the baseline.

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9. Attachments

9.1.1. Percentage contribution of authors to each publication

9.1.2. Publications constituting a doctoral dissertation

9. Attachments

9.1. Percentage contribution of authors to each publication

Aneta Jaszczyk, Grzegorz R. Juszczak, Glucocorticoids, metabolism, and brain activity., Neurosci Biobehav Rev., 2021 Jul;126:113-145

Lp.	Name and Surname	Description of the author's participation	Participation in the publication in %	Institution	Signature
1.	Aneta Jaszczyk	Review of literature, writing the manuscript	50	Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences	Jaszczyk
2.	Grzegorz R. Juszczak	Review of literature, writing the manuscript	50	Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences	Juszczak

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Aneta Jaszczyk, Adrian M. Stankiewicz, Grzegorz R. Juszczak Dissection of mouse hippocampus with its dorsal, intermediate, and ventral subdivisions combined with molecular validation., Brain Sci., 2022 Jun 18;12(6):799

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2.	Adrian M. Stankiewicz	Data analysis	6	Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences	
3.	Grzegorz R. Juszczak	Designing the experiment, hippocampal isolation, preparing the data for publication, writing the manuscript	34	Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences	

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Aneta Jaszczyk, Adrian M. Stankiewicz, Joanna Goscik, Alicja Majewska, Tadeusz Jezierski, Grzegorz R. Juszczak. Overnight corticosterone and gene expression in mouse hippocampus., Int. J. Mol. Sci., 2023 Feb 1;24(3):2828

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4.	Alicja Majewska	Hybridization of samples on microarrays	4	Institute of Veterinary Medicine, Warsaw University of Life Sciences (SGGW)	
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6.	Grzegorz R. Juszczak	Designing and performing the experiment, statistical analysis (weight of internal organs, corticosterone and glucose levels), preparing the data for publication, manuscript writing	20	Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences	

Date 26.06.2023

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Neuroscience and Biobehavioral Reviews 126 (2021) 113–145

Contents lists available at ScienceDirect

Neuroscience and Biobehavioral Reviews

journal homepage: www.elsevier.com/locate/neubiorev

Glucocorticoids, metabolism and brain activity

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ARTICLE INFO

Keywords:
Brain metabolism
Glucocorticoids
Corticosterone
Cortisol
Glucose
Glycogen
Lactate
Hydroxybutyrate
Ketone bodies
c-Fos
Microdialysis
Glutamate
GABA
Noradrenaline
Electrophysiology
fMRI
Oxygen
ATP
Glycolysis
Mitochondria

ABSTRACT

The review integrates different experimental approaches including biochemistry, c-Fos expression, microdialysis (glutamate, GABA, noradrenaline and serotonin), electrophysiology and fMRI to better understand the effect of elevated level of glucocorticoids on the brain activity and metabolism. The available data indicate that glucocorticoids alter the dynamics of neuronal activity leading to context-specific changes including both excitation and inhibition and these effects are expected to support the task-related responses. Glucocorticoids also lead to diversification of available sources of energy due to elevated levels of glucose, lactate, pyruvate, mannose and hydroxybutyrate (ketone bodies), which can be used to fuel brain, and facilitate storage and utilization of brain carbohydrate reserves formed by glycogen. However, the mismatch between carbohydrate supply and utilization that is most likely to occur in situations not requiring energy-consuming activities lead to metabolic stress due to elevated brain levels of glucose. Excessive doses of glucocorticoids also impair the production of energy (ATP) and mitochondrial oxidation. Therefore, glucocorticoids have both adaptive and maladaptive effects consistently with the concept of allostatic load and overload.

1. Introduction

Glucocorticosteroids (GCs) are involved in the regulation of many basic physiological functions both under basal conditions (Kalafatakis et al., 2019) and in response to stress (de Kloet et al., 2019). Furthermore, because of a potent effect on the immune system, GCs are also widely used in the treatment of inflammatory, autoimmune and lymphoproliferative diseases. Effects of endogenously released GCs are divided into three broad categories that is permissive/stimulating, suppressive and preparative actions (Sapolsky et al., 2000). The first category of effects primes defensive mechanisms in basal conditions (permissive) and enhances the first wave of hormonal responses to stress (stimulating). Suppressive effects, in turn, prevent defense reactions from overshooting (Sapolsky et al., 2000) and in this respect can be compared with actions preventing water damage at the time of fire-fighting (Tausk, 1951 as cited in Sapolsky et al., 2000). Suppressive effects are also the main reason for using GCs in pharmacology. Finally, the last category of actions prepares an organism for subsequent stressors enabling, therefore, better responses in future (Sapolsky et al., 2000). All these actions of GCs participate in allostasis which is an active process of adaptation enabling maintaining physiological stability. However, when adaptation mechanisms are overused, they lead to so called allostatic overload causing adverse effects (Gray et al., 2017). This general classification of GC-mediated effects helps to place pharmacological effects in physiological perspective.

Although GCs affect many different physiological and cellular processes, they are in fact intimately connected with energy production because corticosterone and cortisol are mitochondria-derived hormones (mitokines) that mediate mitochondria-to-mitochondria communication among distant sites throughout the organism (Picard et al., 2018). Furthermore, the process of energy production is closely connected to many other cellular processes, even ones that are apparently not related such as gene methylation (Picard et al., 2018). Therefore, the regulation of metabolism is a key process in stress adaptation and contributes both

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https://doi.org/10.1016/j.neubiorev.2021.03.007
Received 30 March 2020; Received in revised form 4 March 2021; Accepted 7 March 2021
Available online 13 March 2021

to adaptive and maladaptive responses (Picard et al., 2018). The effect of glucocorticoids on metabolism of peripheral tissues received a lot of attention (Kuo et al., 2015; Magomedova and Cummins, 2016) but much less is known about the effect of glucocorticoids on brain metabolism. This issue is especially difficult because brain metabolism is highly dynamic due to changes in ongoing electrical signaling (Harris et al., 2012; Watts et al., 2018) and because it depends on the interaction between neurons and astrocytes which have a different metabolic profile (Watts et al., 2018). Furthermore, brain responses should be considered together with the time-course of peripheral metabolic changes to obtain a full picture of GC-induced changes in metabolism. Therefore, we decided to integrate different experimental approaches ranging from *in vitro* biochemical experiments to fMRI and electrophysiological studies to better understand the effect of an elevated level of glucocorticoids on the brain function. This integration also includes the discussion of limitations associated with different methodological approaches.

2. Limitations of the review

2.1. Neuronal activity

First, it should be noted that GCs exert a pleiotropic effect leading to changes in a multitude of cellular functions (Juszczak and Stankiewicz, 2018) and physiological processes ranging from immune responses (Sorrells and Sapolsky, 2007; Sorrells et al., 2009) to neuronal plasticity (Sapolsky et al., 2000; Joels et al., 2012; Gray et al., 2017). Therefore, we only tackle a relatively small aspect of a widespread bodily effect of glucocorticoids. Furthermore, we only focused on changes in activity ranging from the firing of single cells to a net effect observed at the population level. We chose the spiking activity as the lowest considered level because it constitutes the direct measure of changes in the neuronal activity. Obviously, spike generation depends on multiple local changes in membrane potentials that sum up to produce action potential or to block further signal transmission in the network. These processes depend in turn on multiple neurotransmitters and their receptors, ion channels and intracellular signaling pathways which are itself very complex and this complexity is even growing due to plastic changes. These mechanisms are very important but are beyond the scope of this review. Therefore, interested readers are referred to earlier reviews attempting to better understand the effect of GCs on local changes in membrane potentials that are responsible for spike generation (Joels et al., 2012).

2.2. Pharmacological effects

The second limitation results from the fact that most of the available pharmacological studies focused exclusively on the effects of an increased level of GCs without the consideration of interaction with other stress molecules. Such reductionism is a common approach in science because it helps to disentangle the contribution of separate factors to more complex phenomena. In the case of treatments with GCs, it has also a direct relevance for medicine because GCs are commonly used anti-inflammatory drugs. However, it should be stressed that the sum of separate parts studied in isolation is not always equal to the complex system from which they were derived and this issue applies also to glucocorticoids. For example, few available studies suggest that actions induced by GCs vary considerably depending on the presence or absence of noradrenergic stimulation and timing of this interaction (Allaman et al., 2004; van Stegeren et al., 2010; Schwabe et al., 2012; Karst and Joels, 2016). The distinction between treatment with GCs and stress response is also nicely shown by sensitization of the HPA axis to repeated stressful experiences in contrast to inhibition found after administration of exogenous corticosterone (Dallman and Jones, 1973). Another example is a beneficial effect of exercise in contrast to the detrimental effect of stress despite the fact that both of them increase the level of GCs (Chen et al., 2017). Therefore, disentangling the interaction between GCs and other factors such as noradrenaline is crucial for understanding the role of glucocorticoids in more complex physiological phenomena. Furthermore, it can help to better understand the variability in psychiatric side effects associated both with administration of GCs and Cushing's disease. It has also been suggested that differences in experimental procedures leading to inadvertent sympathetic activation may be responsible for variability between studies (van Stegeren et al., 2010). However, due to the shortage of data, future experiments will be needed to properly understand interactions between GCs and other mediators of the stress response (Joels, 2018).

2.3. Adrenalectomy

The third major limitation of the review results from potentially confounding effects of adrenalectomy that was frequently performed before many experiments. The rationale for performing adrenalectomy was to create a situation in which mineralocorticoid (MR) and glucocorticoid (GR) receptors are unoccupied at the beginning of an experiment to enable testing a wide range of doses of glucocorticoids including also the small one (Karst and Joels, 1991). Additionally, this approach solves the problem of stress-induced changes in GCs that may occur during preparation of animals for the experiments. Therefore, the experiments performed on adrenalectomized animals advanced our understanding of the role of MRs and GRs in responses to GCs. However, the adrenalectomy also affects other hormones released by the adrenal gland (Rosol et al., 2001) and leads to a number of metabolic (Kadekaro et al., 1988; Freo et al., 1992; Doyle et al., 1994b; Plaschke et al., 1996) and electrophysiological changes (Rey et al., 1987; Kasai and Yamashita, 1988; Joels and de Kloet, 1989). A peculiar phenomenon observed after adrenalectomy is also translocation of glucocorticoid receptors to nuclei in the absence of corticosterone (Sarabdjitsingh et al., 2009). Importantly, problems associated with adrenalectomy cannot be completely overcome by corticosterone supplementation because a basal level of corticosterone in intact animals is not stable but instead displays ultradian and circadian rhythms (Qian et al., 2012), and there are data suggesting that these naturally occurring fluctuations have functional significance (den Boon et al., 2019). It has also been found that corticosterone supplementation (pellets) results in altered responsiveness of hippocampal neurons in adrenalectomized animals (Beck et al., 1994). Collectively, these data indicate that adrenalectomized animals are not fully representative for the general population of intact subjects. Therefore, we restricted our review to non-adrenalectomised animals as much as possible. However, in case of research topics with few available data, such studies were included with annotation about adrenalectomy as a potential confounding factor.

3. Permeability of the blood-brain barrier

3.1. Differences between glucocorticoids

Both endogenous and synthetic GCs differ in their ability to penetrate the blood-brain barrier because its permeability is high for corticosterone while low for cortisol (Pardridge and Mietus, 1979; Karssen et al., 2001), dexamethasone (De Kloet et al., 1975; Meijer et al., 1998) and prednisolone (Karssen et al., 2002). Cortisol administered to rats enters brain at a low rate that is stable across various brain areas (McEwen et al., 1976). As a result, when comparable doses of cortisol and corticosterone are applied, similar concentrations are found after an hour in brain areas with lower expression of receptors such as hypothalamus, cortex and cerebellum while especially huge differences are found in the hippocampus (McEwen et al., 1976; Karssen et al., 2001). Differences between cortisol and corticosterone are present also in the human brain indicating that preferential uptake of corticosterone is a common phenomenon found in different species (Karssen et al., 2001). Despite lower permeability of human blood-brain barrier for cortisol, its content in brain is comparable with that in plasma indicating that it enters the

brain (Karssen et al., 2001) although the time-course of this process is unknown.

Similarly, a restricted ability to penetrate the blood-brain barrier is found in the case of dexamethasone (De Kloet et al., 1975; Meijer et al., 1998). The threshold for its entry into the brain depends on the applied dose because 50 μg/kg had a negligible central effect after acute (Cole et al., 2000) and repeated treatments (Karssen et al., 2005) in contrast to 250 −500 μg/kg (Reul et al., 1987; Karssen et al., 2005). Responses to lower doses of dexamethasone may also differ between various brain areas because regions with lower expression of glucocorticoid receptors such as hypothalamus are more likely to be saturated with dexamethasone than hippocampus (De Kloet et al., 1975). Similar properties in terms of the restricted ability to cross the blood-brain barrier poses prednisolone but despite clinical significance we have a very limited number of experimental data (Karssen et al., 2002). Low permeability of the blood-brain barrier for some GCs in combination with peripheral effects of GCs and differences in affinity to MRs and GRs (Section 4) may lead to indirect effects confounding interpretation of pharmacological data (Section 5, Fig. 1).

3.2. Corticosterone in rodent brain

3.2.1. Corticosterone - time course

The most precise information about the timing of GCs entrance into the brain has been collected in laboratory rodents subjected to stress or treated with corticosterone, and this knowledge is crucial for the proper interpretation of experimental data. Experiments performed with microdialysis showed that the total blood level of corticosterone, which is usually measured, may not provide an accurate reflection of glucocorticoid concentrations in the brain. It is because brain corticosterone peaks 20 min later than total corticosterone in blood under stress conditions (Droste et al., 2008). The duration of initial period when there are no significant changes in the brain level of corticosterone can only be assumed based on the previously reported data because they either lack a detailed statistical analysis of differences between each time point and the baseline (Venero and Borrell, 1999; Droste et al., 2008, 2009; Heinzmann et al., 2010; Qian et al., 2011) or present rather conservative estimates due to multiple comparisons (Thoeringer et al., 2007). A comparison of means and SEM indicates that during the first 5−10 min the changes are negligible after subcutaneous administration of corticosterone (Droste et al., 2008) or stress exposure in rats while a clear-cut increase occurs after at least 10−15 min (Venero and Borrell, 1999; Droste et al., 2008, 2009; Qian et al., 2011). Microdialysis data are consistent with a finding that 10 min after i.v. injection of corticosterone there is increased nuclear translocation of glucocorticoid receptors (Conway-Campbell et al., 2007). This in turn indicates that such latency is sufficient for the penetration of the blood-brain barrier by the hormone at least in adrenalectomized animals. Similarly, delayed changes in the brain level of corticosterone were also observed in mice after exposure to stress (Thoeringer et al., 2007; Heinzmann et al., 2010) although some studies reported much faster changes (Yau et al., 2015a, b).

The maximum brain level of extracellular corticosterone is recorded 20−40 min after injection (Venero and Borrell, 1999; Droste et al., 2008; Bouchez et al., 2012) and usually 20−67 min after the beginning of the stress procedure in mice (Thoeringer et al., 2007; Heinzmann et al., 2010) and rats (Droste et al., 2008, 2009; Bouchez et al., 2012). Occasionally, very short latencies (10 min) to reach the maximum brain level of corticosterone were also reported in mice after stress (Yau et al., 2015a, b). Available data additionally show that nuclear translocation of glucocorticoid receptors peaks from 15 to 120 min after treatment (Conway-Campbell et al., 2007; Sarabdjitsingh et al., 2009) depending on the route of administration, studied brain area and utilization of either intact or adrenalectomized animals. An alternative approach based on nuclear binding of radiolabeled corticosterone (De Kloet et al., 1975) showed a maximum hippocampal radioactivity in adrenalectomized animals one hour after i.v. injection of a tracer amount of the hormone which is retained in these conditions by the high affinity mineralocorticoid receptors. These scarce data give us a hint of the time-course of a direct interaction between genome and receptor-bound corticosterone with the caveat that there are multiple mechanisms regulating binding of the available receptors to glucocorticoid response elements existing within the DNA (Polman et al., 2013; Mifsud and Reul, 2018).

Elevated brain levels of GCs return to the baseline or are greatly diminished after approximately 60–120 min although in some cases full normalization occurs during the third hour (Venero and Borrell, 1999; Thoeringer et al., 2007; Droste et al., 2008, 2009; Heinzmann et al., 2010; Bouchez et al., 2012; Yau et al., 2015a). A similar time course was observed in case of experiments assessing receptor occupation and nuclear translocation of glucocorticoid receptors in adrenalectomized (Conway-Campbell et al., 2007) and intact animals (Reul et al., 1987; Sarabdjitsingh et al., 2009) after injection of corticosterone or stressful experience. Summing up, available data indicate that the brain level of corticosterone is usually elevated 10−15 min after peripheral injection or stress exposure, peaks after 20−60 min and in most cases returns to baseline after 60−120 min.

3.2.2. Factors affecting brain entrance of corticosterone

The reviewed experiments show that the kinetics of the brain level of corticosterone depends on several factors such as genetic background, pre-exposure to mild stress (Thoeringer et al., 2007), age of animals (Yau et al., 2015a) and the amount of injected or released hormone due to the stress exposure. Higher doses (Bouchez et al., 2012) and more severe

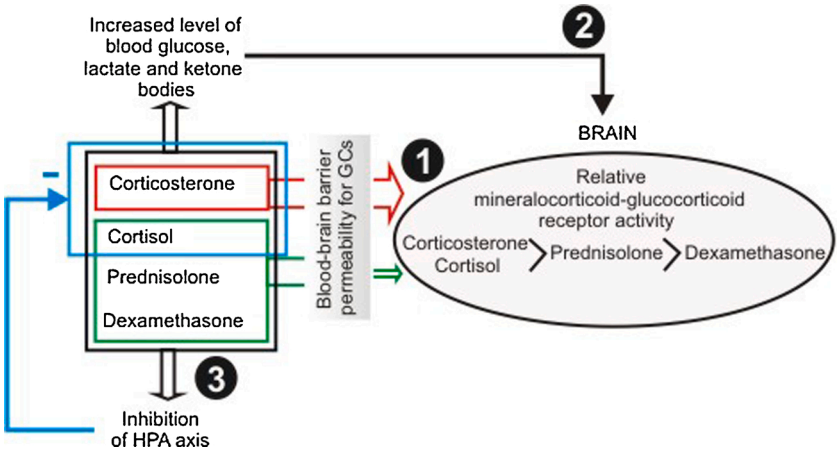

Fig. 1. First, GCs affect the nervous system directly by activation of brain mineralocorticoid and glucocorticoid receptors. These central effects are modulated by properties of GCs that affect their ability to penetrate the blood-brain barrier (Section 3.1) and ability to activate MRs and GRs (Section 4). Second, GCs affect the nervous system indirectly by changes in the blood level of glucose (Section 8.3.1) and other energetic substrates (Section 8.5) leading to altered gradient across the blood-brain barrier and ensuing changes in brain concentration of these substances (Section 8.3.2.4 and 8.5). Third, treatment with GCs inhibits the HPA axis leading to a decreased release of endogenous glucocorticoids during the sleep-waking cycle (Section 5). Therefore, the administration of GCs may also lead to periods of hypocortisolemia (Karssen et al., 2005) that will additionally depend on the properties of applied GCs and time-course of experiments. These three mechanisms complicate the functional interpretation of effects induced by peripheral administration of GCs.

stressors (Thoeringer et al., 2007; Droste et al., 2008, 2009; Qian et al., 2011) result in a more delayed peak level of corticosterone and longer time needed to normalize the level of the hormone. A physiological mechanism contributing to the delay of brain entrance of corticosterone is a concomitant release of Corticosteroid-Binding Globulin from the liver that is most pronounced during moderate and severe stress (Qian et al., 2011). A confounding factor that can also contribute to the variability of results is stress associated with preparation of animals for experiments, for example transport of animals between different rooms. Such inadvertent stress can initiate release of GCs before the start of the procedure intended for inducing the stress response. Finally, significant changes in the brain level of corticosterone are easier for detection in adrenalectomized animals (Venero and Borrell, 1999; Conway-Campbell et al., 2007) because of a negligible basal level of GCs and smaller between-subject variability due to the absence of changes in the level of endogenously released hormone. Therefore, multiple factors affect the timing of elevated levels of GCs and resulting brain responses.

4. Contribution of mineralocorticoid and glucocorticoid receptors

Endogenous GCs (corticosterone/cortisol) bind both to mineralocorticoid (MR) and glucocorticoid (GR) receptors but with different affinity leading to variable contribution of these two types of receptors depending on the level of circulating hormones. Relatively low levels of GCs are released in basal conditions (Qian et al., 2012; Oster et al., 2017) which are additionally associated with circadian and ultradian fluctuations supporting daily activities, while high levels are present during stress response or after treatment with exogenous hormones (Section 3). In morning hours, when rodent HPA axis displays the lowest basal activity, brain MRs are occupied by hormones in about 80 % while GRs only in about 30 % (Reul et al., 1987). Therefore, effects observed after elevated levels of GCs due to stress exposure or pharmacological treatment were mostly attributed to activation of GRs. It should be noted, however, that the activity of MRs is also important. It creates not only a setpoint of the stress system (Joels et al., 2008) but also participates in effects induced by GCs released during the stress response (Joels and de Kloet, 2017; de Kloet et al., 2019). For example, MRs affect appraisal processes, behavioral reactivity, selection of coping style, encoding of new memories and retrieval of previously acquired information (Joels and de Kloet, 2017; de Kloet et al., 2019). Such a role is possible because there is a pool of membrane-associated MRs that has a lower affinity to corticosterone than intracellular receptors and, therefore, are activated at the time of elevated levels of glucocorticoids (Joels and de Kloet, 2017). Activation of GRs, in turn, facilitates behavioral adaptation and memory consolidation (de Kloet et al., 2019).

Taking into account the role of MRs and GRs is also crucial for the interpretation of effects induced by exogenous glucocorticoids that differ in ability to bind and activate these receptors. While different binding assays produced variable results (Lan et al., 1982; Rupprecht et al., 1993), the relative mineralocorticoid – glucocorticoid receptor activity is a most consistent determinant of physiological responses (Lan et al., 1982). Available data indicate that betamethasone and prednisolone that are commonly applied synthetic glucocorticoids have a lower relative mineralocorticoid activity than corticosterone and cortisol but higher than dexamethasone (Lan et al., 1982) which has a negligible effect on MRs in rodent brain after peripheral injections (Reul et al., 1987). These differences in affinity to MRs and GRs constitute an important factor contributing to the variability of results reported after administration of various glucocorticoids (Fig. 1).

5. Interaction between exogenous and endogenous GCs

Functional interpretation of effects induced by *in vivo* treatments with GCs is further complicated by inhibition of the HPA axis (Fig. 1) leading to a decreased release of endogenous GCs such as cortisol and corticosterone (Reul et al., 1987; Juruena et al., 2006). Cortisol is the main glucocorticoid in most of mammals including humans while corticosterone is preferentially released in rodents and rabbits (Bush, 1953; Karssen et al., 2001; Koren et al., 2012; Gong et al., 2015). Importantly, endogenous GCs are released not only in response to stress but also in basal conditions, with the highest level around the time of the sleep-wake transition and the lowest level at the beginning of the sleep period (Qian et al., 2012; Oster et al. 2017). Therefore, the effect of treatment with exogenous GCs can be counterbalanced by a decreased release of endogenous GCs especially in the case of prolonged experiments. In the case of rodents the effect of disrupted circadian rhythm of corticosterone should be considered especially when experimental period extends into the evening period of rising level of corticosterone (Reul et al., 1987). This issue is further complicated by the selection of administered glucocorticoid, doses and investigated species. For example, some older studies applied cortisol in experiments performed in rodents. In such a case, even a restricted range of changes in the basal release of corticosterone can be relevant because of considerable differences in permeability of the blood-brain barrier for these two hormones (Section 3.1). The variability in penetration of the blood-brain barrier will also lead to dissociation between peripheral and central effects of treatment. Another important factor is a difference between endogenous and synthetic GCs in affinity for MRs and GRs (Section 4) in combination with applied doses. For example, lower doses of dexamethasone that are not crossing the blood-brain barrier are expected to produce central hypocorticosteroid state (insufficient activation of both MRs and GRs) while higher doses alter the balance between activation of these two types of receptors in favor of GRs (Karssen et al., 2005). A support for this hypothesis is given by observation of reduced occurrence of neuropsychological symptoms in patients receiving both dexamethasone and cortisol (Warris et al., 2016). Summing up, GCs can induce both direct and indirect effects (Fig. 1) that vary depending on applied drugs, administered doses, treatment durations and investigated species leading to difficulty in interpretation of experimental data.

6. Time-course of non-genomic and genomic effects

Considering latency of responses, GCs have two modes of action: rapid non-genomic and delayed genomic mechanism that depends on changes in gene expression. Both these modes of action involve MRs (Karst et al., 2005; Nasca et al., 2015; van Weert et al., 2017) and GRs (Morsink et al., 2007; Nahar et al., 2015; van Weert et al., 2017) coded by Nr3c2 and Nr3c1 genes, respectively. The non-genomic activity of GCs starts almost immediately after the entrance of GCs into the brain as indicated by changes in the firing rate of neurons (Table 3) and lasts for approximately 60 min (Joels et al., 2012). Because of various mechanisms involved in the non-genomic activity of GCs, there is an additional differentiation between rapid effects occurring almost immediately after the hormone reaches the brain and intermediate effects that peak after 20−60 min (Joels et al., 2012). In contrast, genomic effects start after a delay of about 1 h and last for many hours (Joels et al., 2012) involving a direct and indirect mechanism of gene regulation (Newton, 2000; Popoli et al., 2011). Some early indirect genomic effects rely on interference of GCs with signaling mediated by second messengers as indicated by experiments investigating a negative feedback regulation of HPA axis activity (van der Laan et al., 2009; Evans et al., 2013). In such a case, the effect depends on the level of cellular activation that triggers the second messenger signaling and timing of GC administration (van der Laan et al., 2009). In contrast, direct genomic mechanisms are mediated by cytoplasmic receptors that move to the nucleus after binding the hormone and act as transcription factors (Popoli et al., 2011). Importantly, numerous transcriptomic (Carter et al., 2012) and proteomic (Kamisoglu et al., 2015; Ayyar et al., 2017) effects induced by GCs occur with longer delays peaking at about 4 h–6 h in case of mRNA and 5 h–8 h in case of proteins or even later after treatment. These delays in transcription and translation of genetic information is consistent with the time-course of

changes in blood glucose (Section 8.3.1, Fig. 2) and with alternation in glucose uptake in neuronal and astrocytic cell culture (Horner et al., 1990; Virgin et al., 1991). Collectively, these data points to various mechanisms involved in responses to GCs and expand the time window for studying their effects from minutes to many hours.

7. Methods used to investigate brain metabolism and activity

7.1. Measures of brain metabolism

Brain metabolism is usually measured by the assessment of the local level of 2-deoxyglucose (2DG) which is labeled with isotopes allowing its detection by autoradiography (Sokoloff et al., 1977), scintillation (Delanoy and Dunn, 1978) or Positron Emission Tomography (PET) (Lameka et al., 2016). 2DG is incorporated into cells due to the structural similarity to glucose but cannot be oxidized leading to the cellular accumulation of the isotope (Sokoloff et al., 1977). It should be noted that in case of animal *in vivo* experiments the accumulation of 2DG is measured postmortem and, therefore, transient changes may not be detected because of averaging the uptake over a longer period of time. Another method enabling tracing substances labeled with isotopes is magnetic resonance (MRS) spectroscopy detecting atoms of carbon-13 in various metabolites (Hyder and Rothman, 2017; Rothman et al., 2019). Importantly, this approach enables differentiation between neuronal and astrocytic metabolism but it was not applied to study effects of GCs, thus creating an important gap in available data. Other methods rely on measurement of metabolites such as lactate, energy-carrying molecules (ATP) and oxygen in animal tissues. These methods show a contribution of oxidative and non-oxidative metabolism (lactate) and provide information about the balance between production and utilization of energy (ATP). Together with the assessment of glucose utilization they provide basic information about metabolism of studied tissues.

7.2. Measures of brain activity

Metabolism is tightly coupled with neuronal activity that is responsible for most of the energy expenditures in the brain (Yu et al., 2018). The direct measure of neuronal activity relies on detection of electric

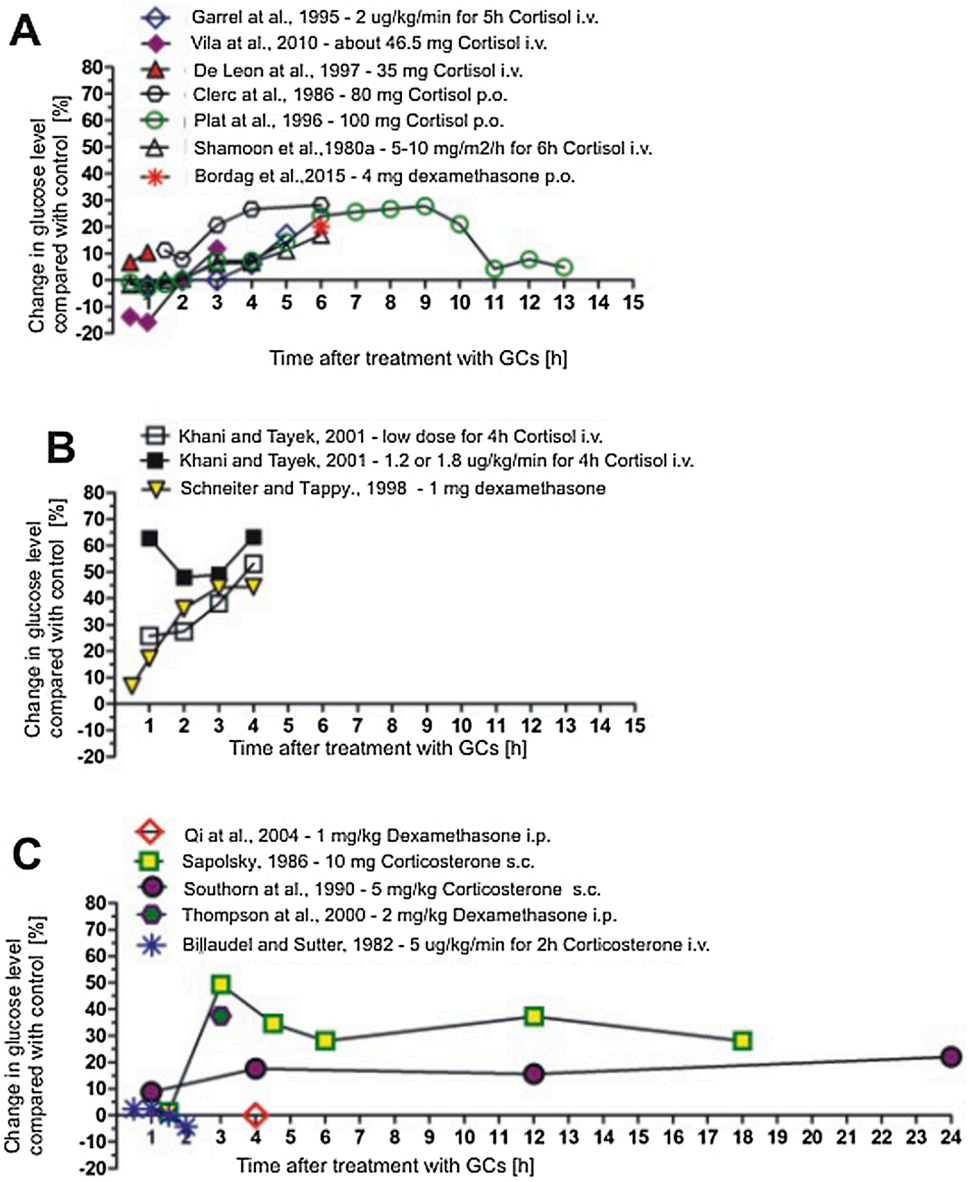

Fig. 2. Change in blood / serum glucose after treatment with GCs. A percentage change was assessed based on data reported in experimental studies. The comparison does not include data from one withdrawn study (Shamoon et al., 1980b).

currents generated by single neurons (single-unit recordings) or small groups of adjacent cells (multi-unit recordings). Such recordings can be performed with electrodes either *in vivo* or in simplified *in vitro* models such as brain slices or cell cultures. They also have the highest temporal resolution compared with other methods. Unfortunately, classical electrophysiological techniques used in the past to study the effect of GCs are restricted to a small number of neurons and brain areas.

An alternative method detects expression of c-Fos protein which is transiently expressed in neurons after synaptic stimulation and, therefore, constitutes an indirect marker of neuronal activity (Hudson, 2018). The advantage of c-Fos is that it allows detecting neuronal activity at cellular resolution in the entire brain. The main drawback of this method is that it has a very low temporal resolution because the expression of c-Fos protein peaks approximately 90−120 min poststimulus and is detected postmortem (Hudson, 2018). It is also less suitable for the detection of inhibited regions (Stark et al., 2006a).

A third method applicable in animal studies is microdialysis combined with HPLC enabling detection of neurotransmitters in extracellular space and, therefore, measuring a synaptic component of neuronal signaling (Linthorst and Reul, 2008). The advantage of microdialysis is that it allows performing experiments in freely moving animals with temporal resolution much better compared with c-fos expression. However, it is restricted to selected brain areas and is not allowing detection of changes at the level of single neurons.

Finally, an indirect method that can be easily applied in humans is functional magnetic resonance imaging (BOLD fMRI) which exploits differences in magnetic properties between oxygenated and deoxygenated hemoglobin (Magistretti and Allaman, 2015). An increased neuronal activity leads to dilation of vessels and increased local blood flow that surpasses the increase in oxygen utilization, resulting in locally increased oxygenation of hemoglobin (Kim and Ogawa, 2012). The main advantage of fMRI is that it is a non-invasive method enabling the analysis of the entire brain although its spatial and temporal resolution are very low compared to the direct measurement with electrodes (Heeger and Ress, 2002; Glover, 2011). This is because the changes in oxygenation of hemoglobin result from the pooled activity of a very large number of cells and are much slower than the spiking activity of neurons. It also means that fMRI is not able to detect changes neither in the activity of small subpopulations of neurons mixed with nonresponsive cells nor in structures composed of neurons displaying opposite responses.

These four methods assessing the brain activity (electrophysiology, c-Fos, microdialysis, and fMRI) together with the measurement of metabolism constitute complementary approaches that enable better understanding of the brain activity. Each method has some limitations and neither of them is able to detect all changes in the brain activity. For example, there is a considerable but not complete overlap between c-fos mapping and fMRI (Stark et al., 2006a) and between c-fos and 2-deoxyglucose uptake (Sharp et al., 1989; Komisaruk et al., 2000; Kaliszewska et al., 2012). Therefore, these data should be considered jointly.

7.3. Limitations of in vitro experiments

Finally, it is important to understand limitations associated with methods used to study brain metabolism and activity in *in vitro* preparations especially when neuronal or astrocytic cultures are used. First of all, they are obtained usually from embryos, pups or immortalized cell lines and, therefore, are not fully representative of an adult brain. Many concerns related to cell cultures and brain slices were expressed previously (Dienel, 2012; Joels et al., 2012) including differences in the rate of glucose metabolism during prenatal/early postnatal stages of development and adulthood (Nehlig, 1996; Dienel, 2012). Second, neurons in the intact brain do not function autonomously because they depend on metabolic cooperation with astrocytes. Therefore, any procedure that disrupts the structural and functional integrity of the network can lead to quantitative and qualitative changes in metabolism (Clarke and Sokoloff, 1999). Finally, the constant and highly standardized milieu applied in *in vitro* experiments may not be representative of dynamic changes in the content of various metabolites and neurotransmitters found *in vivo*. This problem is especially important in the case of glucocorticoids as discussed in greater detail in section 8.3.2.4 and 8.5. Therefore, while *in vitro* experiments provide valuable information, their interpretation is difficult and should be done cautiously.

8. Metabolic effects of GCs

8.1. Overview of GC-induced metabolic effects in peripheral tissues

One of the most known effects of glucocorticoids is an increased level of blood glucose (Fig. 2, Section 8.3.1) due to a decreased uptake in some tissues (Sakoda et al., 2000; Su et al., 2014) and increased gluconeogenesis in the liver (McMahon et al., 1988; Khani and Tayek, 2001; Kuo et al., 2015). Additionally, GCs also stimulate the intake of food containing carbohydrates (Tataranni et al., 1996). Importantly, maintaining high levels of glucose is not feasible without a disabled negative feedback mediated by insulin (Fig. 3), which inhibits glucose production in the liver and stimulates the uptake of blood glucose by muscles and adipose tissue (Wilcox, 2005). This in turn necessitates the mobilization of fatty acids stored in adipose tissue to provide energy for muscles deprived of the carbohydrates (Fig. 3) (Ciaraldi et al., 1995; Hunter and Garvey, 1998). Therefore, although GCs are primarily linked with metabolism of glucose, they also have a profound effect on metabolism of lipids and affect the availability of numerous energetic substrates that are described in more detail in subsequent sections.

8.2.1. Blood insulin

GC-mediated interference with insulin signaling after activation of HPA axis is known as cerebral insulin suppression (Peters and McEwen, 2015). It depends on short-term inhibition of release (Billaudel and Sutter, 1982; Longano and Fletcher, 1983; Plat et al., 1996) that is followed by induction of insulin resistance in muscles (Su et al., 2014) and adipose tissue (Sakoda et al., 2000). The inhibition of release occurs with a delay of about 1 h and is manifested by either a decreased level of insulin or altered proportion between blood insulin and glucose (Billaudel and Sutter, 1982; Longano and Fletcher, 1983; Plat et al., 1996). A factor that modifies the effect of GCs on the insulin release is glucose availability indicating flexibility of the regulatory mechanism (Billaudel and Sutter, 1982; Longano and Fletcher, 1983). In humans the blood insulin concentration returns to the basal level after about 3−4 h and next increases following changes in the blood glucose (Plat et al., 1996). The increased levels of glucose despite an elevated level of insulin (Plat et al., 1996) indicates the development of insulin resistance that is observed as early as 4−6 h after treatment (Plat et al., 1996; Qi et al., 2004) and results from a decreased uptake of glucose in muscles (Weinstein et al., 1995, 1998; Su et al., 2014) and adipocytes (Sakoda et al., 2000). An elevated level of insulin may persist despite the return of the glucose level to the baseline after acute GC treatment (Plat et al., 1996). Insulin resistance is also observed after longer treatments (Doyle et al., 1994a; Chipkin et al., 1998; Severino et al., 2002; Piroli et al., 2007; Su et al., 2014). This indicates that changes in insulin signaling contributes to the effects induced by GCs.

8.2.2. Brain insulin

Because of the GC-induced changes in the level of insulin, we should consider a role of insulin in central effects mediated by glucocorticoids. Such effect is possible because insulin crosses the blood-brain barrier by a saturable transport system (Banks et al., 1997; Rhea et al., 2018) and activates specific receptors that are present in the brain and affect various processes including metabolism, apoptosis, neuronal plasticity and regulation of food intake (Arnold et al., 2018). Although insulin is not necessary for the neuronal uptake of glucose (Heidenrich et al., 1989; Uemura and Greenlee, 2006), it supports an increased uptake

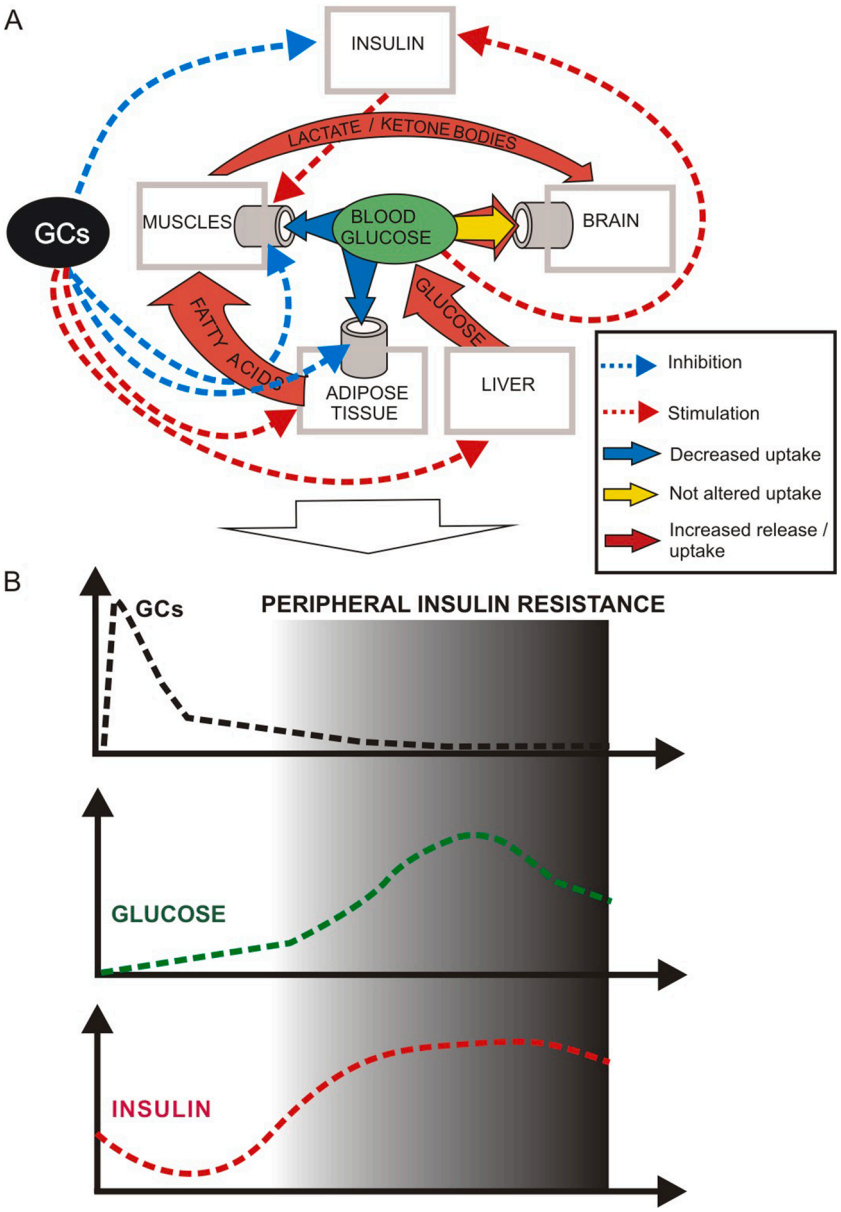

Fig. 3. Mechanisms responsible for GC-induced changes in the blood level of glucose and lipids. The gray color (B) indicated increasing insulin resistance. For more details see Section 8.1 (overview) and subsequent sections discussing in detail regulation of insulin (8.2), glucose (8.3) and other metabolites (8.5).

during periods of neuronal excitation (Uemura and Greenlee, 2006). Despite the importance of this topic, there are few experimental data showing involvement of insulin in brain metabolic effects mediated by GCs. First, acute treatment with dexamethasone impaired the insulin-stimulated glucose uptake in accumbal slices 35 min after treatment (Pinheiro et al., 2016). Second, repeated treatments with corticosterone (7 days) resulted in the impaired hippocampal signaling mediated by the insulin receptor, a decreased level of glucose transporter type 4 (GLUT4) and blocked translocation of GLUT4 to the plasma membrane in response to glucose bolus (Piroli et al., 2007). Third, treatment with dexamethasone for one week attenuated the actions of insulin in arcuate nucleus leading to a decreased sympathoexcitatory response to insulin (Steiner et al., 2014). This effect was specific for some brain areas since the same treatment schedule did not affect insulin signaling in ventral hypothalamus (Steiner et al., 2014). The whole brain insulin resistance was not either present after two days of treatment with dexamethasone (Su et al., 2014) but these data are difficult for interpretation because of specific properties of dexamethasone (Section 3.1, 4 and 5, Fig. 1). On the other hand, there are data showing that chronic hyperinsulinemia is important for induction of an increased brain uptake of glucose after the repeated treatment with dexamethasone (Chipkin et al., 1998) consistently with an increased brain/plasma glucose ratio after joint treatment with dexamethasone and insulin (Thompson et al., 2000). The picture is further complicated by the fact that dexamethasone also impairs the efficiency of brain insulin uptake as indicated by an experiment performed in dogs (Baura et al., 1996). Collectively, these data show that insulin contributes to effects mediated by GCs in brain although the extent of these actions and underlying mechanisms are still poorly understood.

8.3.1. Blood glucose

The time course of changes in blood glucose varies between studies and species. In humans most studies show that glucose does not increase during the first 2 h after treatment with cortisol (Shamoon et al., 1980a; Garrel et al., 1995; Plat et al., 1996; Vila et al., 2010) or changes are small because they are within an about 10 % range (Clerc et al., 1986; de

Leon et al., 1997). A clear increase starts at longer delays (Shamoon et al., 1980a; Clerc et al., 1986; Garrel et al., 1995; Plat et al., 1996; Vila et al., 2010) and plateau is achieved after 4−6 hours when the change in blood glucose is approaching 30 % (Clerc et al., 1986; Plat et al., 1996) (Fig. 2A). It should be noted, however, that much faster and higher increases in blood glucose were found in two studies (Fig. 2B). One of them applied dexamethasone (Schneiter and Tappy, 1998) which is a very potent synthetic glucocorticoid while the other study applied pituitary-pancreatic clamp infusion of somatostatin, insulin, growth hormone and glucagon to maintain all hormone concentrations in the fasting range, except for that of cortisol (Khani and Tayek, 2001). Therefore, both studies are not likely to represent physiological conditions associated with an increased level of endogenous GCs. In rats, the time course of blood glucose response to corticosterone (Sapolsky, 1986; Southorn et al., 1990) is similar to human studies applying cortisol (Fig. 2C). Lack of changes was observed during the first 2 h (Billaudel and Sutter, 1982; Sapolsky, 1986; Southorn et al., 1990) after corticosterone treatment while an increase occurred after 3−4 hours (Sapolsky, 1986; Southorn et al., 1990). An elevated level of glucose was still observed 12 (Sapolsky, 1986; Southorn et al., 1990) and 24 h (Southorn et al., 1990) after a single injection of corticosterone and after repeated treatments with dexamethasone (Thompson et al., 2000). In contrast, mice displayed changes in blood glucose much faster than humans and rats because significant hyperglycemia was observed 30, 60 (Longano and Fletcher, 1983) and 120 min after treatment with cortisol (Watanabe and Passonneau, 1973; Longano and Fletcher, 1983) although some researchers observed this effect only in fasted animals (Watanabe and Passonneau, 1973). It is possible that the time course of metabolic effects depends on the body weight and related rate of metabolism leading to differences between species. It also suggests that rats may be a better model of human metabolic responses to GCs than mice because of a stable blood level of glucose during the first two hours followed by hyperglycemia that develops 3−4 hours after treatment.

8.3.2. *Brain glucose*

8.3.2.1. First 2−3 hours after acute treatment. Glucose is especially important for the brain because it has a very limited ability to oxidize fatty acids and, therefore, requires a constant supply of glucose in contrast to other tissues (Yang et al., 1987; Schonfeld and Reiser, 2013). This severe dependence on glucose supply is demonstrated by the loss of consciousness triggered by a sudden drop of blood glucose due to insulin overdose (Cryer, 2007; Kalra et al., 2013). The only study that tested immediate effects of GCs (15 s) was performed in adrenalectomized and anaesthetized rats (Landgraf et al., 1978). This study reported a decreased incorporation of glucose in most brain areas but because of adrenalectomy and anesthesia these data are not comparable with normal physiological conditions (Section 2.3). Furthermore, very short latency after administration of corticosterone was not sufficient for penetration of the blood-brain barrier (Section 3.2.1). Other experiments applied much longer latencies between treatments and measurements of glucose utilization (Table 1). Most frequently, there were no changes in the brain glucose uptake during the first two hours after treatment with GCs (Table 1), that is at the time when changes in blood glucose are usually negligible or very small (Fig. 2). The lack of significant changes in the uptake of 2-deoxyglucose was found in basal conditions in slices derived from rat nucleus accumbens and treated with dexamethasone (35 min) (Pinheiro et al., 2016), mouse brain including hippocampus in response to corticosterone (55 min) (Delanoy and Dunn, 1978), most of the human brain with an exception of the hippocampus after administration of cortisol (55 min) (de Leon et al., 1997) and in hippocampal astrocytes treated with corticosterone (1 and 2 h) (Virgin et al., 1991). There were neither any changes in the level of glucose in the mouse brain 2 h after treatment with cortisol (Watanabe and Passonneau, 1973). Some studies reported the lack of significant changes even at longer latencies, that is 3 h after administration of dexamethasone (total brain glucose) (Thompson et al., 2000) and after 4 h of corticosterone treatment (2DG in mixed neuronal/glial culture derived from the hippocampus) (Horner et al., 1990). The only positive finding during the first 3 h was a decreased uptake in the human hippocampus after administration of cortisol (55 min) (de Leon et al., 1997) and prevention of the insulin-stimulated glucose uptake in accumbal slices treated with dexamethasone (35 min) (Pinheiro et al., 2016). Collectively, these data indicate that during an initial period of 2−3 hours after treatment with GCs, the changes in the brain glucose level and its utilization are in most cases negligible. Although there is a problem of restricted penetration of the blood-brain by cortisol and dexamethasone (Section 3.1), negative results are confirmed with corticosterone *in vivo* (Delanoy and Dunn, 1978) and *in vitro* both in hippocampal astrocytes (Virgin et al., 1991) and in mixed culture derived from the hippocampus (Horner et al., 1990).

8.3.2.2. 4−12 hours after acute treatment. Longer latencies after a single treatment with GCs were associated with more variable effects that additionally depended on the studied brain area. Experiments performed on hippocampal cell cultures that contained various proportion of astrocytes (from 20 % to more than 95 %) revealed a decreased glucose uptake after treatment with corticosterone and dexamethasone (Horner et al., 1990; Virgin et al., 1991). A significant effect occurred after 4–8 hours (Horner et al., 1990; Virgin et al., 1991), achieving maximum values after 12 h (Virgin et al., 1991). Importantly, the reduced glucose uptake was not associated with a significant change in the level of intracellular glucose in hippocampal astrocytes after treatment with corticosterone (Tombaugh et al., 1992). A potential mechanism responsible for decreased utilization of glucose is increased expression of pyruvate dehydrogenase kinase (Pdk4) which suppresses the oxidation of glucose (Juszczak and Stankiewicz, 2018). The inhibition of glucose uptake depends, however, on the studied brain area because differences existed in hippocampal cells (astrocytes, mixed and neuron-enriched cultures) after corticosterone treatment lasting for 24 h but not in cortical and cerebellar astrocytes and mixed cell cultures derived from cortex, cerebellum and hypothalamus (Horner et al., 1990; Virgin et al., 1991). Other research groups testing dexamethasone found even increased glucose uptake in cortical astrocytes after 9 h (Allaman et al., 2004) and in astrocytes obtained from brain hemisphere after 24 h (Skupio et al., 2019). The significant effect after 9 h was visible only when dexamethasone was administered without the concomitant treatment with noradrenaline (Allaman et al., 2004). This indicates that the effects induced by GCs may vary greatly depending on interaction with other signaling molecules.

There is a question about functional significance of observed changes in the glucose uptake in cell cultures that are maintained in stable milieu because a decreased uptake detected *in vitro* coincides with increased glucose availability *in vivo* (Fig.2). There is a possibility that a decreased rate of glucose uptake is compensated by increased glucose availability. Explaining this issue requires *in vivo* experiments but available data provided discrepant results. First, there is a study performed in rat pups (7 days old) that revealed a decreased uptake in several brain regions (including hippocampus) in basal conditions 6 h after treatment with dexamethasone (Tuor et al., 1997). In contrast, the experiment performed in older rats showed the lack of changes in the hippocampus and in most of other brain areas at comparable latency (5 h) after administration of dexamethasone (Kadekaro et al., 1988) (Table 1). However, both studies suffer from methodological problems such as administration of dexamethasone (Kadekaro et al., 1988; Tuor et al., 1997) characterized by restricted penetration of the blood-brain barrier and a different pattern of affinity to MRs and GRs compared with endogenously released corticosterone (Section 3.1 and 4, Fig.1). There is also a problem of interaction with circadian release of corticosterone (Section 5, Fig.1) although in this case it should play rather a minor role

Table 1
Effect of GCs on brain glucose uptake.

Author	Species	Experiment	Measurement	Brain area	Drug /dose	Latency	Effect
(Landgraf et al., 1978)	Rats (Adx)	*In vivo* Anaest.	[^{14}C]glucose	Most of the brain	Corticosterone 1−100 μg/mL	15 s	Decrease
(Pinheiro et al., 2016)	Rats	*In vitro* slices	[^{3}H]2-deoxyglucose	Nucleus accumbens	Dexamethasone 1−10 μM	35 min	No effect on basal glucose uptake
(Pinheiro et al., 2016)	Rats	*In vitro* slices	[^{3}H]2-deoxyglucose	Nucleus accumbens	Dexamethasone 1−10 μM	35 min	Prevents insulin-stimulated glucose uptake
(Delanoy and Dunn, 1978)	Mice	*In vivo*	[^{3}H]2-deoxyglucose in dissected brain regions	Whole brain and several regions analyzed separately	Corticosterone 2.5 mg/kg	55 min	No effect on basal glucose uptake.
(de Leon et al., 1997)	Human	*In vivo*	2-deoxy-2-[^{18}F]fluoro-D-glucose PET	hippocampus	Cortisol 35 mg	55 min	Reduced brain glucose utilization
(de Leon et al., 1997)	Human	*In vivo*	2-deoxy-2-[^{18}F]fluoro-D-glucose PET	Brain with the exception of hippocampus	Cortisol 35 mg	55 min	No significant changes in brain glucose utilization
(Fishman and Reiner, 1972)	Rats	*In vivo*	3−0-methyl-D-[^{14}C] glucose	Pons, cerebellum, brain hemisphere	Cortisol 75 mg/kg	1 h	No effect
(Virgin et al., 1991)	Rats	*In vitro*	[^{14}C]2-deoxy-D-glucose	Hippocampal astrocytes	Corticosterone 1 μM	1−2 h	No effect
(Watanabe and Passonneau, 1973)	Mice	*In vivo*	Total glucose	Whole brain	Cortisol 25 mg/kg i.p.	2 h	No effect
(Horner et al., 1990)	Rats	*In vitro*	[^{14}C]2-deoxy-D-glucose	Mixed (neuronal/glial) hippocampal culture	Corticosterone 1μM	4 h	No effect
(Virgin et al., 1991)	Rats	*In vitro*	[^{14}C]2-deoxy-D-glucose	Hippocampal astrocytes	Corticosterone 1μM	4, 8, 12 and 24 h	Decreased glucose uptake
(Tuor et al., 1997)	Rats (pups)	*In vivo*	[^{14}C]2-deoxy-D-glucose	Hippocampus, ventrolateralthalamus, parietal cortex, mid caudate nucleus	Dexamethasone 0.1 mg/kg	6 h	Decreased glucose uptake
(Tuor et al., 1997)	Rats (pups)	*In vivo*	[^{14}C]2-deoxy-D-glucose	Corpus callosum, hippocampus, ventrolateral thalamus, hypothalamus, parietal cortex, mid caudate nucleus	Dexamethasone 0.1 mg/kg + hypoxia	7.5 h-9 h	Increased glucose uptake
(Kadekaro et al., 1988)	Rats (sham-operated)	*In vivo*	[^{14}C]2-deoxy-D-glucose in slices	Most of brain	Dexamethasone 0.25 mg/kg	5 h	No effect
(Kadekaro et al., 1988)	Rats (sham-operated)	*In vivo*	[^{14}C]2-deoxy-D-glucose in slices	Locus ceruleus	Dexamethasone 0.25 mg/kg	5 h	Increase
(Kadekaro et al., 1988)	Rats (sham-operated)	*In vivo*	[^{14}C]2-deoxy-D-glucose in slices	Median eminence, pituitary anterior lobe, superior cervical ganglion	Dexamethasone 0.25 mg/kg	5 h	Decrease
(Horner et al., 1990)	Rats	*In vitro*	[^{14}C]2-deoxy-D-glucose	Mixed (neuronal/glial) hippocampal culture	Corticosterone 1 μM	8, 12 and 24 h	Decreased glucose uptake
(Allaman et al., 2004)	Mice	*In vitro*	[^{14}C]2-deoxy-D-glucose	Cerebral cortical astrocytes	Dexamethasone 100 nM	9 h	Increased glucose uptake
(Allaman et al., 2004)	Mice	*In vitro*	[^{14}C]2-deoxy-D-glucose	Cerebral cortical astrocytes	Dexamethasone 100 nM + noradrenalinę	9 h	No effect
(Horner et al., 1990)	Rats	*In vitro*	[^{14}C]2-deoxy-D-glucose	Mixed (neuronal/glial) hippocampal culture	Dexamethasone 10nM-1 μM Corticosterone (100 nM-1 μM)	24 h	Decreased glucose uptake
(Virgin et al., 1991)	Rats	*In vitro*	[^{14}C]2-deoxy-D-glucose	Hippocampal astrocytes	Dexamethasone 100 nM-10 μM	24 h	Decreased glucose uptake
(Tombaugh et al., 1992)	Rats	*In vitro*	[^{14}C]2-deoxyglucose	Hippocampal Astrocytes	Corticosterone 100nM	24 h	Decreased glucose uptake
(Virgin et al., 1991)	Rats	*In vitro*	[^{14}C]2-deoxy-D-glucose	Cortical and cerebellar astrocytes	Corticosterone 1 μM	24 h	No effect
(Horner et al., 1990)	Rats	*In vitro*	[^{14}C]2-deoxy-D-glucose	Mixed cortical, cereberral/brainstem, hypothalamic (neuronal/glial) culture	Corticosterone 1 μM	24 h	No effect
(Horner et al., 1990)	Rats	*In vitro*	[^{14}C]2-deoxy-D-glucose	Neuronal (80 %) hippocampal culture	Corticosterone 1 μM	24 h	Decreased glucose uptake
(Skupio et al., 2019)	Mice	*In vitro*	2-deoxyglucose-6-phosphate	Cultured astrocytes from brain hemispheres	Dexamethasone 100 nM	24 h	Increased glucose uptake
(Thompson et al., 2000)	Rats	*In vivo*	Total glucose	Whole brain	Dexamethasone 2 mg/kg i.p.	1−4 days	Increased glucose content
(Doyle et al., 1994a)	Rats	*In vivo*	[^{14}C]2-deoxy-D-glucose	hippocampus, septal nuclei, caudate putamen, median eminence	Corticosterone pellets 70 mg	2 days	Increased brain glucose utilization
(Doyle et al., 1994a)	Rats	*In vivo*	[^{14}C]2-deoxy-D-glucose	Lateral habenula, mediodorsal thalamic nuclei, the dorsomedial and ventromedial hypothalamic nuclei	Corticosterone pellets 70 mg	2 days	Reduced brain glucose utilization
	Rats	*In vivo*	[^{14}C]2-deoxy-D-glucose			2 days	No change

(*continued on next page*)

Table 1 (*continued*)

Author	Species	Experiment	Measurement	Brain area	Drug /dose	Latency	Effect
(Doyle et al., 1994a)				Number of other brain areas and total cerebral utilization	Corticosterone pellets 70 mg		
(Fishman and Reiner, 1972)	Rats	*In vivo*	2-deoxy-D-[^{14}C]glucose or 3−0-methyl-D-[^{14}C] glucose	Pons, cerebellum, brain hemisphere	Cortisol 75 mg/kg	5–7 days	No effect
(Chipkin et al., 1998)	Rats	*In vivo*	[^{14}C]-D-glucose	Whole brain	Dexamethasone 1 mg/day + sucrose feeding	7 days	Increased uptake
(Chipkin et al., 1998)	Rats	*In vivo*	[^{14}C]-D-glucose	Whole brain	Dexamethasone 1 mg/day	7 days	Insignificant
(Thurston et al., 1980)	Mice	*In vivo*	Total glucose	Whole brain	Cortisol 50 mg/kg s.c.	10 days	Increased content
(Thurston et al., 1980)	Mice	*In vivo*	3-O-[14C]methyl-D-glucose	Whole brain	Cortisol 50 mg/kg s.c.	10 days	Increased brain/plasma ratio
(Thurston and Pierce, 1969)	Mice	*In vivo*	Total glucose	Whole brain	Cortisol 50 mg/kg s.c.	10 days	Increased content
(Liu et al., 2018)	Human	*In vivo*	[^{18}F]-fluorodeoxyglucose positron emission tomography	Basal ganglia, limbic lobe, parahippocampal gyrus, hippocampus, amygdala, thalamus, precentral cortex, and cerebellum	Cushing's disease		Increased brain glucose utilization
(Liu et al., 2018)	Human	*In vivo*	[^{18}F]-fluorodeoxyglucose positron emission tomography	Medial and lateral frontal cortex, superior and inferior parietal lobule, medial occipital cortex, and insular cortex	Cushing's disease		Reduced brain glucose utilization
(Brunetti et al., 1998)	Human	*In vivo*	[^{18}F]-fluorodeoxyglucose PET	Entire brain with the exception of striatum	Cushing's disease		Reduced brain glucose utilization

* *in vitro* culture derived from fetuses derived from mothers that were either treated with corticosterone or adrenaloctomized and treated with metyrapone to inhibit GC synthesis in fetuses (control).

considering the duration of experiments and the time course of circadian rhythm of corticosterone release (Reul et al., 1987). Finally, generalization of results is further compromised by utilization of pups (Tuor et al., 1997) that differ from mature animals in terms of brain metabolism (Nehlig, 1996; Dienel, 2012). Therefore, we still cannot unequivocally state what happens with the brain glucose uptake *in vivo* during the period ranging from approximately 4–12 hours after acute elevation of GCs when at least some genomic effects are expected to reach a maximum level (Section 6).

8.3.2.3. Repeated treatments with GCs. Repeated treatments with GCs are associated with an increased whole brain level of glucose as indicated by experiments performed in rats treated with dexamethasone for 1–4 days (Thompson et al., 2000) and young mice treated with cortisol for 10 days (Thurston and Pierce, 1969; Thurston et al., 1980). There are also some data showing an increased uptake of radiolabeled derivates of D-glucose although the results are more variable (Table 1). On the one hand, there was increased brain to plasma ratio of radiolabeled derivate of D-glucose after treatment with cortisol lasting for 10 days (Thurston et al., 1980), increased whole brain uptake in some conditions (high sugar intake) after 7 days of treatment with dexamethasone (Chipkin et al., 1998) and in some brain areas after two days of treatment with corticosterone (Doyle et al., 1994a). On the other hand, the same treatments did not affect the whole brain uptake in standard feeding conditions (Doyle et al., 1994a; Chipkin et al., 1998) while in some brain areas caused even a decrease in glucose uptake (Doyle et al., 1994a). Finally, there is one study that yielded negative results in the brain both after acute and repeated treatments (5–7 days) in mature and immature rats (Fishman and Reiner, 1972). However, this study also failed to find differences in the blood glucose level and glucose uptake in other tissues including muscles and liver even after repeated treatments with a high dose of cortisol (75 mg/kg) (Fishman and Reiner, 1972). Therefore, such unexpected negative results in all tissues suggest methodological problems.

Considering all available data, we can conclude that most experiments showed increases in uptake or the total brain level of glucose at least in some experimental conditions after repeated treatment with corticosterone, cortisol and dexamethasone (Thurston and Pierce, 1969; Thurston et al., 1980; Doyle et al., 1994a; Chipkin et al., 1998; Thompson et al., 2000) while only one study showed concomitant local decreases after a relatively short period of treatment with corticosterone lasting for two days (Doyle et al., 1994a). To further disentangle these data, it is important to separate data concerning the total brain glucose from the uptake of its derivates marked with isotopes. The total brain glucose that was consistently increased by GCs (Thurston and Pierce, 1969; Thurston et al., 1980; Thompson et al., 2000) is informative on its own. In contrast, the uptake of radiolabeled derivates of glucose depends not only on brain metabolism but also on other factors such as route of injection, timing of measurements and concentration of blood glucose competing for transport across the blood-brain barrier. Therefore, the increased total brain glucose constitutes a basis for further consideration of the effects of repeated treatments with GCs on the whole brain metabolism (the next section).

8.3.2.4. Mechanism of increased brain glucose. Available data suggest two potential mechanisms responsible for the increased total brain glucose after repeated treatments with dexamethasone and cortisol (Thurston and Pierce, 1969; Thurston et al., 1980; Thompson et al., 2000). First, the transport of glucose across the blood-brain barrier is proportional to the blood level as indicated for example by diabetic animals (Gandhi et al., 2010). Therefore, the likely explanation is that an increased brain level of glucose results from peripheral effects of glucocorticoids triggering rise in blood glucose (Section 8.3.1, Fig. 2). Such a possibility is supported by observation that changes in the total brain glucose in rats treated with dexamethasone were indeed proportional to levels of blood glucose without significantly altered brain-to-plasma glucose ratio (Thompson et al., 2000).

Second, GCs can also affect efficiency of glucose transport across the blood-brain barrier because changes in brain glucose are not always associated with concomitant increases in blood glucose (Thurston and Pierce, 1969; Thurston et al., 1980). Changes in the brain up-take of 3−0-[^{14}C]methyl-D-glucose after i.p. injection suggest increased transport in animals treated with cortisol but interpretation of these data is confounded by differences between groups in blood and the brain level of glucose (Thurston et al., 1980). However, experiments performed with [14C]deoxy-D-glucose confirmed that at least in some conditions

glucocorticoids may affect the transport of glucose across the blood-brain barrier. Chipkin et al. (1998) tested incorporation of radiolabeled D-glucose after single cerebral circulatory passage (5 s) in rats treated with dexamethasone for 3 and 7 days. All animals received a single dose of D-glucose together with $^{3}H2O$ as an extraction marker and the solution was injected after transient interruption of the blood circulation in carotid artery. Therefore, detected brain uptake of the deoxy-D-glucose should not be affected by GC-induced changes in the level of blood glucose. The experiment showed that dexamethasone increased the uptake but only after longer treatments (7 days) combined with a high sugar intake (Chipkin et al., 1998). Changes in brain D-glucose uptake were associated with increased expression of glucose transporter GLUT1 in cerebral microvessels and required chronic hyperinsulinemia (Chipkin et al., 1998) consistently with the observation that joint treatment with dexamethasone and insulin increases the brain-to-plasma glucose ratio (Thompson et al., 2000). Therefore, prolonged treatment with GCs may affect the transport of glucose across the blood-brain barrier especially in cases when glucocorticoids are combined with elevated glucose consumption and chronic hyperinsulinemia.

Obviously, there is a question about benefits of an increased level of brain glucose. In fact brain has privileged access to glucose and even in most extreme conditions like starvation receives sufficient amount of nutrients to maintain its structure in contrast to other organs (Peters, 2011). Increased blood glucose facilitates entrance of glucose into the brain and this additional fuel can be used at the time of increased energy expenditures. However, unused glucose that accumulates in the brain due to its increased blood level or potentiated transport constitutes a metabolic stress and therefore is not beneficial (Gandhi et al., 2010; Rowan et al., 2018).

8.3.2.5. Cushing's disease. Previous studies were restricted to several days of treatment with GCs and, therefore, there are no experimental data concerning a chronic effect of glucocorticoids on brain glucose utilization and the only source of information comes from patients with Cushing's disease. PET experiments provided, however, inconsistent results because both widespread decrease (Brunetti et al., 1998) and mixed results including an increase in numerous brain areas such as hippocampus were reported (Liu et al., 2018) (Table 1). One of the possible explanation of discrepancies is a difference in number of tested subjects because Brunetti et al. (1998) tested only 13 patients while Liu et al. (2018) investigated 92 patients and, therefore, the later study is more representative. This is important because Cushing's disease is not homogenous in terms of glucose homeostasis. It is estimated that up to 70 % of patients with Cushing's disease have impaired glucose metabolism including diabetes mellitus (20–45 %) and defective glucose tolerance (10−30%) although in many cases these patients display normal fasting glucose levels (Scaroni et al., 2017). Previous PET studies indicated a normal level of glucose in tested patients but the measurement procedure was not clearly described and probably was restricted to standard fasting condition (Brunetti et al., 1998; Liu et al., 2018) that is not sufficient to unequivocally identify abnormalities in glucose metabolism found in Cushing's disease (Scaroni et al., 2017). These data indicate that chronic exposure to cortisol affects both peripheral and brain metabolism of glucose although there is a considerable variability between patients and studies. Identification of subgroups displaying comparable glucose impairments in blood constitute a potential avenue for understanding variability between patients in brain glucose utilization.

8.4. Brain glycogen

Glucose entering the brain can be either catabolized or stored in the form of glycogen which is accumulated by astrocytes (Brown and Ransom, 2007, 2015). There is a growing body of evidence that astrocytic glycogen is not only an energy storage activated in pathological conditions but also has an important function in normal brain physiology. It is stored in astrocytic processes contacting neurons and, therefore, can be quickly mobilized to provide 'fast' ATP at times of locally increased energy demand over intervals too short to be met by changes in delivery of blood glucose (Dienel and Carlson, 2019; Wu et al., 2019). Therefore, the blood-born glucose is used to replenish the local pools of glycogen during periods of lower activity (glycogen shunt) and the glycogenolysis is activated during bursts of neuronal activity to buffer against rapid changes in energy demands (Dienel and Rothman, 2019).

Administration of radiolabeled glucose in fasted mice showed that acute cortisol increases both synthesis and utilization of brain glycogen (Watanabe and Passonneau, 1973). This explains the variability of results that showed both an increased level of brain glycogen after acute (0.5−5 h) and repeated (6–10 days) administration of cortisol in mice, rats and rabbits (Timiras et al., 1956; Coxon et al., 1965; Watanabe and Passonneau, 1973; Thurston et al., 1980), an insignificant effect of cortisol (Thurston and Pierce, 1969; Watanabe and Passonneau, 1973) and dexamethasone (Klepac, 1985; Thompson et al., 2000) after acute and repeated treatments and, finally, a decreased level of glycogen in astrocytic cultures treated with corticosterone and dexamethasone for 9–24 h (Tombaugh et al., 1992; Allaman et al., 2004; Skupio et al., 2019). Differences between *in vivo* (Timiras et al., 1956; Coxon et al., 1965; Thurston and Pierce, 1969; Watanabe and Passonneau, 1973; Thurston et al., 1980) and *in vitro* (Tombaugh et al., 1992; Allaman et al., 2004; Skupio et al., 2019) experiments also suggest that there are metabolic differences between these conditions that preferentially uncover one component of responses to GCs in astrocytic cultures. A potential mechanism supporting accumulation of glycogen is increased expression of glycogenin (Gyg1) after treatment with GCs (Juszczak and Stankiewicz, 2018). This effect can be further potentiated by increased availability of ketone bodies derived from lipid catabolism that constitute an alternative source of energy for brain (Thurston et al. 1980). Finally, increased brain synthesis of glycogen can also result from increased availability of blood glucose. In the experiment performed by Watanabe and Passonneau (1973), increased brain glycogen was associated with increased blood glucose in fasted animals in contrast to negative effects of cortisol in normally fed animals that displayed both insignificant changes in brain glycogen and blood glucose. Therefore, an increased level of brain glycogen can result both from peripheral effects of GCs leading to an increased level of blood glucose and central effects facilitating synthesis of glycogen. In contrast, there are no clues suggesting the mechanism responsible for facilitated utilization of glycogen. Nonetheless, it is clear that the double effect of GCs, which both increase synthesis and utilization of brain glycogen (Watanabe and Passonneau, 1973), clearly supports a metabolic flexibility of neuronal networks.

8.5. Other metabolites

An increased blood level of glucose is associated with an increased level of lactate (Thompson et al., 2000; Bordag et al., 2015), pyruvate (Bordag et al., 2015), mannose, 3-hydroxybutyrate (ketone bodies) and other metabolites (Thurston et al., 1980; Dardzinski et al., 2000; Bordag et al., 2015). These effects occur usually as early as 6 h after treatment with dexamethasone (Bordag et al., 2015). Importantly, mentioned metabolites cross the blood-brain barrier (Oldendorf, 1971; Fuglsang et al., 1986; Miller and Oldendorf, 1986; Bhattacharya and Boje, 2004; Knudsen, 2012) and are used by the brain as an alternative source of energy (Dringen et al., 1994; Smith et al., 2003; Zielke et al., 2009; Wyss et al., 2011; Achanta and Rae, 2017; Achanta et al., 2017; Rastedt et al., 2017).

Ketone bodies are produced mainly in the liver from free-fatty acids (Evans et al., 2017) while the source of glucocorticoid-induced mannose is not clear. It is known that the blood level of mannose is closely corelated with glucose and that it is increased in diabetic subjects (Sone

et al., 2003; Mori et al., 2009). Therefore, it is not surprising that mannose is also increased at the time of GC-induced insulin resistance.

The precise source of increased blood lactate and pyruvate found after treatment with dexamethasone is also not well defined (Thompson et al., 2000; Bordag et al., 2015). One of the involved mechanisms is probably increased expression of pyruvate dehydrogenase kinase (Pdk4) in muscles (Salehzadeh et al., 2009) and brain astrocytes (Juszczak and Stankiewicz, 2018) consistently with local changes in levels of these metabolites in muscles and cultured astrocytes in response to dexamethasone (Ardawi and Jamal, 1990; Allaman et al., 2004; Skupio et al., 2019). The activity of Pdk4 suppresses the influx of glycolytic metabolites into mitochondria leading to decreased incorporation of pyruvate into Krebs cycle and increased production of lactate (Liu et al., 2017; Juszczak and Stankiewicz, 2018). This in turn constitutes a part of mechanism responsible for the switch from utilization of glucose to fatty acids as an energy source (Connaughton et al., 2010). Increased production of lactate is present after acute treatment with dexamethasone in astrocytic cell culture in basal conditions (Allaman et al., 2004; Skupio et al., 2019) or can be unmasked in brain by special conditions such as hypoxic-ischemic insult occurring 24 h after a single dose of dexamethasone *in vivo* (Tuor et al., 1997). Although the interpretation of actions triggered by dexamethasone is complicated (Section 5, Fig. 1), the simplest explanation for these delayed brain effects is increased availability of blood glucose that was also unmasked by injury (Tuor et al., 1997). While acute treatments with dexamethasone increased production of lactate, such effect was not observed in the brain after repeated treatment with cortisol *in vivo* (Thurston and Pierce, 1969; Thurston et al., 1980). It is not clear, however, whether the negative data result from adaptation to elevated levels of GCs, different properties of dexamethasone and cortisol (Section 4, Fig. 1) or from disrupted release of endogenous corticosterone counterbalancing the effects of administered cortisol (Section 5, Fig. 1).

Acute changes in blood levels of lactate that were found after treatment with dexamethasone (Thompson et al., 2000; Bordag et al., 2015) can significantly affect brain metabolism. First, there are data showing that lactate is preferred over glucose when both substrates are available (Wyss et al., 2011) and, therefore, intravenous infusion of lactate leads to decreased glucose brain uptake (Smith et al., 2003). Second, according to the model of astrocyte-neuron lactate shuttle, astrocytes release lactate at the time of increased brain activity to provide fuel for neurons (Belanger et al., 2011). While there is an ongoing controversy regarding the fate of lactate released during brain activation (Dienel and Cruz, 2016), it is also known that brain oxidation of lactate increases at the time of altered concentration gradient due to an elevated blood level of this metabolite (Quistorff et al., 2008; van Hall et al., 2009; Rasmussen et al., 2011). Therefore, GC-induced rise in blood level of lactate due to peripheral effects in muscles is expected to increase the utilization of lactate in brain.

Summing up, available data show that GCs increase blood level of a number of metabolites such as 3-hydroxybutyrate (ketone bodies), mannose, pyruvate, and lactate that can be used by the brain to produce energy. These changes together with increased blood glucose constitute a metabolic context that is important for interpretation of brain effects of GCs especially in *in vitro* preparations that are maintained in a standard milieu.

8.6. Oxygen consumption

It is estimated that neurons produce about 80 % of total brain oxidative ATP (Hyder et al., 2006) while astrocytes rely largely but not exclusively on glycolysis (Bolanos, 2016). Although neurons and glial cells differ in metabolism, there is paucity of studies testing the effect of GCs on oxygen utilization in different cell types that are present in the nervous system. Only one study tested neuronal mitochondria (Du et al., 2009) while the remaining experiments used either anaesthetized animals (Liu and Zhou, 2012) or mitochondria obtained from a homogenized brain tissue containing all types of cells (Bottoms and Goetsch, 1968; Morin et al., 2000; Katyare et al., 2003; Pandya et al., 2007).

Available data obtained in rats indicate that GCs induce a biphasic or even triphasic response. Shortly after treatment (15 min - 2 h) with various GCs (cortisol, corticosterone, dexamethasone, and prednisolone), a decrease in mitochondrial oxygen consumption was reported (Morin et al., 2000; Katyare et al., 2003). After 5 h there were no differences in adrenalectomized rats (see also Section 2.3) treated with corticosterone (Bottoms and Goetsch, 1968) while longer treatments lasting for 1–3 (Du et al., 2009) or 40 days (Liu and Zhou, 2012) resulted in increased mitochondrial oxidation in cortical neurons (Du et al., 2009) and reduced oxygen partial pressure in the rat brain suggesting increased *in vivo* oxygen utilization (Liu and Zhou, 2012). Finally, high doses of corticosterone (1μM) decreased mitochondrial oxidation after 3 days, indicating the occurrence of toxic effect (Du et al., 2009). Mechanisms responsible for these effects are not well understood. Considering the time-course of responses ranging from minutes to days, both non-genomic and genomic actions of GCs should be considered (Section 6) including direct regulation of mitochondrial genes (Picard et al., 2018).

Presence of triphasic response may explain variable results obtained after repeated subcutaneous treatments (Katyare et al., 2003; Pandya et al., 2007) that involved different drugs (corticosterone *vs* dexamethasone), treatment schedules (consecutive days *vs* every second day), time of analysis after the last treatment (24 h *vs* 48 H), age of animals and metabolic substrates added to mitochondrial preparations (Katyare et al., 2003; Pandya et al., 2007). However, because of the large number of variables, these data are difficult to interpret.

Although the short-term (15 min−2 h) decrease in oxygen consumption following treatment with GCs may seem maladaptive, it can be easily understood considering a metabolic response to an increased neuronal activity observed for example during intense sensory stimulation or mental effort. Such activation is associated with highly increased nonoxidative glycolytic metabolism despite an excessive supply of oxygen due to locally increased blood flow (Dienel, 2012; Dienel and Cruz, 2016). Therefore, the biphasic effect consisting of initially decreased and then increased oxygen consumption in response to GCs may constitute an adaptation for initial increase in glycolytic metabolism associated with task-related brain activation followed by delayed compensation for a metabolic debt. On the other hand, delayed disruption of mitochondrial oxidation contributes to toxic effects induced by prolonged exposition to high doses of GCs.

8.7. Synthesis of ATP

8.7.1. Basal conditions

ATP is a molecule responsible for the transfer of energy needed for various processes taking place in cells. Medium doses of GCs applied for a restricted period of time (from 1 h to 10 days) usually did not affect the brain level of ATP or less frequently increase it. Lack of effect was observed *in vitro* after administration of corticosterone (100 nM) to astrocytes and mixed hippocampal cultures maintained in standard milieu (Tombaugh and Sapolsky, 1992; Brooke et al., 1998) and *in vivo* after treatment with cortisol (25−50 mg/kg) (Watanabe and Passonneau, 1973; Thurston et al., 1980). The same doses of cortisol occasionally increased the brain level of ATP *in vivo* (Thurston and Pierce, 1969; Watanabe and Passonneau, 1973) and in some cases the effect depended on the feeding status with lack of changes in fasted animals and an increased ATP level in fed mice (Watanabe and Passonneau, 1973). Lack of changes in the total pool of ATP was also reported *in vivo* after treatment with dexamethasone in basal conditions (Adlard and De Souza, 1974; Tuor et al., 1997; Adachi et al., 2001; Namba et al., 2002; Yorozuya et al., 2015). However, interpretation of results obtained with cortisol and dexamethasone is complicated by restricted entrance into the brain, differences in affinity to MRs and GRs and interaction with

endogenous release of glucocorticoids (Section 3.1, 4 and 5, Fig. 1). Nonetheless, negative results obtained with corticosterone in standard *in vitro* conditions (Tombaugh and Sapolsky, 1992; Brooke et al., 1998) support the notion that medium doses of GCs applied for a restricted period of time do not impair synthesis of ATP while some *in vivo* data obtained after cortisol treatment suggest that at least in some situations GCs may even increase the production of ATP (Thurston and Pierce, 1969; Watanabe and Passonneau, 1973).

8.7.2. Brain ischemia

Another common finding is that pretreatment with dexamethasone and cortisol slows ATP loss during severe but short-term brain ischemia lasting for 30 s–60 s after decapitation (Thurston et al., 1980; Adachi et al., 2001; Namba et al., 2002) or during more prolonged but gradual asphyxia leading almost to death (Adlard and De Souza, 1974). Such effect was found in mice (3 h and 10 days) (Thurston et al., 1980; Namba et al., 2002), mongolian gerbils (1 h) (Adachi et al., 2001) and rats (2 h) (Adlard and De Souza, 1974) while one study showed insignificant suppression of the ATP loss (3 h) (Yorozuya et al., 2015). Additionally, one study showed that dexamethasone (24 h) significantly slowed the loss of ATP during locally induced hypoxia (Tuor et al., 1997). Comparable results were also obtained after dexamethasone treatment (22 h) in the experiment applying ^{31}P magnetic resonance spectroscopy that allows for *in vivo* measurement of the total pools of inorganic phosphate and nucleoside triphosphate that includes ATP (Dardzinski et al., 2000). The limitation of these results is that they are based on treatment with dexamethasone (Adlard and De Souza, 1974; Tuor et al., 1997; Dardzinski et al., 2000; Adachi et al., 2001; Namba et al., 2002) and cortisol (Thurston et al., 1980), which penetrate the blood-brain barrier much less effectively than corticosterone and, therefore, may lead even to central hypocorticosteroid state (Section 3.1 and 5, Fig. 1). Therefore, interpretation of these results in terms of underlying mechanism is difficult, especially in the case of longer latencies between drug administration and metabolic testing (section 5). In contrast to short-term ischemia, the prolonged oxygen/glucose deprivation combined with corticosterone treatment leads to an opposed effect in cortical astrocytes (Tombaugh and Sapolsky, 1992). However, relevance of this *in vitro* finding for *in vivo* conditions has not been proven (see also Section 7.3). Collectively, available data show that pretreatment with GCs has a beneficial effect in case of short-term ischemia resembling a situation that can be found during strangling although significance of these findings for endogenously released GCs is not clear due to methodological limitations of performed experiments.

8.7.3. Toxic doses of GCs

A decreased level of ATP was observed in basal conditions after application of very high doses of corticosterone *in vitro* (10 μM) (Zheng et al., 2015; Zhao et al., 2018) or after prolonged *in vivo* treatments with corticosterone lasting for 7–21 days in mice (Zhao et al., 2008) and 40 days in rats (cortex and hippocampus) (Hoyer and Lannert, 2008). These data are consistent with the finding that longer treatments and high doses of corticosterone (1μM) impair mitochondrial oxidation (Du et al., 2009). It should be noted, however, that the level of ATP returned to the baseline after 5 weeks of treatment with corticosterone (Zhao et al., 2008). Therefore, this study shows that there is an adaptation process counteracting toxic effects of prolonged treatment with corticosterone. Collectively, available data show that excessive doses of GCs disrupt production of energy in the brain.

8.7.4. Contribution of mitochondrial and glycolytic metabolism

ATP is synthetized both during cytosolic glycolysis and mitochondrial oxidative metabolism. In case of glycolysis that is increased by GCs at least in some conditions (Tuor et al., 1997; Allaman et al., 2004; Skupio et al., 2019) we can only assume the increased production of ATP but there are no studies quantifying contribution of glycolytic metabolism to total production of energy after treatment with GCs. In contrast, we have some reports testing contribution of mitochondrial metabolism but the data are anyway fragmentary. The most consistent data show that mitochondrial synthesis of ATP is decreased 10 min - 2 h after treatment with corticosterone (Katyare et al., 2003; Fujita et al., 2009) consistently with decreased oxygen utilization at this period of time (Morin et al., 2000; Katyare et al., 2003). Other data are less consistent and more difficult for interpretation. For example, after repeated treatments (3 days) there were frequently reported decreases in mitochondrial synthesis of ATP but the results were variable because in some groups no changes or even increases were found (Katyare et al., 2003; Pandya et al., 2007). The mitochondrial synthesis of ATP varied depending on the treatment schedule (consecutive days *vs* every second day), time of analysis after the last treatment (24 h *vs* 48 H), injected drug (corticosterone *vs* dexamethasone), age of animals and metabolic substrates added to mitochondrial preparations (Katyare et al., 2003; Pandya et al., 2007). Because of the large number of variables, these data are difficult for interpretation. Finally, there is a study that found no effect 5 h after treatment with corticosterone (Bottoms and Goetsch, 1968) but the experiment was performed on adrenalectomized animals which restricts our ability to draw firm conclusions (section 2.3). Therefore, we still have very limited information about contribution of oxidative and nonoxidative metabolism to energy production in conditions of elevated levels of GCs and the most consistent data are restricted to first two hours after administration of corticosterone that results in decreased production of mitochondrial ATP.

8.8. Summary: the most consistent finding and existing gaps

Acute metabolic responses to GCs can be divided into early (first 2 h) and delayed ($\geq$ 4 h) effects based on the time-course of concomitant changes in blood glucose (section 8.3.1, Fig. 2). During the first two hours after administration of GCs there is a decrease in oxidative production of energy (Morin et al., 2000; Katyare et al., 2003; Fujita et al., 2009) without concomitant decreases in total energy production (ATP) (Watanabe and Passonneau, 1973) and glucose utilization (section 8.3.2.1, Fig. 4). Unimpaired production of energy suggests that decreased oxidative metabolism is probably compensated by increased glycolysis but there are no data confirming this assumption. However, glycolysis is known to be increased by GCs at longer delays (Allaman et al., 2004; Skupio et al., 2019). GCs also promote syntheses and utilization of glycogen (Coxon et al., 1965; Watanabe and Passonneau, 1973) supporting metabolic flexibility (Fig. 4). The delayed phase starting after about 4 h is poorly understood. It is associated with increased blood availability of glucose (Section 8.3.1, Fig. 2) and other metabolic substrates (Section 8.5) that can be used to diversify sources of energy but the fate of these substances is unknown. Their utilization in brain metabolism can be assessed with MR spectroscopy after injection of substrates labeled with carbon-13 (Hyder and Rothman, 2017) but no such studies concerning GCs are available. *In vitro* experiments indicate changes in brain glucose utilization but there is a paucity of *in vivo* data that can be unequivocally interpreted (Section 8.3.2.2). Nonetheless, we can assume that during the delayed phase there is an increase in total blood glucose because its transport is proportional to blood levels (Gandhi et al., 2010) that achieve maximum concentration 4–6 hours after treatment with glucocorticoids (Section 8.3.1, Fig. 2). Although there are no data for this time window, it is known that the increased total brain level of glucose is present after repeated treatments (Thurston and Pierce, 1969; Thurston et al., 1980; Thompson et al., 2000). Longer treatments with GCs are also known to increase mitochondrial oxidative metabolism (Du et al., 2009) although excessive doses and prolonged treatments can lead to toxic effects disrupting mitochondrial oxidation (Du et al., 2009) and energy production (Section 8.7.3, Fig. 4). Available *in vivo* data also indicate that metabolic effects of GCs are affected by feeding status of animals (Watanabe and Passonneau, 1973; Chipkin et al., 1998) indicating flexibility of responses. One of the main weakness of available data is that they are

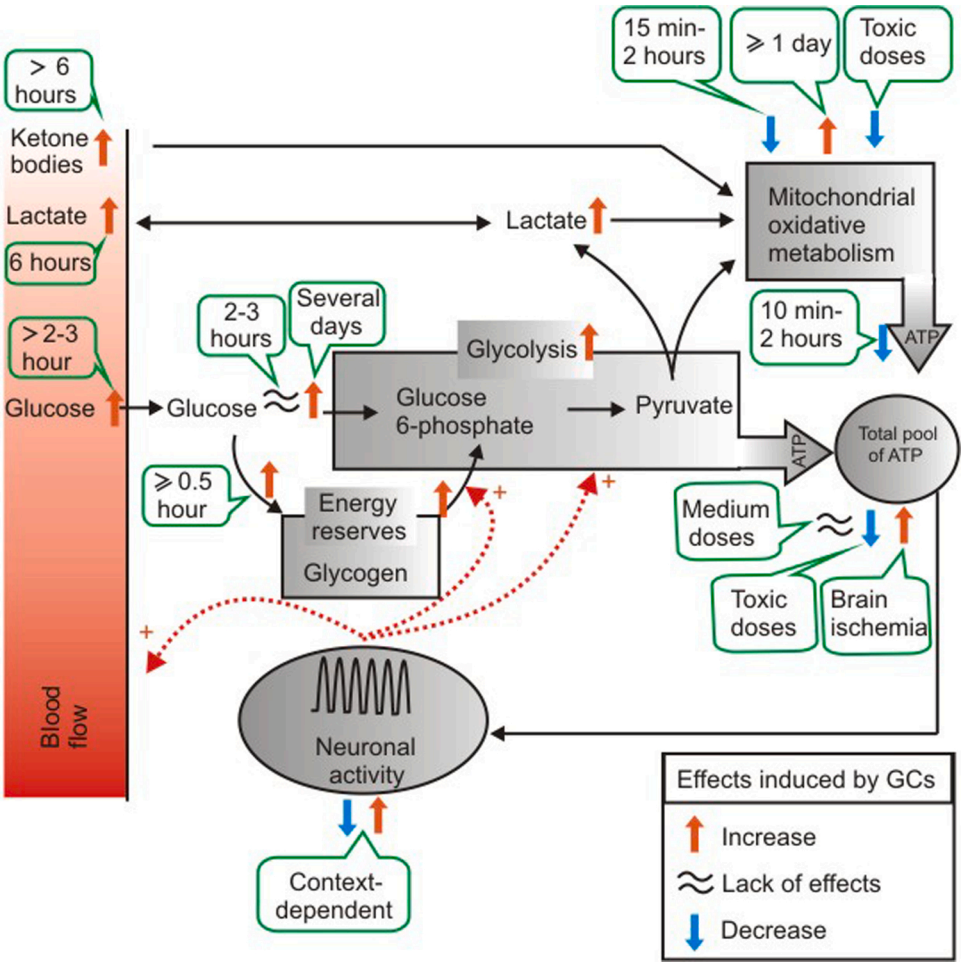

Fig. 4. Summary of the most important effects of GCs (Section 10) on brain metabolism and activity. Comments with green frames indicate time of occurrence of the effect after treatment with GCs or modifying factors. Metabolic effects of GCs include increased availability of energetic substrates in blood, facilitated synthesis and utilization of glycogen, increased glycolysis and time-dependent changes in oxidative metabolism. The total pool of energy (ATP) is not decreased by GCs in standard conditions, indicating maintained balance between energy production and utilization. However, prolonged treatments with excessive doses of GCs disrupt the production of energy, leading to toxic effects. A crucial limitation of available data is that metabolic experiments are restricted to resting or ischemic conditions and, therefore, have no behavioral relevance (Section 11). This is important because the brain activity varies considerably depending on the state of the organism and is regulated in a context-dependent manner by GCs (Section 10.3). Furthermore, the brain activity is intimately connected with metabolism because most of brain energy is consumed by neuronal signaling which in turn regulates regional blood flow, glycogen utilization and glycolysis.

restricted to resting conditions and often were performed in *in vitro* conditions with stable milieu. This is an important gap because brain metabolism is dynamic and depends on the neuronal activity that regulates blood flow leading to local changes in availability of metabolic substrates that are additionally modulated by peripheral effects of GCs (Section 8.3.1 and 8.5, Fig. 2). Additionally, available data contains many gaps concerning the time-course of observed effects and are frequently difficult for interpretation because of usage of exogenous GCs (Section 5, Fig. 1).

9. Brain activity

9.1. Electrophysiology

9.1.1. Activation vs inhibition of neuronal activity

First observations of altered brain excitability after treatment with glucocorticoids come from experiments investigating mechanisms of epilepsy (Hall, 1982). This line of research showed that GCs increase brain excitability as indicated by an increased propensity for occurrence of seizures after treatments with cortisol and corticosterone (Feldman and Davidson, 1966; Conforti and Feldman, 1975; Reddy, 2013). Similar conclusions were drawn from a number of experiments that found a cortisol-induced increase in the amplitude of evoked potentials (Table 2) which represent a summated activity of large populations of neurons. These observations indicate increased excitability leading to the lowering of the threshold for synaptic transmission (Feldman et al., 1961). However, the later experiment showed that cortisol affected the brain in two opposite ways because they both increased ascending activation of the brain stem in response to peripheral stimulation shortly after treatment and facilitated forebrain inhibitory influence on the brain stem activating system that developed during the second hour (Endroczi et al., 1968). These early observations made with crude electrophysiological methods are consistent with a modern concept of the brain response to stress emphasizing biphasic and reciprocal regulation of brain salience and executive control networks leading to initial hypervigilant state facilitating detection of sensory stimuli (Hermans et al., 2014). Furthermore, the dual effect characterized by induction of facilitatory and inhibitory effects should be considered as a hallmark of actions induced by GCs because it is visible with different recording tools at different levels of brain circuitry.

First, both inhibitory and excitatory effects were observed in single-unit recordings when neurons were treated individually using microelectrophoresis (Table 3). Importantly, opposite effects were triggered with short latency after application of the same doses of GCs and with the same experimental set-up as indicated by experiments reporting both excitatory and inhibitory responses after administration of cortisol and corticosterone (for example Kelly et al. 1977; Papir-Kricheli and Feldman, 1981, 1982) (see also Table 3). This indicates that the opposite effects were not resulting from different doses, variable time of recording or other procedural and technical differences between studies. Similarly, both excitatory and inhibitory responses to GCs were also frequently reported by *in vivo* studies which additionally showed some percentage of cells with biphasic responses to cortisol indicating time-related changes (for example Slusher et al., 1966; Phillips and Dafny, 1971; Nagler et al., 1973) (see also Table 5).

Although the variability in responses is clearly cell type specific as indicated by microelectrophoresis (Table 3), it also results from altered signal transmission within the neural network. Zeise et al. (1992)

Table 2
Effect of GCs on brain evoked potentials.

Author	Species	Experiment	Drug	dose	Brain area	Measurement	Effect	Additional information
(Feldman et al., 1961)	Cats	*In vivo* anaest	Cortisol	25 mgi. v.	Hypothalamus, intralaminar nuclei of thalamus, midbrain reticular formation	Amplitude of evoked potentials triggered by stimulation of sciatic nerve.	↑	
(Endroczi et al., 1968)	Rats	*In vivo* anaest	Cortisol	5–10 mg i.p.	Brain stem reticular formation, ventromedial hypothalamic nucleus	Amplitude of evoked potentials triggered by stimulation of sciatic nerve.	↑	Detected after 30–45 min
(Endroczi et al., 1968)	Rats	*In vivo* anaest	Cortisol	5–10 mgi.p.	Brain stem reticular formation, ventromedial hypothalamic nucleus	Amplitude of evoked potentials triggered by stimulation of sciatic nerve.	↓	Detected after 90–150 min
(Endroczi et al., 1968)	Rats	*In vivo* anaest	Cortisol	5–10 mgi.p.	Specific sensory pathways (lemniscus)	Amplitude of evoked potentials triggered by stimulation of sciatic nerve.	≈	
(Endroczi et al., 1968)	Rats	*In vivo* anaest	Cortisol	100 ug i. v.	Brain stem reticular formation, ventromedial hypothalamic nucleus	Amplitude of evoked potentials triggered by stimulation of sciatic nerve combined with conditioning stimulation of the medial forebrain bundle.	↓	Developed in the second hour after treatment
(Feldman et al., 1973)	Rats	*In vivo* anaest	Cortisol	5 mg i.p.	Hypothalamus	Amplitude of evoked potentials triggered by photic stimulation, stimulation of sciatic nerve, reticular formation, septum, and hippocampus	↑/≈	
(Kavushansky and Richter-Levin, 2006)	Rats	*In vivo* anaest	Corticosterone	10–25 mg/kg	Basolateral amygdala	Amplitude of evoked potentials triggered by stimulation of the entorhinal cortex	↑	
(Feldman and Davidson, 1966)	Rabbits	*In vivo*	Cortisol	25 mg i. v.	Septum, hippocampus	Amplitude of evoked potentials obtained by stimulating the midbrain reticular formation	↑	
(Feldman and Davidson, 1966)	Rabbits	*In vivo*	Cortisol	25 mg i. v.	Hypothalamus	Amplitude of evoked potentials obtained by photic stimulation or stimulation of septum and midbrain reticular formation	↑	
(Marcus et al., 1966)	Cats	*In vivo* Paralysed	Cortisol	1% topical	Lateral gyrus	Amplitude of evoked potentials induced by photic stimulation	↑	
(Covian et al., 1963)	Cats	*In vivo* anaest	Cortisol	topical	Cortex (gyrus marginalis)	Amplitude of evoked potentials induced by photic stimulation	↑	

≈ - lack of effect; ↑ - increased amplitude; ↓ - decreased amplitude.

showed that corticosterone reduces both GABAergic inhibition and excitability of hippocampal pyramidal neurons (Zeise et al., 1992). This pattern of changes suggests that corticosterone inhibits hippocampal responses to single stimulation but increases activity in response to repetitive excitatory stimulations (Zeise et al., 1992). The importance of the input signals on responsiveness of neurons to GCs (cortisol, corticosterone, dexamethasone) is also visible in experiments testing effects of glucocorticoids on resting and sensory-evoked activity (Feldman and Dafny, 1970b, a; Nagler et al., 1973; Mandelbrod et al., 1981; Feldman et al., 1983; Lei et al., 2014). For example, hypothalamic neurons that are not sensitive to locally applied cortisol (with background stimulation with glutamate) become sensitive at the time of distal (sensory and hippocampal) stimulation (Mandelbrod et al., 1981). Even more strikingly, some hypothalamic neurons may even change the direction of responses to corticosterone and cortisol (inhibition / excitation) depending on the presence or absence of sensory stimulation (Feldman and Dafny, 1970a; Feldman et al., 1983). Similarly, a more recent study found that locally applied dexamethasone both increase and decrease neuronal activity in resting conditions but induce a general increase in the firing of auditory neurons at the time of sensory stimulation (Lei et al., 2014).

Summing up, these data indicate that in normal nonepileptic brain GCs alter dynamics of the neuronal activity instead of inducing exclusively inhibitory or excitatory effects. Therefore, depending on interaction with other factors, GCs may lead to variable neuronal responses including both excitation and inhibition. This, in turn, is expected to support task-related activity in responses to environmental challenges. These conclusions are consistent with variable responses observed after treatment with cortisol (Henckens et al., 2010, 2012b; Sudheimer et al., 2013; Bos et al., 2014; Montoya et al., 2015) and prednisolone (Buades-Rotger et al., 2016) in humans. For example, differential responses were reported by fMRI studies depending on the threat escapability (Montoya et al., 2015) and emotional valence of presented stimuli (Henckens et al., 2010, 2012b; Sudheimer et al., 2013; Bos et al., 2014; Buades-Rotger et al., 2016). These observations also indicate that it is important to consider the effect of GCs both on resting and task-evoked activity. This is especially important for interpretation of metabolic studies that were performed mainly in resting conditions or less frequently during severe brain ischemia but not during a task-related activity.

9.1.2. *Responses of single cells and networks*

Pharmacologically-induced changes in the neuronal activity can result from altered responsiveness of individual neurons, altered interaction between neurons composing local networks and from interaction between distant brain areas. Responsiveness of individual neurons can be studied using microelectrophoresis (Table 3) while local networks are studied after topical administration *in vivo* or in *in vitro* preparation (Table 4). In contrast, effects observed after systemic administrations (Table 5) represent a net effect of individual responses and interactions between neurons composing local and distal networks.

Microelectrophoresis applies micropipettes to release small amounts of drugs in the direct vicinity of selected neurons. A number of studies using this technique showed that at least some neurons scattered across the brain respond within seconds to corticosterone, cortisol and dexamethasone (Table 3). The responses include both GC-induced excitation and inhibition of firing during a spontaneous or glutamate facilitated activity in anesthetized animals (Table 3). Furthermore, the short-term

Table 3
Changes in the neuronal activity after local administration of GCs in direct vicinity of individual neurons (microelectrophoresis).

Author	Species	Drug	Dose [M]	Brain area	n	Neuronal activity			Latency of response
						≈	↓	↑	
(Barak et al., 1977)	Rats	Cortisol	0.05	Dorsal hippocampus	236	100 %	0%	0%	
(Barak et al., 1977)	Rats	Corticosterone	0.05	Dorsal hippocampus	125	100 %	0%	0%	
(Barak et al., 1977)	Cats	Cortisol	0.05	Dorsal hippocampus	142	100 %	0%	0%	
(Ruf and Steiner, 1967)	Rats	Dexamethasone	0.5	Hippocampus	?	100 %	0%	0%	
(Steiner et al., 1969)	Rats	Dexamethasone	0.5	Dorsal hippocampus	17	100 %	0%	0%	
(Segal, 1976)	Rats	Dexamethasone	0.5	Dorsal hippocampus (CA1-CA3)	24	42 %	58 %	0%	
(Ruf and Steiner, 1967)	Rats	Dexamethasone	0.5	Cortex	?	100 %	0%	0%	
(Steiner et al., 1969)	Rats	Dexamethasone	0.5	Cortex	26	100 %	0%	0%	
(Ruf and Steiner, 1967)	Rats	Dexamethasone	0.5	Thalamus	?	100 %	0%	0%	
(Steiner et al., 1969)	Rats	Dexamethasone	0.5	Thalamus	75	100 %	0%	0%	
(Saphier and Feldman, 1988)	Rats	Corticosterone	0.025	Paraventricular nucleus	102	36 %	36 %	27 %	Almost immediate
(Saphier and Feldman, 1988)	Rats	Cortisol	0.025	Paraventricular nucleus	143	32 %	36 %	32 %	Almost immediate
(Saphier and Feldman, 1990)	Rats	Cortisol	0.025	Paraventricular nucleus	24	33 %	67 %	0%	Usually within a few seconds
(Kasai et al., 1988)	Rats	Cortisol	0.0001	Paraventricular nucleus	83	65%	5%	30 %	Excitatory effects appeared rapidly
(Chen et al., 1991)	Rats	Cortisol	0.15	Paraventricular nucleus	97	22 %	70 %	8%	Several seconds
(Chen et al., 1991)	Rats	Dexamethasone	0.2	Paraventricular nucleus	100	63%	7%	30 %	
(Kasai et al., 1988)	Rats	Cortisol	0.0001	Periventricular hypothalamic nucleus	13	69 %	31 %	0%	
(Barak et al., 1977)	Rats	Cortisol or Corticosterone	0.05	Hypothalamus	17	76%	24 %	0%	
(Papir-Kricheli and Feldman, 1981)	Rats	Cortisol	0.05	Mediobasal hypothalamus	30	37 %	50 %	13 %	
(Mandelbrod et al., 1974)	Rats	Cortisol	0.05	Mediobasal hypothalamus	356	50 %	41 %	9%	Usually within 1–5 sec.
(Steiner et al., 1968)	Rats	Dexamethasone	0.5	Medial and anterior basal hypothalamus	49	76%	18 %	6%	Inhibition present after 2–20 sec or more
(Steiner et al., 1969)	Rats	Dexamethasone	0.5	Midline hypothalamus and midbrain	337	82%	17 %	1%	Some neurons responded almost instantaneously, others after 15–20 sec or even later.
(Ruf and Steiner, 1967)	Rats	Dexamethasone	0.5	Periventricular gray (hypothalamus and mesencephalon)	115	87%	13 %	0%	Inhibition of hypothalamic neurons was almost instantaneous. Inhibition of mesencephalic neurons was somewhat more delayed.
(Papir-Kricheli and Feldman, 1983)	Rats	Cortisol	0.05	Central gray	16	25 %	50 %	25 %	5–10 sec.
(Papir-Kricheli and Feldman, 1983)	Rats	Cortisol	0.05	Midbrain reticular formation	15	40%	47 %	13 %	5–10 sec.
(Avanzino et al., 1983)	Rats	Corticosterone	0.013	Brainstem reticular formation	98	59%	17 %	23 %	Excitatory effects were usually maximum by 10–40 sec. Inhibitory effects appeared with a slightly longer delay.
(Avanzino et al., 1983)	Rats	Cortisol	0.025	Brainstem reticular formation	169	59%	15 %	26 %	
(Avanzino et al., 1987b)	Rats	Corticosterone	0.013	Rostral part of reticular formation	74	55%	38 %	7%	Excitatory effects were maximum after 10–30 sec. Inhibitory effects appeared after 15–40 sec.
(Avanzino et al., 1987b)	Rats	Corticosterone	0.013	Caudal part of reticular formation	78	59%	4%	37 %	
(Avanzino et al., 1984)	Rats	Corticosterone	0.013	Raphe nuclei	54	39%	0%	61 %	Usually the excitatory effect was maximum within 20–30 sec. In some cases the effect appeared within 60–70 sec.
(Avanzino et al., 1987a)	Rats	Corticosterone	0.013	Locus coeruleus	48	27 %	0%	73 %	Maximum excitation within 1–5 sec.
(Papir-Kricheli and Feldman, 1981)	Rats	Cortisol	0.05	Medial septal nucleus	48	48%	21 %	31 %	5–10 sec.
(Papir-Kricheli and Feldman, 1981)	Rats	Corticosterone	0.05	Medial septal nucleus	29	38 %	34 %	28 %	5–10 sec.
(Papir-Kricheli and Feldman, 1982)	Rats	Cortisol	0.05	Medial preoptic Area	64	42 %	30 %	28 %	5–10 sec.
(Kelly et al., 1977)	Rats	Cortisol	0.05	Preoptic-septal area	166	73 %	19 %	8%	

≈ - nonresponsive cells; ↓ - cells displaying a decreased activity; ↑ - cells displaying an increased activity. Some results obtained after exposure to GCs and sensory stimuli are not summarized in this table because of too complex pattern of responses (Mandelbrod et al., 1981).

response to GCs (cortisol, corticosterone and dexamethasone) is cell-type specific in terms of the direction of change (Table 3, Fig. 5) and firing mode (Papir-Kricheli and Feldman, 1981, 1982). It should be noted, however, that these experiments cannot identify all neurons responsive to glucocorticoids. First, microelectrophoresis is associated with sampling bias. Some neurons may not be detectable due to their slow rate of discharge, whereas others may be detected preferentially because of a high rate of firing induced by experimental conditions

Table 4
Changes in neuronal activity after administration of GCs affecting only restricted region of brain (*in vitro* preparations or topical administration in *in vivo* experiments).

Author	Species	Experiment	Drug	Dose	Brain area	Activity	N (cells)	Neuronal activity		
								≈	↓(%)	↑(%)
(Chen et al., 1991)	Rats	*In vitro*	Corticosterone	0.01−1 μM	Paraventricular nucleus of the Hypothalamus	Spontaneous	104	66%	27 %	7%
(Kasai and Yamashita, 1988)	Rats	*In vitro*	Cortisol	0.1−1 μM	Paraventricular nucleus of the Hypothalamus	Spontaneous	43	100 %	0%	0%
(Kasai and Yamashita, 1988)	Rats	*In vitro*	Cortisol	10−100 μM	Paraventricular nucleus of the Hypothalamus	Spontaneous	69	91%	3%	6%
(Liebmann et al., 2008)	Mice	*In vitro*	Corticosterone	100 nM	Basolateral Amygdala	Spikes elicited by depolarizing pulse	11−12	≈		
(Liebmann et al., 2008)	Mice	*In vitro*	Corticosterone	100 nM	Hippocampus (CA1)	Spikes elicited by depolarizing pulse	11−12	≈		
(Duvarci and Pare, 2007)	Rats	*In vitro*	Corticosterone	100 nM	Basolateral amygdala	Neuronal activity evoked by depolarizing current	10			↑
(Duvarci and Pare, 2007)	Rats	*In vitro*	Corticosterone	100 nM	Basolateral amygdala	Neuronal activity evoked by depolarizing current	6	≈		
(Maggio and Segal, 2009)	Rats	*In vitro*	Corticosterone	100 nM	Dorsal hippocampus	Neuronal activity evoked by depolarizing current	12	≈		
(Maggio and Segal, 2009)	Rats	*In vitro*	Corticosterone	100 nM	Ventral hippocampus	Neuronal activity evoked by depolarizing current	12			↑
(Lei et al., 2014)	Rats	*In vivo* anaest	Dexamethasone	1−10 μM	Auditory cortex	Sound-evoked single-unit activity	103			↑
(Lei et al., 2014)	Rats	*In vivo* anaest	Dexamethasone	1−10 μM	Auditory cortex	Spontaneous single-unit activity	103	≈		
(Vidal et al., 1986)	Rats	*In vitro*	Corticosterone	1−10 μM	Hippocampus (CA1)	Amplitude of evoked population spikes (stimulation of Schaffer collaterals)	6−9		↓	
(Vidal et al., 1986)	Rats	*In vitro*	Dexamethasone	10 μM	Hippocampus (CA1)	Amplitude of evoked population spikes (stimulation of Schaffer collaterals)		≈		
(Rey et al., 1987)	Mice	*In vitro*	Corticosterone	0.2 nM	Hippocampus (CA1)	Amplitude of evoked population spikes (stimulation of Schaffer collaterals)	4−6			↑
(Rey et al., 1987)	Mice	*In vitro*	Corticosterone	5−10 nM	Hippocampus (CA1)	Amplitude of evoked population spikes (stimulation of Schaffer collaterals)	4−6		↓	
(Doi et al., 1991)	Rats	*In vitro*	Corticosterone	1 μM	Hippocampus (CA1)	Amplitude of evoked population spikes triggered by stimulation of CA3		≈		
(Doi et al., 1991)	Rats	*In vitro*	Corticosterone	1 μM	Hippocampus (CA3)	Amplitude of evoked population spikes triggered by stimulation of DG		≈		
(Doi et al., 1991)	Rats	*In vitro*	Corticosterone	1 μM	Hippocampus (DG)	Amplitude of evoked population spikes triggered by stimulation of performant pathway		≈		
(Cho and Little, 1999)	Rats	*In vitro*	Corticosterone	50 nM-2 μM	Ventral tegmental area	Spontaneous single-unit activity	4−7	≈		
(Cho and Little, 1999)	Rats	*In vitro*	Corticosterone	50 nM-2 μM	Ventral tegmental area	Dopamine-induced single-unit activity	4−7	≈		
(Cho and Little, 1999)	Rats	*In vitro*	Corticosterone	100 nM-1 μM	Ventral tegmental area	NMDA-induced single-unit activity	4−7			↑
(Cho and Little, 1999)	Rats	*In vitro*	Corticosterone	2 μM	Ventral tegmental area	NMDA-induced single-unit activity	4−7		↓	
(Cho and Little, 1999)	Rats	*In vitro*	Corticosterone	100 nM	Ventral tegmental area	AMPA-induced single-unit activity	4−7		↓	
(Cho and Little, 1999)	Rats	*In vitro*	Corticosterone	500 nM-2 μM	Ventral tegmental area	AMPA-induced single-unit activity	4−7			↑
(Cho and Little, 1999)	Rats	*In vitro*	Corticosterone	100 nM and 2 μM	Ventral tegmental area	Kainic acid-induced single-unit activity	4−7		↓	
(Cho and Little, 1999)	Rats	*In vitro*	Corticosterone	500 nM-1 μM	Ventral tegmental area	Kainic acid-induced single-unit activity	4−7			↑
(Michal, 1974)	Rats	*In vivo* anaest	Dexamethasone	0.01−1 nM	Dorsal hippocampus	Multi-unit activity	3−9		↓	

≈ - lack of effect; ↓ - decreased activity; ↑ - increased activity.

(Steiner, 1970). Second, most studies recorded a spontaneous activity that can be supported with background application of glutamate. However, there are data showing that some neurons are not affected by locally applied cortisol at such conditions but respond to glucocorticoids at the time of additional sensory or electric stimulation (Mandelbrod et al., 1981).

Data obtained with microelectrophoresis can be further extended by experiments applying peripheral treatments and longer periods of recording that detected an additional population of neurons displaying a biphasic response to cortisol during the first hour after treatment (Slusher et al., 1966; Phillips and Dafny, 1971; Nagler et al., 1973). Peripheral treatments with cortisol (Feldman and Dafny, 1970a) also trigger different patterns of firing (regular *vs* burst activity) (Fig. 6) consistently with studies using microelectrophoresis (Papir-Kricheli and Feldman, 1981, 1982). Recordings made in freely moving rats also revealed that more than 80 % of hypothalamic and midbrain neurons are responsive to cortisol (Phillips and Dafny, 1971).

An important observation derived from electrophysiological experiments is that changes in the activity of single cells after acute administration of GCs do not always result in significant changes in the firing rate at the level of local population of neurons due to the heterogeneity of responses. Such situation was found in auditory cortex in resting conditions after local treatment with dexamethasone (Lei et al., 2014) and in hypothalamus after peripheral administered cortisol (Nagler et al., 1973). Additionally, the reanalysis of data provided by (Feldman et al., 1983) and (Feldman and Dafny, 1970a) also shows the lack of changes at the population level of studied neurons at least in some experimental conditions (Supplementary file 1) despite changes in the activity of single cells detected in original studies. These observations are important for interpretation of fMRI imaging studies which usually show rather small and restricted changes in the brain activity after acute treatments (see Section 9.2.1.2). Furthermore, the detected brain activity is not always consistent with behavioral output. This issue can be exemplified by an fMRI study that found a propensity for striatal-dependent behavior without concomitant changes in activity in striatum after joint treatment with cortisol and yohimbine (Schwabe et al., 2012). Collectively, these data show limitations of brain imaging methods and indicate that some responses to GCs are detectable at the level of single cells but not at the level of averaged responses of larger populations of cells.

9.1.3. Latency of responses

Although GCs affect the neuronal activity almost immediately as evidenced by direct application, the effects observed after peripheral administration are rather much more delayed. The shortest responses of individual neurons to peripheral treatments were reported immediately in some neurons after i.p. injection of cortisol (Nagler et al., 1973), 1−2 min after i.v. injection of corticosterone (Avanzino et al., 1987b) and 1−5 min in some neurons after i.v. administration of cortisol (Slusher et al., 1966; Feldman and Dafny, 1970a) with mean latencies ranging, however, from almost 6–9.5 min depending on the analyzed brain area (Feldman and Dafny, 1970a). Importantly, all fast responses occurring during the initial 5 min after peripheral administration of GCs were obtained in acute preparations. In such cases, recording was performed shortly after surgical implantation of electrodes which inevitably leads to disruption of the blood-brain barrier due to physical injuries. Other studies including experiments performed several days after surgery (Pfaff et al., 1971; Dafny et al., 1973) reported neuronal responses to GCs (corticosterone, cortisol and dexamethasone) that occurred within 10 min (Kasai et al., 1988) or that started 10−15 min after drug administration (Pfaff et al., 1971; Ondo and Kitay, 1972; Dafny et al., 1973; Zhang et al., 2013). Such latencies are consistent with data concerning the permeability of the blood-brain barrier for corticosterone (Section 3.2.1). Comparable time-course was also reported in case of evoked potentials recorded in acute preparations. First effects were noticeable 5−15 min after i.v. injection of cortisol while a maximum effect occurred after 30−60 min (Feldman et al., 1961). Collectively, these data indicate that neuronal responses usually occur with a delay of about 10 min after peripheral administration of GCs.

9.1.4. Gaps in electrophysiological data

Although the electrophysiological experiments provided indispensable information about effects induced by GCs, there are still large gaps in the available data. First, almost all experiments were performed in *in vitro* preparations or in anaesthetized/paralyzed animals and the only modification of experimental conditions relied on administration of sensory or electric stimulation. It means that we do not have electrophysiological data about the effect of GCs during real life situations requiring escape, problem solving *etc.* Furthermore, the available information is restricted to a short time window after administration of GCs. For example, most studies investigating the firing rate after local administration of cortisol, corticosterone and dexamethasone (Table 4) were performed during the first hour and only one study (Liebmann et al., 2008) recorded the activity during a period longer than two hours. Similarly, effects induced by peripheral tratements with corticosterone, cortisol and dexamethasone (Table 5) were usually reported for the first or second hour and in some cases an available description is not sufficent to precisely determine the total duration of recording in groups of interest. Although some researchers performed prolonged recordings (Phillips and Dafny, 1971) or used longer delays such as 3 h (Hesen and Joels, 1996) or 24 h (Koranyi et al., 1971b), the data are scarce and there is no systematic comparison of short-term non genomic effects and delayed genomic effects on neuronal firing especially after the first 2 h. This is important because a number of transcriptomic, proteomic and metabolic processes develop over the course of several hours after treatment (see Section 6 for more details).

Finally, some older *in vivo* studies applied cortisol and dexamethasone for peripheral treatments administered to rodents (Table 5), which utilize corticosterone as a main endogenous glucocorticoid (Section 5). Positive results occuring with short latencies (Section 9.1.3) indicate efectivenes of these treatments consistently with data showing that cortisol and dexamethasone enter the brain at a constant rate leading to concentrations comparable with corticosterone in some brain areas (McEwen et al., 1976). However, because of differences in concentration of these GCs in brain areas containig high levels of GRs (Section 3.1) and variability in affinity to MRs and GRs (Section 4), the data obtained after peripheral administation of cortisol and dexamethasone may differ from effects induced by corticosterone. Furthere complications are present when effects of cortisol or dexamethasone are tested after longer intervals (Section 5, Fig. 1) but this issue is less relevant for available electrophysiological experiments that usually applied short recording periods. Overall, these data indicate that there is a need for testing the effects of corticosterone, which is more relevant for physiological conditions in rodents than cortisol and dexamethasone, and the analysis of the neuroanl activity should be extended to enable detection of delayed genomic effects (Section 6). Finally, it is important to study the effects of corticosterone in conditions allowing the collection of behaviourally relevant data. Such a possibility is offered by recent advancements in imaging methods and virtual reality (Aronov and Tank, 2014; Weisenburger and Vaziri, 2018; Piatkevich et al., 2019).

9.2. Functional magnetic resonance imaging (fMRI)

Magnetic resonance imaging is the most important source of information about the effects of GCs on the human brain activity. In case of most commonly applied BOLD fMRI, the level of activity is inferred from changes in oxygenation of blood hemoglobin resulting from altered blood flow that is in principle coupled with changes in the firing rate of local populations of neurons (Section 7.2). In this section we also included a PET study that applied $H^{15}{}_2O$ as a tracer (de Quervain et al., 2003) and a continuous arterial spin labelling MRI study (Strelzyk et al., 2012). Both these studies tested changes in cerebral blood flow and,

Table 5
Effect of peripheral administration of GCs on single and multi-unit activity in *in vivo* experiments.

Author	Species	Animals	Drug	Dose	Brain area	Activity	N (cells)	Neuronal activity			
								≈	↓(%)	↑(%)	↕(%)
(Phillips and Dafny, 1971)	Rats	Freely moving	Cortisol	8 mg/kg	Hippocampus (CA3)	Spontaneous single-unit	18	44%	22 %	28 %	6%
(Pfaff et al., 1971)	Rats	Freely moving/ Anaesthetized	Corticosterone	0.5–1 mg	Dorsal hippocampus	Spontaneous single-unit	?		↓		
(Dafny et al., 1973)	Rats	Freely moving	Cortisol	1–64 mg/kg	Dorsal hippocampus	Spontaneous single-unit	28	57 %	18 %	25 %	0%
(Slusher et al., 1966)	Cats	Paralyzed	Cortisol	25 mg	Hypothalamus	Spontaneous single-unit	11	0%	18 %	64 %	18 %
(Nagler et al., 1973)	Rats	Anaesthetized	Cortisol	5 mg	Tuberal Hypothalamus	Spontaneous single-unit	39	2,5%	205%	28 %	49 %
(Feldman and Dafny, 1970a)	Cats	Anaesthetized	Cortisol	5 mg/kg	Anterior-tuberal hypothalamus	Spontaneous single-unit	19	42 %	0%	58 %	
(Feldman and Dafny, 1970b)	Rats	Anaesthetized	Cortisol	5 mg	Anterior-tuberal hypothalamus	Spontaneous single-unit	54			↑	
(Phillips and Dafny, 1971)	Rats	Freely moving	Cortisol	8 mg/kg	Anterior hypothalamus	Spontaneous single-unit	18	17 %	11 %	61 %	11 %
(Dafny et al., 1973)	Rats	Freely moving	Cortisol	1–64 mg/kg	Anterior hypothalamus	Spontaneous single-unit	35	14 %	17 %	69 %	11 %
(Ondo and Kitay, 1972)	Rats	Anaesthetized	Dexamethasone	200ug/ kg	Basal hypothalamus	Spontaneous single-unit	22	23 %	41 %	36 %	
(Ondo and Kitay, 1972)	Rats	Anaesthetized	Dexamethasone	200ug/ kg	Basal hypothalamus island	Spontaneous single-unit	22	41 %	50 %	9%	
(Kasai et al., 1988)	Rats	Anaesthetized	Cortisol	0.5 mg	Paraventricular nucleus of the hypothalamus	Spontaneous single-unit	11	55%	9%	36 %	0%
(Feldman and Dafny, 1970a)	Cats	Anaesthetized	Cortisol	5 mg/kg	Posterior hypothalamus	Spontaneous single-unit	21	38 %	5%	57 %	
(Feldman and Dafny, 1970b)	Rats	Anaesthetized	Cortisol	5 mg	Posterior hypothalamus near the midline.	Spontaneous single-unit	44			↑	
(Phillips and Dafny, 1971)	Rats	Freely moving	Cortisol	8 mg/kg	Ventromedial hypothalamus	Spontaneous single-unit	18	17 %	61 %	22 %	0%
(Dafny et al., 1973)	Rats	Freely moving	Cortisol	1–64 mg/kg	Ventromedial hypothalamus	Spontaneous single-unit	28	18 %	50 %	32 %	0%
(Slusher et al., 1966)	Cats	Paralyzed	Cortisol	25 mg	Zona incerta	Spontaneous single-unit	6	17 %	33 %	50 %	0%
(Dafny et al., 1973)	Rats	Freely moving	Cortisol	1–64 mg/kg	Midbrain reticular formation	Spontaneous single-unit	31	13 %	19 %	68 %	26 %
(Phillips and Dafny, 1971)	Rats	Freely moving	Cortisol	8 mg/kg	Midbrain reticular formation	Spontaneous single-unit	18	11 %	28 %	56 %	6%
(Avanzino et al. 1987b)	Rats	Anaesthetized	Corticosterone	0.05 mg/kg	Brain stem reticular formation	Spontaneous single-unit	27	26 %	37 %	37 %	0%
(Feldman et al., 1983)	Rats	Freely moving	Corticosterone	5 mg	Medial hypothalamus	Spontaneous multi-unit	15	13 %	27 %	60 %	0%
(Feldman et al., 1983)	Rats	Freely moving	Corticosterone	5 mg	Lateral hypothalamus	Spontaneous multi-unit	12	25 %	0%	75 %	0%
(Feldman et al., 1983)	Rats	Freely moving	Corticosterone	5 mg	Midbrain reticular formation	Spontaneous multi-unit	17	24 %	24 %	53 %	0%
(Feldman et al., 1983)	Rats	Freely moving	Corticosterone	5 mg	Amygdala	Spontaneous multi-unit	19	32 %	26 %	42 %	0%
(Feldman et al., 1983)	Rats	Freely moving	Corticosterone	5 mg	Hypothalamus	Spontaneous single-unit	23	35%	4%	61 %	0%
(Koranyi et al., 1971b)	Cats	Freely moving	Cortisol	10 mg/ kg	Medial forebrain Bundle	Multi-unit activity			↓		
	Cats	Freely moving	Cortisol		Medial preoptic area	Multi-unit activity			↓		

(*continued on next page*)

Table 5 (*continued*)

Author	Species	Animals	Drug	Dose	Brain area	Activity	N (cells)	Neuronal activity			
								≈	↓(%)	↑(%)	↕(%)
(Koranyi et al., 1971b)				10 mg/kg							
(Koranyi et al., 1971b)	Cats	Freely moving	Cortisol	10 mg/kg	Mesencephalic reticular formation	Multi-unit activity			↓		
(Mor et al., 1986)	Rats	Freely moving	Corticosterone	0.5–5 mg/kg	Hypothalamic paraventricular nucleus	Multi-unit activity triggered by photic and acoustic stimulation			↓		
(Koranyi et al., 1971a)	Cats	Slow-wave sleep	Cortisol	10 mg/kg	Mesencephalic reticular formation	Multi-unit activity (spontaneous and induced by electrical stimulation)	6		↓		
(Koranyi et al., 1971a)	Cats	Slow-wave sleep	Cortisol	10 mg/kg	Medial forebrain Bundle	Multi-unit activity (spontaneous and induced by electrical stimulation)	6		↓		
(Koranyi et al., 1971a)	Cats	Slow-wave sleep	Cortisol	10 mg/kg	Medial preoptic area	Multi-unit activity (spontaneous and induced by electrical stimulation)	6		↓		
(Koranyi et al., 1971a)	Cats	Slow-wave sleep	Cortisol	10 mg/kg	Nucleus centromedianus of the thalamus	Multi-unit activity (spontaneous and induced by electrical stimulation)	6		↓		
(Zhang et al., 2013)	Rats	Anaesthetized	Dexamethasone	10 mg/kg	Lateral habenula, cocaine-up neurons	Spontaneous single-unit activity	9			↑	
(Zhang et al., 2013)	Rats	Anaesthetized	Dexamethasone	10 mg/kg	Lateral habenula, cocaine-down neurons	Spontaneous single-unit activity	6	≈			
(Hesen and Joels, 1996)	Rats	Slices	Corticosterone	1–10 mg/kg	Dorsal hippocampus	Percentage of neuron showing activity during carbachol perfusion				↑	

≈ - nonresponsive cells; ↓ - cells displaying decreased activity; ↑ - cells displaying increased activity; ↕ - cells displaying biphasic response. Some results obtained after exposition to GCs and sensory stimuli (Nagler et al., 1973) are not summarized in this table because of a too complex pattern of responses.

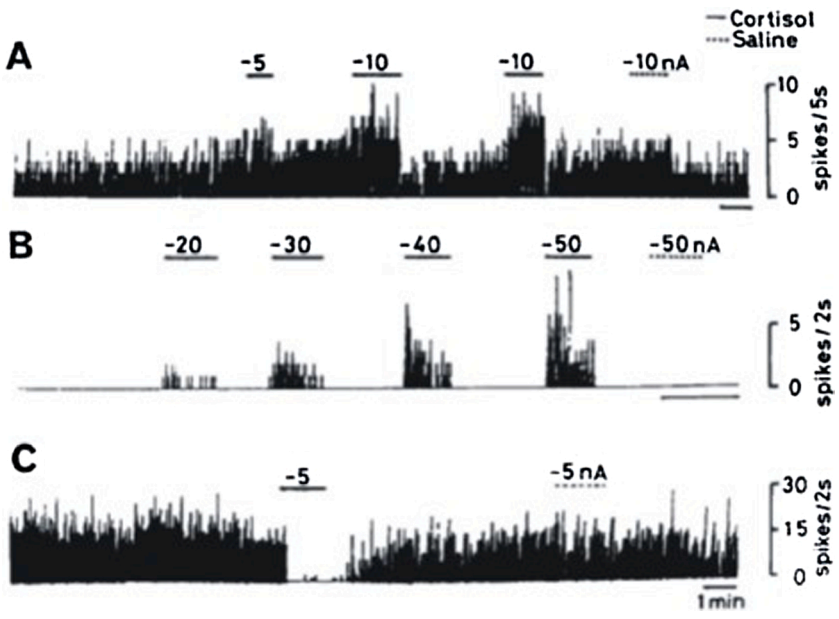

Fig. 5. Examples of excitatory and inhibitory effects induced by cortisol administered with iontophoresis in vicinity of TI-neurons in the PVN and the periventricular hypothalamic neurons. A - a spontaneously firing TI neuron which was excited by cortisol. B - a silent TI neuron which was excited by cortisol in a current dependent manner. C - spontaneously firing neuron in the periventricular hypothalamic nucleus which was inhibited by cortisol. Black bars and broken bars indicate the period of application of cortisol and saline, respectively. Numbers show the amount of ejection currents (nA). Reprinted with permission from Kasai et al. (1988).

therefore, detected the same physiological response as BOLD fMRI. Due to the number of limitations (Section 7.2), human brain imaging data should be considered jointly with other sources of information including biochemical and electrophysiological studies to obtain a more comprehensive picture of GC-induced changes in the brain activity (Section 10). Human studies investigating the effects of GCs on the brain activity applied two approaches. First, the acute effect of cortisol (hydrocortisone), cortisone or prednisolone (Supplementary file 2) were studied mainly in healthy volunteers although there are also data collected in depressed patients (Abercrombie et al., 2011) and spider phobic patients (Nakataki et al., 2017). Second, chronic effects of GCs were studied in patients with Cushing's disease that is caused by tumors triggering a

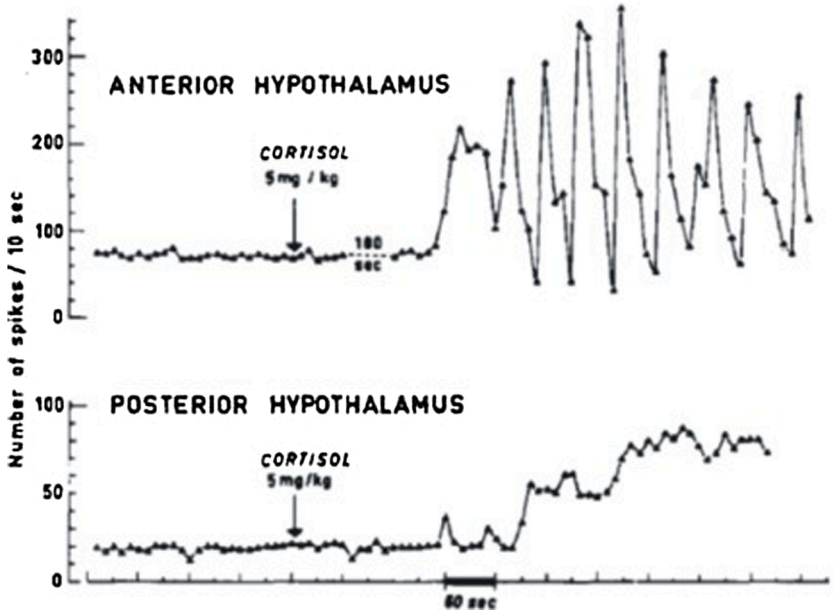

Fig. 6. Example of burst and regular activity induced by cortisol in cat hypothalamus after i.v. injection. Reprinted with permission from Feldman and Dafny (1970a).

continuous release of cortisol (Andela et al., 2015; van der Werff et al., 2015). These two lines of research are discussed in more detail in subsequent Sections 9.2.1 and 9.2.2.

9.2.1. *Pharmacological fMRI experiments*

9.2.1.1. General characteristic. GCs were administered usually within 80 min (de Quervain et al., 2003; Stark et al., 2006b; Oei et al., 2007; Henckens et al., 2010; Lovallo et al., 2010; Merz et al., 2010; Tabbert et al., 2010; van Stegeren et al., 2010; Abercrombie et al., 2011; Henckens et al., 2011, 2012a; Henckens et al., 2012b; Merz et al., 2012; Schwabe et al., 2012; Strelzyk et al., 2012; Symonds et al., 2012; Merz et al., 2013; Bos et al., 2014; Merz et al., 2014; Montoya et al., 2014, 2015; Kinner et al., 2016; Nakataki et al., 2017; Kinner et al., 2018; Merz et al., 2018; Fleischer et al., 2019) or two hours (Sudheimer et al., 2013; Ma et al., 2017) before the beginning of brain scanning while the minority of studies applied longer latencies such as 3 h (Henckens et al., 2012a), 4 h (Henckens et al., 2011; Buades-Rotger et al., 2016; Serfling et al., 2019), 4.5–4.75 h (Henckens et al., 2010, 2012b), 16 h (van Marle et al., 2013), 2.5 days (Brown et al., 2013) and one week (Merz et al., 2018) (for more details see Supplementary file 2). Considering the delayed penetration of the blood-brain barrier (Section 3) and the time-course of gene expression (Section 6), it should be assumed that most of the brain imaging studies investigated mostly non-genomic and early genomic effects of GCs. In contrast, effects occurring at longer latencies that are associated with the number of transcriptomic, proteomic and metabolic changes (Section 6) were rarely tested in fMRI experiments.

Almost all reviewed studies tested a single dose of GCs (Supplementary file 2) with an exception of two studies that applied treatments repeated for 2.5 and 4 days (Brown et al., 2013; Sudheimer et al., 2013). The most frequently used glucocorticoid was cortisol that was administered usually at a dose of 30 mg (Supplementary file 2) although the doses ranged from 10 (Henckens et al., 2010, 2011; Henckens et al., 2012b; Fleischer et al., 2019) to 100 mg (Sudheimer et al., 2013; Ma et al., 2017) in case of oral administration and from 4 (Strelzyk et al., 2012) to 100 mg (Symonds et al., 2012) in case of i.v. injections. Some brain imaging studies investigated the basal/resting activity in humans (Lovallo et al., 2010; Strelzyk et al., 2012; Symonds et al., 2012) and rats (Ferris and Stolberg, 2010) but most of the published experiments tested exclusively an effect of GCs on the task-related activity. Experimental protocols at the time of brain scanning included a working memory task (Henckens et al., 2011; Symonds et al., 2012), memory encoding (Henckens et al., 2012a) and retrieval (de Quervain et al., 2003; Oei et al., 2007; Brown et al., 2013; van Marle et al., 2013; Fleischer et al., 2019), fear conditioning (Stark et al., 2006b; Merz et al., 2010; Tabbert et al., 2010; Merz et al., 2012, 2013; Merz et al., 2014; Kinner et al., 2018; Merz et al. 2018), instrumental learning task (Schwabe et al., 2012), emotional distraction task (Henckens et al., 2012b), exposition to neutral and emotional stimuli such as infant crying (Bos et al. 2014), pictures (Henckens et al., 2010; van Stegeren et al., 2010; Sudheimer et al., 2013; Buades-Rotger et al., 2016; Ma et al., 2017; Nakataki et al., 2017) and words (Abercrombie et al., 2011), anticipation of reward (Montoya et al., 2014; Kinner et al., 2016) or threat (Montoya et al., 2015) and finally the Go/NoGo task with food and neutral targets (Serfling et al., 2019). In some experiments the exposure to stimuli was intended as a part of a memory test (van Stegeren et al., 2010; Abercrombie et al., 2011). Summing up, the fMRI studies were focused on acute effects occurring within 80 min after administration of cortisol and usually involved tasks relevant for mechanisms of declarative memory, fear conditioning and responses to emotion-charged stimuli. In contrast, underrepresented aspects include longer treatments, measurement latencies extending beyond the first 2 h after treatment and assessments of the resting brain activity.

9.2.1.2. Magnitude of acute effects. In many cases cortisol-induced changes in the brain activity were not associated with significant cognitive and emotional effects at the time of scanning (Oei et al., 2007; Henckens et al., 2012a, b; Symonds et al., 2012; Bos et al., 2014; Montoya et al., 2015; Fleischer et al., 2019). Henckens et al. (2012b) showed that this dissociation between the brain activity and cognition at least in some cases is time-dependent because the lack of significant cognitive effects was observed at longer latencies (4 h) but not one hour after administration of cortisol. Changes in the brain activity detected by fMRI after acute treatment are rather subtle. In fact, the only study that revealed widespread changes in the brain activity after administration of GCs was performed in rats treated with corticosterone (Ferris and Stolberg, 2010) but this experiment differs from all other reviewed studies due to the specificity of animal fMRI procedures as discussed in Section 9.2.1.4. In contrast, human fMRI experiments detected less pronounced

effects of cortisol in terms of magnitude of responses (Symonds et al., 2012) and size of affected brain areas (for example (Oei et al., 2007; Henckens et al., 2011; Montoya et al., 2014; Ma et al., 2017; Nakataki et al., 2017) or even failed to detect a significant effect (Schwabe et al., 2012). The change in the resting activity of human hippocampus, that is the most frequently studied brain area after administration of GCs, was reported to be within 5% (Symonds et al., 2012), indicating a small range of changes during the first 20 min after administration of cortisol. The restricted effect of acute treatments with cortisol is also visible when we compare stimulus/task related changes with drug effects (for example (Oei et al., 2007; Merz et al., 2010; van Stegeren et al., 2010; Montoya et al., 2014; Ma et al., 2017). These studies show that experimental procedures usually including exposure to various stimuli induce much more widespread changes in the brain activity than the acute elevation of the stress hormone. It has also been found that the ability to detect the effect of cortisol at least in some cases depends on the applied method of data analysis (Henckens et al., 2012a). For example, changes in the hippocampal activity were below the detection threshold of the voxel-wise analysis that was used specifically to test this brain area but were detected when BOLD signal from the entire area was averaged (Henckens et al., 2012a). This is probably because an altered activity was widely distributed across the entire hippocampus and, therefore, was not detected by a method that is most suitable for identification of focal effects (Henckens et al., 2012a). It is also symptomatic that almost all reviewed human studies applied a ROI (Region Of Interest) approach in addition to the whole brain analysis or as the exclusive method of data analysis. The comparison between a priori selected regions of interest increases sensitivity of a statistical analysis because it avoids the problem of multiple comparisons and about half of the reviewed fMRI studies provided only information about GC-induced changes in the activity of declared ROIs even if the whole brain analysis was performed (for example: (Henckens et al., 2010, 2011; Bos et al., 2014; Fleischer et al., 2019). This restricted effect of cortisol detected in fMRI experiments can be easily reconciled with electrophysiological data (Table 3 and 5) because GCs both increase and decrease the activity of some subpopulations of neurons and, therefore, the net effect may be small or even absent (Sections 9.1.1 and 9.1.2).

9.2.1.3. Contribution of female subjects to human fMRI data. A male bias is common in various disciplines of biomedical research including neuroscience (Beery and Zucker, 2011). As expected, male subjects were included in most of the reviewed fMRI experiments with few exceptions (Tabbert et al., 2010; Fleischer et al., 2019). However, there is also a number of studies investigating female subjects as separate groups (Stark et al., 2006b; Merz et al., 2010; Abercrombie et al., 2011; Merz et al., 2012, 2013; Kinner et al., 2016; Ma et al., 2017; Kinner et al., 2018) or jointly with males (Lovallo et al., 2010; Schwabe et al., 2012; Symonds et al., 2012; Brown et al., 2013; Sudheimer et al., 2013; Nakataki et al., 2017). A number of experiments showed that brain responses to cortisol are affected by sex (Stark et al., 2006b; Merz et al., 2010; Abercrombie et al., 2011; Kinner et al., 2016; Ma et al., 2017; Kinner et al., 2018) and hormones as indicated by differences observed in women taking oral contraceptives (Merz et al., 2012). Importantly, a comparison between cortisol and placebo showed even opposite responses in some areas (amygdala, prefrontal cortex, hippocampus, cingulate gyrus and other) of male and female brains at the time when participants were engaged in a task involving aversive stimuli (Stark et al., 2006b; Merz et al., 2010; Kinner et al. 2016; Ma et al., 2017; Kinner et al., 2018). These data indicate that we should be careful with generalization of animal data that are obtained mostly in males.

9.2.1.4. Basal/resting activity. Resting activity consumes most of the energy produced in the brain and has vital functions such as monitoring the internal and external environment, preparation for actions and offline processing of information (Miall and Robertson, 2006; Raichle, 2010). Nonetheless, very few MRI studies provided information about the effect of cortisol on the resting brain activity in humans (Lovallo et al., 2010; Strelzyk et al., 2012; Symonds et al., 2012) and rats (Ferris and Stolberg, 2010). Furthermore, these studies were restricted to the initial 5 min (Ferris and Stolberg, 2010), 19 min (Symonds et al., 2012), 27 min (Strelzyk et al., 2012) and 45 min (Lovallo et al., 2010) after drug administration. Therefore, available data are restricted to short-term effects relying exclusively or mostly on non-genomic mechanism.

The rat study detected a significantly increased activity in four brain areas already within the first minute and even more robust increases in most of the brain within five minutes after the treatment with corticosterone (Ferris and Stolberg, 2010). Maximum changes in BOLD signal were within the range of 10–14% including hippocampus and cortex, which displayed about 8% increase after 1 min and 12–14 % after 5 min. Such fast and widespread changes were not detected in any other fMRI experiment testing acute effects of cortisol in humans. Importantly, the rat experiment (Ferris and Stolberg, 2010) is different from other studies because the procedure included adrenalectomy combined with corticosterone replacement therapy (see also Section 2.3), application of transient anesthesia preceding the brain imaging, immobilization of animals, administration of corticosterone that differs from cortisol in terms of blood-brain permeability (Section 3.1) and utilization of hydroxypropyl-cyclodextrin to dissolve corticosterone. Despite a unique character of the rat fMRI study, the immediate effect of corticosterone that was visible already after 1 min is puzzling, considering the delayed penetration of the blood-brain by corticosterone (Section 3.2.1). A possibility that was not discussed previously is that this central effect of glucocorticoids detected during first few min could result from rapid peripheral effects altering sensory signaling transmitted to the brain. It is known that GCs affect the peripheral nervous system (Hua and Chen, 1989; Shaqura et al., 2016) and such effect can be especially important in this case because of application of body restrainers and head holders with the animal's canines secured over a bite bar and ears positioned with adjustable screws (Ferris and Stolberg, 2010). Despite the usage of topical lidocaine in most sensitive areas of ears and bridge of the nose, application of such procedure means massive tactile stimulation that is not present in human studies.

An increased hippocampal activity but with different time-course was found in humans after a high dose of cortisol (100 mg) which triggered a gradual rise in BOLD signal during 19 min after the treatment (Symonds et al., 2012). However, the rise in BOLD signal was much slower than in the aforementioned rat study because it changed about 2–2.5% after 5 min and maximally about 5% after 19 min (Symonds et al., 2012). Therefore, this time course is comparable with data showing delayed entrance of GCs into the brain (Section 3.2.1). In another study, a ten times lower dose of cortisol induced a transient increase in hippocampal BOLD signal after 5−10 min (medium effect) that was followed by return to the baseline (10−20 min) and a subsequent large decrease that achieved a maximum value after 30−35 min (Lovallo et al., 2010). A similar decrease was found in amygdala but not in thalamus, insula and posterior parahippocampal gyrus, which were selected as additional ROIs (Lovallo et al., 2010). The discrepancies in the time course of hippocampal changes between these two human studies (Lovallo et al., 2010; Symonds et al., 2012) can be easily explained by the difference in applied doses since both the peak concentration of brain GCs and the return to the baseline occurs faster after administration of lower doses of the hormone (Bouchez et al., 2012). Although Lovallo et al (2010) found no changes in the thalamus after administration of 10 mg of cortisol, another study found that even a lower dose (4 mg) decreased the thalamic activity during the first 7−17 min together with some decreases and increases in frontal, occipital and parietal lobes that were analyzed separately (Strelzyk et al., 2012).

Summing up, these studies showed a number of different brain areas that increased or decreased the activity in resting conditions after administration of GCs. The most consistent change was an increased hippocampal activity (Ferris and Stolberg, 2010; Lovallo et al., 2010;

Symonds et al., 2012) that can be followed by more pronounced inhibition (Lovallo et al., 2010). However, our understanding of GC-induced changes in the resting brain activity is still fragmentary due to a limited amount of published data, restriction of analysis to few brain areas and a short period of recording time. Additionally, existing studies are difficult to compare because of differences in applied experimental procedures including detection methods (BOLD/CASL), applied doses of cortisol, duration of measurements blocks that were used for averaging data and other aspect that were reviewed earlier in this section.

9.2.1.5. Memory. An interest in the role of GCs in memory started with the finding that radiolabeled corticosterone is accumulated in the hippocampus (for historical perspective please see McEwen et al., 2015 and Joels and de Kloet, 2017) and this topic evolved together with the better understanding of mechanisms underlying memory formation. This issue is, however, complicated because there are multiple memory systems that frequently operate in parallel but serve different functions and depend on different neural circuits (Squire, 2004; Cowan, 2008; Norris, 2017). Furthermore, memory is decomposed into acquisition, consolidation and retrieval and the last process can be tested while using different approaches, for example free recall, recognition and cued recall that are not fully comparable (Dobbins et al., 1998; Nobel and Shiffrin, 2001; Yonelinas et al., 2010). Therefore, the problem of GC-induced alterations in memory and associated changes in the brain activity is a complex issue.

9.2.1.5.1. Working memory. Very few fMRI studies tested the effect of GCs on working memory. It has been found that both high (100 mg) and low (10 mg) dose of cortisol had no effect on working memory 30 min after the treatment (Henckens et al., 2011; Symonds et al., 2012) while after 4 h there was a slight improvement that approached the level of significance (Henckens et al., 2011). Despite the lack of significant cognitive effects, the high dose increased the activity in hippocampus, prefrontal cortex and precentral gyrus after 30 min (Symonds et al., 2012) while the low dose had only a delayed (4 h) effect characterized by an increased activity in dorsolateral prefrontal cortex at the time when subjects performed the working memory task (Henckens et al., 2011). Therefore, although GCs affected the brain activity, significance of these effects for working memory is not clear.

9.2.1.5.2. Long-term declarative memory. More brain imaging studies tested the effect of GCs on long-term declarative memory including the process of memory encoding (van Stegeren et al., 2010; Abercrombie et al., 2011; Henckens et al., 2012a), consolidation (van Marle et al., 2013) and retrieval (de Quervain et al., 2003; Oei et al., 2007; Fleischer et al., 2019). One study also tested an effect of cortisol administered for 2.5 days on memory retrieval (Brown et al., 2013). Cortisol administered 30–180 min before memory encoding had a variable effect on subsequent recollection. On the one hand, there was no effect on performance in free and cued recall tests (van Stegeren et al., 2010; Abercrombie et al., 2011; Henckens et al., 2012a) while cortisol improved recognition memory (van Stegeren et al., 2010). Cortisol also differentially affected performance of depressed subjects in a sex specific way because they impaired encoding of positive words (but not neutral and negative) in females but improved in depressed men (Abercrombie et al., 2011). Finally, cortisol administered after viewing pictures improved consolidation of emotional memory during subsequent sleep (van Marle et al., 2013). Changes in the brain activity during memory encoding were time-dependent and in some but not all experiments were related to subsequent performance in recall tests (van Stegeren et al., 2010; Abercrombie et al., 2011; Henckens et al., 2012a). Cortisol had no effect on the brain activity in case when encoding was performed 30 min after the treatment (Henckens et al., 2012a) but affected the brain after longer intervals ranging from 45−60 min (van Stegeren et al., 2010; Abercrombie et al., 2011) to 180 min (Henckens et al., 2012a). Cortisol administered 45 min before encoding increased activation in hippocampus and frontal cortex (van Stegeren et al., 2010) while at longer latencies they had an opposite effect (Henckens et al., 2012a). Cortisol also affected the brain activity in depressed subjects in a sex-specific way (Abercrombie et al., 2011).

In case of memory retrieval, prednisolone (de Quervain et al., 2003) and cortisol (Oei et al., 2007) administered one hour before memory testing had no effect on performance in recognition task but impaired cued recall (de Quervain et al., 2003). The effects on the brain activity at the time of memory retrieval were variable, considering affected brain areas but in all cases they were restricted to significant decreases. A lower activity was found for example in hippocampus (Oei et al., 2007) and prefrontal cortex (Fleischer et al., 2019) after administration of cortisol and in parahipocampal gyrus after administration of prednisolone (de Quervain et al., 2003). Decreased activity was also found in hippocampus at the time of memory retrieval in case when cortisol was administered for 2.5 days before testing (Brown et al., 2013). Changes in the brain activity were found even in cases when cortisol did not significantly affect performance of tested subjects (Oei et al., 2007; Brown et al., 2013).

Although experiments testing an effect of GCs on declarative memory yielded variable results, it should be noted that there are numerous methodological differences such as learned material (images *vs* words), awareness of participants about the purpose of watched stimuli (intentional *vs* unintentional encoding), measurement latency and testing methods (recognition or free and cued recollection tasks) that could contribute to differences in obtained results. Nonetheless, it is striking that with similar doses and latencies, cortisol differentially affected the brain activity depending on the performed task. For example, cortisol increased the activity in working memory task (Henckens et al., 2011) while decreased it at the time of encoding the long-term declarative memory (Henckens et al., 2012a). This shows that GCs are not inducing exclusively inhibitory or excitatory responses. Instead, the effect depends on the performed task.

9.2.1.5.3. Nondeclarative memory. Only one study tested the effect of GCs on the brain activity at the time of instrumental learning but it produced negative results when cortisol was administered without concomitant stimulation of adrenergic receptors with yohimbine (Schwabe et al., 2012). However, combination of both drugs decreased the activity in prefrontal cortex and made instrumental behavior insensitive to changes in the value of the goal. This effect means a shift from goal-directed to habitual responses that involve striatal circuits. Later experiments in stressed subjects implicated in this process mineralocorticoid receptors (Schwabe et al., 2013; Wirz et al., 2017). Such switch favoring striatal pathways may render other areas, such as hippocampus, more vulnerable to stress (Wirz et al., 2017).

Relatively well documented is the effect of cortisol on fear conditioning that is also classified as nondeclarative memory (Squire, 2004). It has been found that cortisol affects fear conditioning in a sex-specific way (Stark et al., 2006b; Merz et al., 2010, 2012) and that the direction of changes depends additionally on contingency awareness (explicit *vs* implicit learning). Cortisol also affects extinction of conditioned fear responses but the effect depended on the timing between conditioning, extinction and the treatment. Cortisol administered immediately after fear conditioning and 45 min before fear extinction increased skin responses indicating impaired fear extinction (Merz et al., 2014). However, cortisol administered at similar interval before fear extinction but one day after conditioning had an opposite effect (Merz et al., 2018). This shows that a consolidation phase between acquisition and extinction is important for effects induced by GCs (Merz et al., 2018). Finally, cortisol also affected the return of extinguished fear but again the effect depended on the timing between fear extinction, reinstatement and the treatment (Kinner et al., 2018; Merz et al., 2018). The crucial role of timing found in brain imaging experiments is consistent with other studies that tested a relationship between stress hormones and memory (de Quervain et al., 2017).

Effects of cortisol on brain responses to fear conditioning were complex and sex-specific as already described in Section 9.2.1.3. The

variability in detected brain responses was likely to result at least partly from differences in experimental protocols which were associated with aforementioned behavioral responses. In general, cortisol both increased and decreased the activity in numerous brain areas involved in memory, emotions, pain responses and behavioral control such as hippocampus, amygdala, cingulate cortex, insula and prefrontal cortex (Stark et al., 2006b; Merz et al., 2010; Tabbert et al., 2010; Merz et al., 2012). A similar set of brain areas was also involved in fear extinction (Tabbert et al., 2010; Merz et al., 2014, 2018) and the return of fear responses (Kinner et al., 2018; Merz et al., 2018). Collectively, these data show a complex effect of GCs on fear conditioning and indicate involvement of neuronal circuits controlling a wide range of processes such as pain responses, emotions, memory and behavioral control.

9.2.1.6. Exposition to neutral and emotional stimuli. Several studies tested an effect of GCs on brain responses to emotional stimuli. Experiments employing angry faces and aversive words suggest that cortisol facilitate responses to highly emotional stimuli in both sexes (Ma et al., 2017), interrupting at the same time the processing of information that is not related to emotions in men (Henckens et al., 2012b; Ma et al., 2017). Cortisol also decreased fear responses to spiders in patients with spider phobia but the effect was not generalized to fear ratings for other stimuli (Nakataki et al., 2017). Finally, cortisol increased arousal evoked by sad stimuli compared with happy and neutral stimuli but the effect was restricted to treatment repeated for four days (Sudheimer et al., 2013). Therefore, the subjective effects were rather mild because they were confined to specific stimuli triggering phobic reactions or to repeated treatments. Brain imaging revealed two general patterns of responses to GCs during exposure to emotional stimuli. On the one hand, some researchers reported decreases in the brain activity in various areas when subjects treated with cortisol watched fearful/happy faces (Henckens et al., 2010), sad faces (Sudheimer et al., 2013) and spiders (Nakataki et al., 2017). On the other hand, increases in the brain activity after treatment with cortisol (Henckens et al., 2012b; Bos et al., 2014) and prednisolone (Buades-Rotger et al., 2016) were reported during the task requiring participants to identify colors of aversive words (Henckens et al., 2012b) and in response to both negative socio-emotional stimuli (Buades-Rotger et al., 2016) and infant crying (Bos et al., 2014). Additionally, the responses were modified by past experiences (Bos et al., 2014) and sex of participants (Buades-Rotger et al., 2016; Ma et al., 2017).

Variability in responses to aversive and threatening situations may result from the perceived ability to control the situation. Such scenario was tested in an experiment that combined images of a rapidly approaching virtual predator with a female scream occurring at the end of the attack (Montoya et al., 2015). In trials with an escapable threat, subjects could terminate the attack by pressing a button. The experiment showed that cortisol decreased the brain activity during inescapable threat while opposite changes were observed during an escapable threat. This context-dependent changes help to explain variability between studies using various stimuli and protocols that can affect the perception of experimental environment. They are also important for the general understanding of effects induced by GCs that are not purely excitatory or inhibitory but rather context-dependent. This conclusion is also supported by a number of studies showing a variable effect of GC on the brain responses to emotionally charged stimuli (Henckens et al., 2010, 2012b; Sudheimer et al., 2013; Bos et al., 2014; Buades-Rotger et al., 2016).

9.2.2. Imaging of chronic effects in Cushing's disease

Acute treatments with cortisol had mild subjective and cognitive effects and many of these studies failed to reveal any significant behavioral effects (Section 9.2.1.2). In contrast, a prolonged elevation in the level of GCs has a much stronger effect on emotions and cognition as indicated both by side effects of GCs administered as anti-inflammatory drugs and symptoms observed in patients with Cushing's disease. GCs administered at therapeutic doses induce a number of psychiatric side effects that occur usually after about 4 days (early onset) or 3 weeks (late onset) of treatment (Hall et al., 1979; Lewis and Smith, 1983; Sirois, 2003) and affect 1–62 % of patients depending on the dose and inclusion criteria (Program, 1972; Lewis and Smith, 1983; Naber et al., 1996; Wada et al., 2001; Bolanos et al., 2004). Most of this data concern synthetic GCs that exert complex effects on the brain because of combination of peripheral and central effects (Section 5, Fig. 1). Serious psychiatric symptoms are also very common in patients with Cushing's disease that are exposed to sustained long-term hypercortisolemia lasting for months or even years (Starkman et al., 1981; Starkman, 2013; Pivonello et al., 2015).

Despite the fact that GCs are commonly used as anti-inflammatory drugs, fMRI testing of chronic effects was performed only in patients with Cushing's disease. This means an important gap in knowledge because patients with Cushing's disease differ from those receiving exogenous steroids in terms of duration of hypercortisolemia (Starkman, 2013). fMRI experiments showed both an increased and decreased resting activity in various brain areas (Jiang et al., 2017) and mainly increased activity during memory and recognition tasks (Maheu et al., 2008; Langenecker et al., 2012). Significant changes in the resting brain activity were detected by the whole brain analysis with multiple comparisons (Jiang et al., 2017), suggesting more pronounced changes in Cushing's disease compared with the brain activity after acute treatments in humans that were detected mostly by direct comparisons in preselected regions of interest (Section 9.2.1.2). Patients with Cushing's disease had an increased resting activity in precuneus, cingulate and prefrontal cortex while decreases were found in thalamus, cerebellum, occipital cortex and postcentral gyrus (Jiang et al., 2017). In contrast, recognition of facial expressions was associated mainly with increased activity in several brain areas including frontal areas, cingulate cortex and left hippocampus while lower activity was found in right middle hippocampus compared with healthy control (Langenecker et al., 2012). The higher activity in hippocampus and amygdala was also found in Cushing's patients during an encoding phase of a memory task (Maheu et al., 2008). Preferential increases in the task-related brain activity in patients with Cushing's disease are consistent with changes in neuronal activity assessed with the c-fos expression in rats treated for 25 days with corticosterone and next subjected to fear conditioning (Skorzewska et al., 2006).

Summing up, chronic exposition to cortisol in Cushing's patients triggers variable effects in resting conditions and mainly increased task-related activity. Normalization of the cortisol level leads to partial restoration of altered spontaneous brain activity in remitted patients (Jiang et al., 2017) but some changes are present even in patients with long-term remission of the disease (Bas-Hoogendam et al., 2015; Ragnarsson et al., 2017) consistently with persistent structural changes found in brains of Cushing's patients (Andela et al., 2015). These data indicate that changes in the brain activity observed after long term exposure to cortisol depend both on ongoing signaling mediated by GRs and persistent structural changes.

9.3. c-fos expression

c-Fos protein is a molecular marker of the neuronal activity that is applied postmortem in animal studies (see Section 7.2 for more details). It should be noted that the c-Fos expression is not always able to capture functional changes because the analysis of slices averages activations during a prolonged period of time spanning between the treatment and brain dissection. Actually, the c-Fos expression simply shows that some populations of neurons were activated during an investigated period of time but it is not providing quantitative information about the frequency and duration of evoked activity. This problem is illustrated by an experiment that tested an effect of acute corticosterone on the activity of the hypothalamic-pituitary-adrenal axis during prolonged

immobilization stress lasting for one hour (Ginsberg et al., 2003). This study showed considerable inhibition of the hypothalamic-pituitary-adrenal axis by GCs (corticosterone and RU28362) as indicated by an amount of released hormones and hypothalamic CRH transcription but not at the level of hypothalamic c-Fos expression measured three hours after treatment (Ginsberg et al., 2003). Despite these limitations, detection of c-Fos expression can provide valuable information about the brain activity. In case of GCs, available c-Fos data can be divided into immediate (Briski et al., 1997; Zhang et al., 2013) and delayed (Skorzewska et al., 2007b) responses to acute treatment and responses observed after repeated treatment (Skorzewska et al., 2006; Sasaki-Hamada et al., 2013).

Acute treatment with dexamethasone revealed activated neurons in a priori selected parts of brain such as lateral habenula (Zhang et al., 2013), hypothalamus and preoptic area (Briski et al., 1997) in resting conditions 1−2 hours after drug administration. The main shortcoming of these experiments is usage of dexamethasone that is characterized by restricted penetration of the blood-brain barrier compared with corticosterone and may exert a complex effect on the brain (Section 3.1 and 5, Fig. 1). Nonetheless, detected activations of hypothalamic neurons are consistent with animal electrophysiological experiments (Table 3 and 5). Interestingly, such an effect was not reported in human fMRI studies that failed to detect hypothalamic changes after treatment with cortisol (Supplementary file 2). One likely reason is that the fMRI signal averages the activity of all neurons present in a relatively large volume of tissue leading to low sensitivity toward changes in dispersed or small subpopulations of local neurons. Second, human studies commonly applied the analysis based on preselected brain areas such as hippocampus, amygdala and cerebral cortex but not hypothalamus showing bias in research interests of cognitive neuroscientists.

In addition to immediate c-Fos responses assessed in resting conditions after treatment with dexamethasone (Briski et al., 1997; Zhang et al., 2013) there is also one study that tested c-Fos expression 27 h after acute treatment with corticosterone in combination with fear conditioning (Skorzewska et al., 2007b). This study failed to detect a significant effect in resting conditions which is not surprising while considering the long delay. However, despite negative results in resting conditions, corticosterone decreased the expression of fear responses on the next day after treatment and increased the concomitant activity in paraventricular hypothalamic nucleus, medial amygdala and cingulate while a number of other areas including hippocampus were not affected (Skorzewska et al., 2007b). This shows that corticosterone administered before fear conditioning leads to long lasting changes affecting delayed responses to context associated with previous noxious stimulation.

An opposite behavioral and c-Fos responses in paraventricular hypothalamic nucleus were observed after a prolonged treatment with corticosterone lasting for 25 days (Skorzewska et al., 2006). Additionally, a long-term treatment increased the task-evoked activity in the hippocampal CA2 area, central and medial amygdala and motor cortex while decreased the activity in dentate gyrus (Skorzewska et al., 2006). Therefore, the long-term treatment with corticosterone had much more widespread effects on the brain activity than acute treatment. In contrast, mostly negative results (infralimbic cortex, amygdala, and hippocampus) were found in resting conditions after 10 days of treatment with dexamethasone (Sasaki-Hamada et al., 2013). In this case, increased c-Fos expression was found only in dorsomedial hypothalamic nucleus that is involved in the regulation of arterial pressure and heart rate during acute psychological stress (Sasaki-Hamada et al., 2013). Unfortunately, conclusions drawn from experiments using dexamethasone are restricted by the limited entrance into the brain in combination with other properties of this drug (Section 3.1, 4 and 5 Fig. 1). Collectively, available c-Fos studies indicate that GCs activate some populations of hypothalamic, habenular and preoptic neurons in basal conditions although the data are restricted to few predefined regions of interest and dexamethasone treatments. c-Fos experiments show also task-specific effects that are more pronounced after prolonged treatment compared with single administration of corticosterone.

9.4. Microdialysis

9.4.1. Acute effects on glutamate / GABA balance

Microdialysis allows tracking local changes in the extracellular level of neurotransmitters that are responsible for signal transmission between neurons. Two most important neurotransmitters for the brain activity is glutamate and GABA which are responsible for excitation and inhibition, respectively. Acute treatments with corticosterone increased the hippocampal level of glutamate (Venero and Borrell, 1999; Skorzewska et al., 2007a) with latency ranging from 10 to 15 min but not after 5 min (Venero and Borrell, 1999). The peak was achieved after 25−60 min and the level of glutamate returned to the baseline after 45−120 min depending on the dose ranging from 2.5−20 mg/kg (Venero and Borrell, 1999; Skorzewska et al., 2007a). This time course is consistent with the resting changes in hippocampal BOLD signal detected by human fMRI experiments but not with very rapid changes observed in the rat fMRI experiment (for more details see Section 9.2.1.4). An increased level of glutamate detected *in vivo* (Venero and Borrell, 1999; Skorzewska et al., 2007a) is also consistent with *in vitro* experiments performed on isolated nerve terminals that showed increased glutamate release after treatment with corticosterone, dexamethasone and methylprednisolone (Wang and Wang, 2009; Neiva et al., 2020). The experiment performed by Skorzewska et al. (2007a) also included a long recording period that extended beyond the time of initial normalization of the glutamate level. This experimental setup revealed a fluctuation of the glutamate level that repeatedly increased and returned to the baseline during 220 min of the recording period (Skorzewska et al., 2007a). This fluctuation can be one of the factors contributing to the variability of experimental data collected at single time points after treatment.

An increased level of glutamate was associated with a less pronounced increase in the level of GABA that achieved significance during some time points after acute treatment with corticosterone (Skorzewska et al., 2007a). Importantly, the glutamate / GABA ratio was not significantly altered which indicates that there was no marked change in the balance between excitatory and inhibitory processes in hippocampus (Skorzewska et al., 2007a). The mechanism of this effect is, however, not clear. Methylprednisolone, a synthetic GC, did not affect GABA release from isolated nerve terminals in contrast to glutamate release (Neiva et al., 2020). This indicates that the effect observed *in vivo* (Skorzewska et al., 2007a) requires a preserved structure of neuronal network that is destroyed during preparation of isolated nerve terminals (Neiva et al., 2020). Summing up, microdialysis data show that acute treatment with GCs increase excitatory neurotransmission but it is balanced by concomitant inhibitory effects. Therefore, the net effect is small consistently with fMRI experiments (Section 9.2.1.2).

9.4.2. Glutamate / GABA balance after repeated treatments

In animals chronically pretreated with corticosterone (25 days), both the baseline concentrations of glutamate and the Glu/GABA ratio were increased, indicating an enhancement of excitatory processes in the hippocampus (Skorzewska et al., 2007a). However, a challenge dose of corticosterone administered to rats chronically pretreated with corticosterone almost completely depleted hippocampal glutamate, and decreased the glutamate/GABA ratio (Skorzewska et al., 2007a). It has been suggested that this phenomenon results from enhancement of local feedback mechanisms, operating to eliminate excess of extracellular glutamate from the synaptic cleft to maintain the equilibrium between the excitatory and inhibitory processes (Skorzewska et al., 2007a). Collectively, these data indicate that prolonged treatment with corticosterone leads to increased hippocampal excitability but this effect is reversed by acute elevation of GCs. Importantly, these findings help to reconcile apparently contradictory results obtained not only during the brain imaging of Cushing's syndrome but also in acute treatment in

healthy population. Small groups of volunteers recruited in some social groups such as students may share the history of stress experienced over weeks prior to the imaging experiment affecting the apparently acute effects of cortisone.

9.4.3. Serotonin and noradrenaline

Two other tested neurotransmitters were serotonin and noradrenaline. Serotonin was increased in the hippocampus 2 h after treatment with corticosterone (10−40 mg/kg) but not earlier (Li et al., 2019). Acute treatment with corticosterone (3 mg/kg, i.p.) also induced a transient increase in the noradrenaline level in the amygdala 15 min after the inhibitory avoidance training while no effect was observed in resting conditions (McReynolds et al., 2010). The repeated treatment lasting for 1 week had an opposite effect in paraventricular nucleus and the differences were much higher during immobilization stress that during basal conditions showing a well-known inhibitory effect of GCs on the hypothalamic-pituitary-adrenal axis (Pacak et al., 1995). However, a longer treatment (2 weeks) administered in the form of implanted pellets had no effect on the basal release of noradrenaline in two other brain areas (prefrontal cortex and locus coeruleus) (Horrillo et al., 2016). Collectively, these data indicate that it is important to test the effect of GCs in different conditions (resting and task/stimulus evoked) because some effects may be less pronounced or even absent in basal conditions as evidenced by the effect on the release of noradrenaline. This observation is consistent with data from electrophysiological (Section 9.1.1) and c-Fos experiments (Skorzewska et al., 2007b).

10. Integration of experimental data

10.1. Production of energy

The most crucial issue for interpretation of metabolic data is the total amount of available energy in the brain as indicated by the level of ATP (Section 8.7). Modest doses of GCs applied for up to several days do not decrease the total amount of brain ATP and in some cases they even increase it (Section 8.7.1). Additionally, GCs slow ATP loss during a short-term brain ischemia resembling a situation that can be found during strangling (Section 8.7.2). In contrast, a decreased level of ATP was observed after application of very high doses of corticosterone or after prolonged treatments (Hoyer and Lannert, 2008; Zhao et al., 2008). This shows that elevated levels of GCs are not impairing production of energy with an exception of prolonged treatments and high doses that induce a toxic effect consistently with the concept of allostatic load and overload (McEwen, 2020). Production of energy is maintained despite an initial decrease in mitochondrial oxidative metabolism occurring during the first 2 h after treatment (Morin et al., 2000; Katyare et al., 2003; Fujita et al., 2009) probably because of increased glycolysis in astrocytes (Allaman et al., 2004; Juszczak and Stankiewicz, 2018; Skupio et al., 2019). Although the short-term decrease in mitochondrial oxidative metabolism may seem maladaptive, it can be easily understood, considering a metabolic response to an increased neuronal activity observed for example during intense sensory stimulation or mental effort. Such activation is associated with highly increased nonoxidative glycolytic metabolism despite an excessive supply of oxygen due to a locally increased blood flow (Dienel, 2012; Dienel and Cruz, 2016). Therefore, the biphasic effect of GCs on oxygen consumption (Section 8.6) may constitute an adaptation for initial increase in glycolytic metabolism associated with a task-related brain activation followed by delayed compensation for a metabolic debt.

10.2. Energetic substrates

During the first two hours there are no changes in the brain glucose uptake at least in resting conditions while increases are frequently found *in vivo* after longer treatments that lead to insulinemia (Section 8.3.2, Table 1). GCs also increase the blood level of several metabolites such as lactate, pyruvate, mannose and hydroxybutyrate (ketone bodies) (Thurston et al., 1980; Thompson et al., 2000; Bordag et al., 2015) that can be used by the brain as an alternative source of energy (Section 8.5). Increased availability of ketone bodies means that brain energetics can benefit from increased metabolism of lipids (Section 8.1. Fig. 3) although it has a negligible ability to directly oxidize fatty acids (Dhopeshwarkar and Mead, 1969; Yang et al., 1987). Therefore, GCs not only increase an amount of available blood glucose but also lead to diversification of available sources of energy that can be used to fuel brain at the time of increased energy expenditures associated with stress response (Picard et al., 2018). These changes in the level of various energetic substrates can be classified as allocative brain-pull mechanisms enabling the brain to actively demand energy from the body (Peters, 2011). Finally, GCs increase both synthesis and utilization of glycogen (Watanabe and Passonneau, 1973) enabling flexible storage and utilization of surplus glucose depending on the local neuronal activity. Such flexibility is important because brain responses to stress are variable. In fact, depending on the type of stressful experience, brain metabolism may either increase or decrease (Bryan, 1990; Warnock and Steckler, 2011). The differences in energy utilization also lead to important consequences in situations not requiring energy-consuming activities because a prolonged elevation of glucose and lipids together with insulin resistance constitutes metabolic stress for the organism (Gandhi et al., 2010; Picard et al., 2018; Rowan et al., 2018).

10.3. Brain activity

10.3.1. Acute effects of GCs

Brain metabolism and activity are intimately connected with each other because most of the brain energy is consumed by neuronal signaling (Yu et al., 2018) which in turn regulates local blood flow and transport of glucose across the blood-brain barrier (Kim and Ogawa, 2012; Koepsell, 2020), glycolysis (Dienel, 2012; Dienel and Cruz, 2016) and utilization of glycogen (Dienel and Rothman, 2019) (Fig. 4). Acute effects of GCs on the net brain activity are modest as indicated by the proportion of excited and inhibited neurons (Table 3 and 5), ratio of released excitatory and inhibitory neurotransmitters (Skorzewska et al., 2007a) and magnitude of effects detected by fMRI (Section 9.2.1.2). Instead of inducing purely excitatory or inhibitory effects, GCs rather alter dynamics of the neuronal activity leading to context-specific changes that are expected to support responses to environmental threats.

Available data also indicate that changes in the activity of single neurons after treatment with GCs do not always lead to gross changes in the activity of studied brain area because of concomitant excitatory and inhibitory effects in local subpopulations of cells (Section 9.1.2). This indicates that some functional effects may occur even without gross changes in the local brain activity. Restricted acute effects of GCs on brain metabolism and activity are consistent with absent or mild subjective effects reported by humans after acute administration of the hormone (Section 9.2.1.2).

Methodological approaches enabling the assessment of a net effect of GCs on the brain activity such as fMRI and microdialysis with concomitant measurement of excitatory and inhibitory neurotransmitters help to put various mechanisms implicated previously in actions induced by GCs in a broader context. Such mechanisms include changes in neurotransmitter clearance (Zschocke et al., 2005; Autry et al., 2006; Popoli et al., 2011), altered excitability depending on expression and trafficking of glutamatergic receptors (Liu et al., 2010; Popoli et al., 2011; Yuen et al., 2011; Nasca et al., 2015) and increased probability of neurotransmitter release that can be either MR-dependent (Karst et al., 2005) or GR-dependent (Wang and Wang, 2009). Furthermore, GC-induced changes in neurotransmission also involve retrograde endocannabinoid release (Di et al., 2003, 2005) and both increased (Di et al., 2005) and decreased GABA release (Hill et al., 2011). These mechanisms studied in isolation are not informing us, however, about

the net effect of altered excitatory and inhibitory neurotransmission at the level of entire brain. However, reviewed fMRI and microanalysis studies show that these various mechanisms are in fact roughly balanced after acute treatments with GCs leading to restricted changes both in terms of size of affected brain areas and magnitude of the excitatory and inhibitory effects.

10.3.2. Chronic effects of GCs

In contrast to acute effects, prolonged elevation in the level of GCs has a much stronger effect on brain metabolism, activity and function as indicated by animal studies (Skorzewska et al. 2006; Skorzewska et al., 2007a) and patients with Cushing's disease (Section 9.2.2). Chronic effects are associated with region-specific changes in resting blood flow (Jiang et al., 2017) and glucose utilization including both increases and decreases (Brunetti et al., 1998; Liu et al., 2018) and mainly an increased task-related activity (Skorzewska et al., 2006; Maheu et al., 2008; Langenecker et al., 2012). The altered brain activity observed after a long-term exposure to high levels of GCs results to some extent from structural changes as indicated by only partial restoration of altered brain activity in remitted patients (Bas-Hoogendam et al., 2015; Jiang et al., 2017; Ragnarsson et al., 2017).

11. Major gaps and future directions

There are many gaps in available data that were discussed in detail in previous Sections (2.2, 8.8, 9.1.4, 9.2.1.1) and methodological shortcomings (2.3, 5, 7, 9.3) frequently preventing us from drawing firm conclusions. A general weakness of available data is that both animal and human studies were focused mainly on brain responses during the first two hours after acute treatments while longer latencies (Section 6) and repeated treatments are underrepresented in the scientific literature (Section 8.8, 9.1.4, 9.2.1.1). Furthermore, animal *in vivo* studies were usually performed in resting conditions and frequently applied GCs characterized by a restricted ability to cross the blood-brain barrier (Section 5, Fig. 1). Therefore, a bulk of animal studies is difficult for interpretation and has no behavioral relevance. This is an important gap because a number of different methodological approaches such as fMRI (Section 9.2), microdialysis (Pacak et al., 1995; McReynolds et al., 2010) and postmortem c-Fos immunohistochemistry (Skorzewska et al., 2007b) show that the effect of GCs on the brain activity depends on exposure to sensory stimuli and their emotional valence, task engagement and conditions affecting escapability of aversive stimulation. Additionally, we are not able to assess the replicability of many findings because of restricted number of studies. A final major gap results from the fact that most of available studies focused exclusively on the effects of an increased level of GCs without the consideration of interaction with other stress molecules (Section 2.2). This issue is important because few available studies suggest that actions induced by GCs vary considerably depending on the presence or absence of noradrenergic stimulation and timing of this interaction (Allaman et al., 2004; van Stegeren et al., 2010; Schwabe et al., 2012; Karst and Joels, 2016). Therefore, despite the fact that GCs are studied extensively for many decades, there are still considerable gaps in our knowledge. One of the most promising research opportunities is offered by the development of new methods enabling detection of neurotransmitters with biosensors (Leopold et al., 2019) and analysis of large populations of neurons in behaving animals (Aronov and Tank, 2014; Weisenburger and Vaziri, 2018; Piatkevich et al., 2019). The second major opportunity is offered by recent advancements in magnetic resonance spectroscopy and other related imaging methods enabling measurement of brain metabolism (Hyder and Rothman, 2017; Rothman et al., 2019). Application of these methods will advance our understanding of effects induced by GCs in brain. Furthermore, linking functional effects of glucocorticoids with specific subpopulations of MRs and GRs differing in binding affinity, cellular localization and interacting partners involved in genomic and non-genomic effects will help to better understand their role in adaptive and maladaptive responses. Especially interesting in this context are still poorly understood interactions between GRs and mitochondrial genome (Du et al., 2009; Weger et al., 2020). It can be expected that this topic will attract more attention in the future, considering the role of mitochondria in cellular metabolism that affect the function of the entire organism (Picard et al., 2014, 2018).

Declaration of Competing Interest

None.

Acknowledgments

This work was supported by the National Science Centre [grant number 2017/27/B/NZ2/02796]. The editorial assistance of Ewa Pajak is highly appreciated.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neubiorev.2021.03.007.

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Protocol

Dissection of Mouse Hippocampus with Its Dorsal, Intermediate and Ventral Subdivisions Combined with Molecular Validation

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Abstract: Many research methods applied in molecular neuroscience require the collection of hippocampal samples, but a still poorly recognized problem is contamination with the choroid plexus during brain dissection. Because of a distinct pattern of gene expression, its inclusion in brain samples can obscure or even confound conclusions drawn from molecular studies. Therefore, we tested our dissection method designed for removal of tissue contamination using expression of the transthyretin gene (*Ttr*) as a marker of the choroid plexus. Additionally, we also validated dissection of the entire hippocampus into its dorsal, intermediate and ventral subdivisions using the expression of *Trhr* and *Lct* genes as molecular markers of anatomical subdivisions. The PCR analysis showed that *Ttr* is expressed at a residual level in hippocampal samples that display an mRNA level several hundred lower than the adjacent control tissue colocalized with the choroid plexus. This indicates that the applied method for dissecting the hippocampus from a fresh brain allows for replicable removal of the majority of choroid plexus from hippocampal samples. In turn, differences in expression of *Lct* and *Trhr* confirmed the proper dissection of dorsal, intermediate and ventral subdivisions from fresh brain tissue. Therefore, a special emphasis on the removal of tissue contamination and avoidance of tissue distortions makes our protocol especially suitable for molecular experiments performed either on the entire hippocampus or its subdivisions.

Keywords: hippocampus; mice; dissection; dorsal; intermediate; ventral

Citation: Jaszczyk, A.; Stankiewicz, A.M.; Juszczak, G.R. Dissection of Mouse Hippocampus with Its Dorsal, Intermediate and Ventral Subdivisions Combined with Molecular Validation. *Brain Sci.* **2022**, *12*, 799. https://doi.org/10.3390/brainsci12060799

Academic Editor: Martina Bocchetta

Received: 4 May 2022
Accepted: 7 June 2022
Published: 18 June 2022

1. Introduction

The hippocampus is involved in various processes ranging from learning and memory to control of emotions and motivation [1]. It is also responsive to stress hormones [2] and is implicated in pathogenesis of various disorders such as depression [3], post-traumatic stress disorder [4], schizophrenia [5], epilepsy [6] and neurodegenerative diseases [7]. Therefore, it is not surprising that the hippocampus is one of the most frequently studied brain areas in rodents. Many research methods applied in molecular neuroscience require dissection of the hippocampus, but a still poorly recognized problem is tissue contamination during brain dissection. This issue has been raised in the past by several authors, especially in the context of inadvertent inclusion of the choroid plexus [8–10], which is located in the vicinity of hippocampus but is poorly visible in a mouse brain. Because of a distinct pattern of gene expression [9], its inclusion in brain samples can obscure or even confound conclusions drawn from molecular studies [10,11]. Our recent meta-analysis showed that the transcriptomic signature of the choroid plexus is common in microarray and RNA-seq experiments because it is present in about 25% of rodent studies, and the brain area most frequently affected is the mouse hippocampus [11]. Despite the importance of this issue, there is no validated method enabling dissection of the hippocampus from fresh mouse

brain with precision sufficient for reliable removal of the choroid plexus. An alternative approach providing perfect precision is laser microdissection from frozen brain slices [8], but this method is very expensive and time-consuming, which severely limits its application. Therefore, we tested our dissection method designed for removal of tissue contamination. The dissection precision was assessed using expression of the transthyretin gene (*Ttr*) as a marker of the choroid plexus [8,9]. Additionally, we also validated dissection of the entire hippocampus into its dorsal, intermediate and ventral subdivisions. This is important because of the functional heterogeneity of different parts of the hippocampus [1] and because there is no validated dissection protocol for fresh brains obtained from adult mice, despite the availability of molecular markers differentiating the hippocampus across the dorsal-to-ventral axis [1,12].

2. Materials and Methods

2.1. Animals

The assessment of dissection precision was performed on tissues obtained from 5 male mice (Swiss-Webster) that were 3.5 months old and weighed 35.7 ± 1.3 g (mean ± SEM). Mice were obtained from the breeding colony located at the Institute of Genetics and Animal Biotechnology (Jastrzebiec). From each mouse, we dissected two hippocampi that constituted separate samples and a part of brain tissue constituting a positive control for expression of choroid plexus marker gene (*Ttr*). The final number of samples was 5 in case of *Ttr* positive control samples and 9 in case of hippocampi (one hippocampus was lost due to dissection failure). The sample quality assessment was performed on spare tissues collected during project 2017/27/B/NZ2/02796, which was performed with the permission of the Second Local Ethical Committee in Warsaw (permit number: WAW2/090/2018) and in accordance with the Polish Act of 15 January 2015 on the protection of animals used for scientific and educational purposes and the 3Rr principle.

2.2. Dissection Tools and Materials

- Scalpels with small (nb. 15) and large (nb. 24) blades (Figure 1A,B).
- Bent dissecting needle (Figure 1C).
- Stainless steel spatula with narrow blade (Figure 1D).
- Stainless steel spatula with flat round and tapered arrow ends (Figure 1E).
- Small surgical scissors (straight) with sharp tips (Figure 1F).
- Large surgical scissors (Figure 1G).
- A large paper clip that is used to prepare a loop restricting movement of dissected hippocampus at the time of rinsing with water (optional; Figure 1H).
- Single edge razor blade (optional; Figure 1I).
- Cutting form made from metal strip. The form helps to make precise vertical cuts (optional; Figure 1J).
- Convex cover of the Petri dish that serves as a dissection table. Convex surface is important because it enables water to flow out of the dissection surface. The cover can be painted black with mat waterproof paint to increase contrast between the background and the dissected tissue (Figure 1K).
- Wash bottle.
- Styrofoam box.
- Tabletop Illuminated Magnifier (3×).
- Filter paper.
- Millimeter paper.

2.3. Brain Dissection

Mice were euthanized by cervical dislocation. Head was separated from the rest of the body with large scissors while remnants of tissues (muscles and cervical vertebrae) were removed with small scissors. Skin covering the head was cut with small scissors along the midline starting from the occipital part to the interorbital constriction of the

skull (approximately half of the total length of the skull). Muscles covering the skull were shoved aside with the round end of spatula. Next, bones were removed from the skull starting from the occipital part (Figure 2; Supplementary Video S1 File). The first cut was carried out by insertion of the tip of the small scissors into the foramen magnum (opening in the occipital bone of the skull), and bones were removed with the round end of spatula inserted gently under bones. The sequence of cutting and removing bones was repeated as illustrated in Figure 2 and Supplementary Video S1 File. At the time of cutting the bones covering hemispheres (Figure 2E,I), the lower tip of scissors should press the bones from the internal side of the skull so that the pressure is applied away from brain tissue. A crucial step is removal of meninges (Figure 2H) because they are very durable and easily damage brain. Frequently, the meninges are not visible after the removal of bones, although in some cases they are partly disrupted and therefore can be easily noticed at this stage of dissection (Supplementary Video S1 File). In both cases, meninges were removed as described below. The dissecting needle was slightly inserted into the interhemispheric fissure to pierce the meninges (or in front of already existing disruption) and moved in the posterior direction along the interhemispheric fissure and downward along the posterior edge of the hemisphere as indicated in Figure 2H. At the end of this movement, the needle was inserted deeply between the cortex and remnants of the skull and moved away from the brain to tear apart the meninges. Next, the needle was moved along the edge of the skull (Figure 2H), and the same procedure was repeated on the other side of the brain. After removal of all remaining bones (Figure 2I,J), the head was moved to vertical position and slightly tilted so the exposed brain was facing downwards. The nerves were cut with the tapered arrow end of the spatula starting from the occipital part of the brain. Finally, the spatula was inserted to separate olfactory bulb from olfactory nerves, and the brain was removed on a spatula (Supplementary Video S1 File).

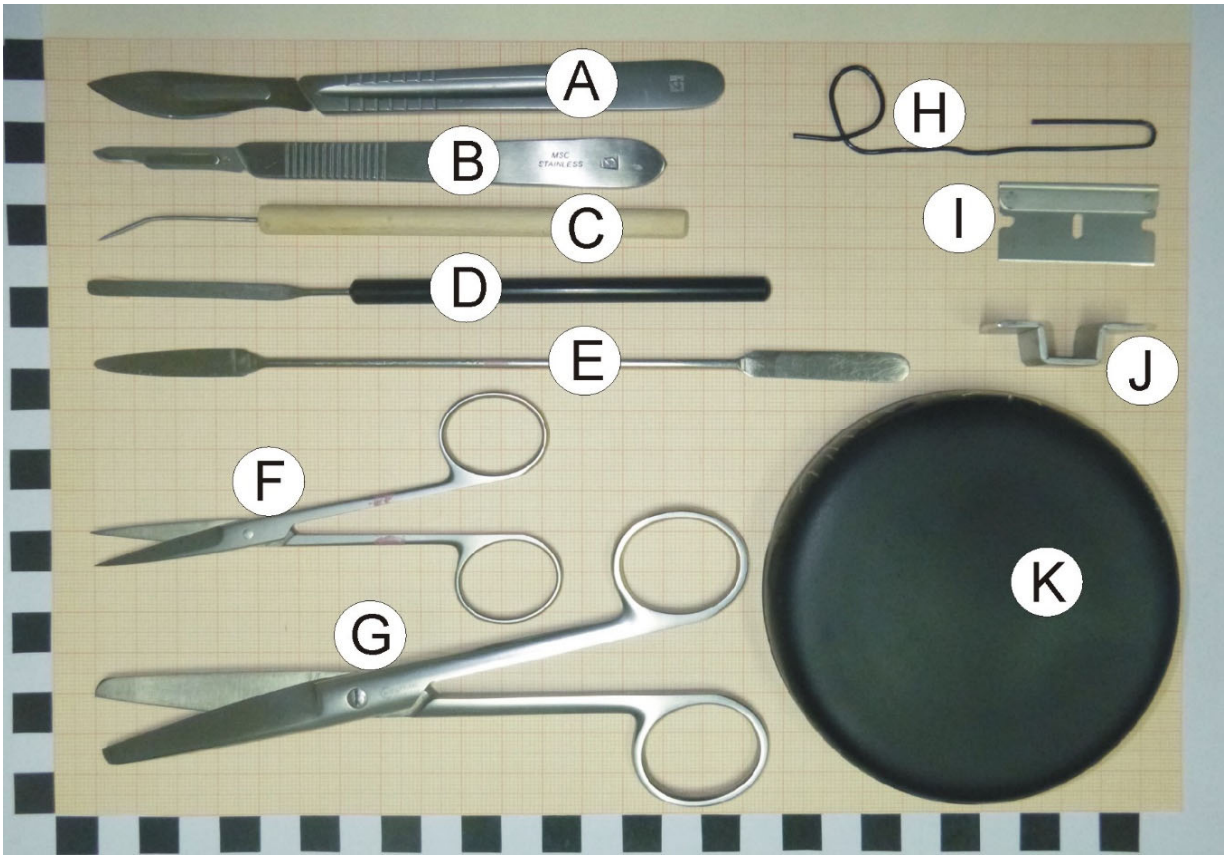

Figure 1. Dissection tools placed on millimeter paper. The black and white scale on the borders of the image is expressed in centimeters. For more information, please see Section 2.2.

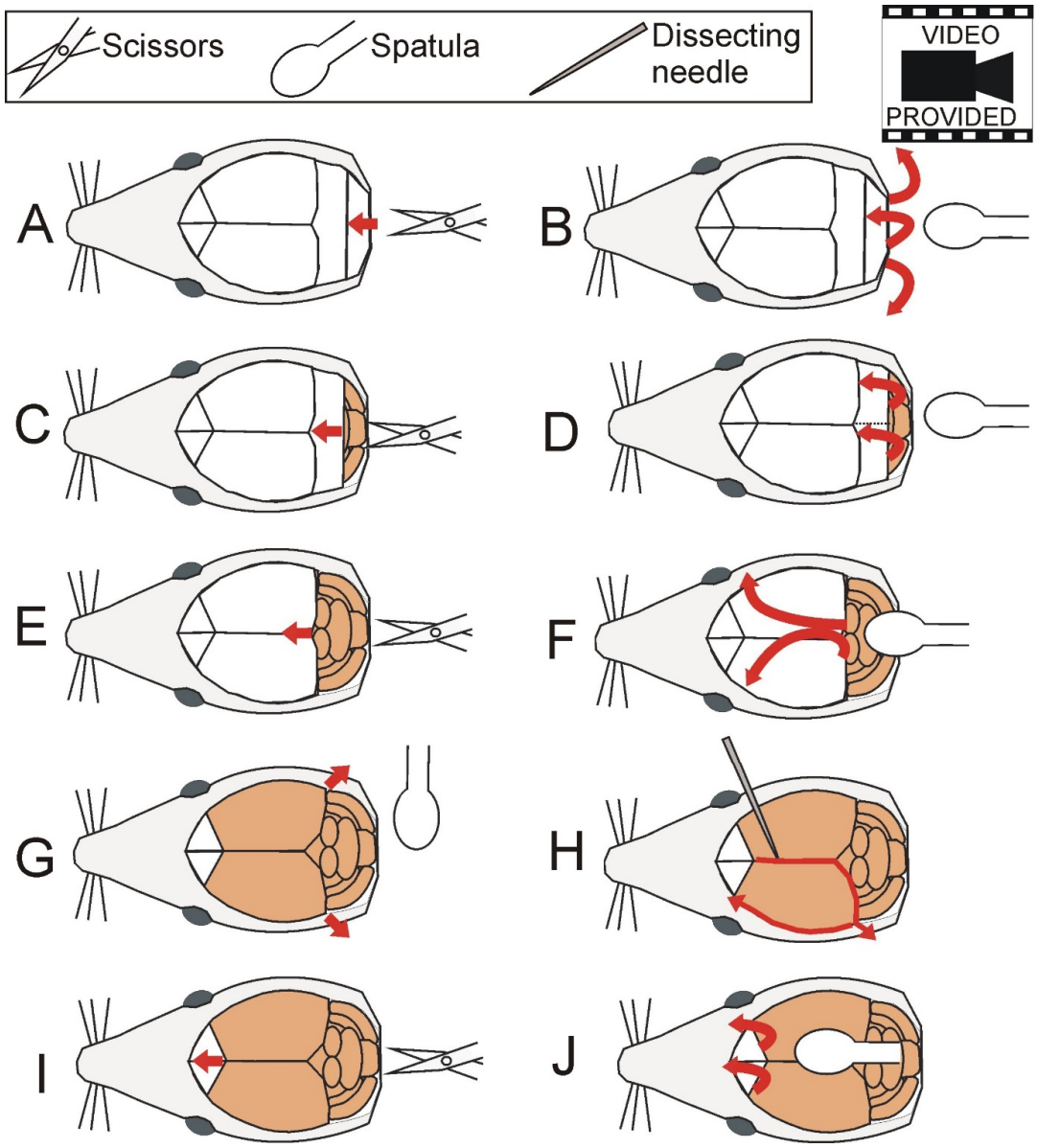

Figure 2. Removal of bones (**A**–**F** and **I**,**J**) and meninges (**H**). Additionally, bones on both sides of the brain (in the vicinity of occipital cortex) can be pulled apart (**G**) to facilitate disruption of the meninges and final brain removal. Bones are removed with small scissors and the round end of spatula while meninges are removed with a dissecting needle. Removal of meninges is shown only for one brain hemisphere for simplicity, but the procedure is performed on both hemispheres. The video is available in Supplementary Video S1 File.

2.4. Hippocampal Dissection

Dissection site constitutes Styrofoam box filled with crushed ice. The convex cover of the Petri dish that served as a dissection table was located on top of the ice. Convex surface is important because it enables water to flow out of the dissection surface. Depending on the need, the cover of the Petri dish can be turned around or tilted. Additionally, the cover of the Petri dish can be painted black (Figure 1K) to increase the contrast between dissected tissues and the background. The dissection was supported with the Tabletop Illuminated Magnifier (3×).

First, a square of filter paper was placed on the Petri dish lid to prevent sliding of the brain during dissection. The filter paper was soaked with ice-cold sterile water. The dorsal and ventral parts of the brain were rinsed thoroughly with ice-cold sterile

water (Supplementary Figure S1A,B). Next, the olfactory bulb was removed with tapered arrow end of spatula (Supplementary Figure S1C). The brain was placed on a square of millimeter paper to make a vertical cut with single edge razor blade to separate the anterior part containing frontal cortex and the posterior part containing hippocampus, thalamus and hypothalamus. The cutting line was 3–4 mm from the frontal pole (Supplementary Figure S1D). The anterior part containing frontal cortex can be used for collection of other brain areas or discarded while the posterior part (Figure 3) is used for dissection of hippocampi. The cortex overlying dorsal hippocampi was removed with dissecting needle and spatula (with a narrow blade) starting from the interhemispheric fissure (Figures 3 and S1H–J). The removal of cortex was preceded by partial disruption of the corpus callosum (Supplementary Figure S1D). The spatula was placed gently on the cortex from one side of the fissure to hold the brain in place at the time when the cortex on the other side was shoved aside with a dissecting needle (Figures 3B and S1I). Next, the spatula was placed on partly removed cortex and the needle was used to shove aside contralateral cortex (Supplementary Figure S1J). Released parts of cortex were cut off (Figures 3C and S1K,L) and collected into vials to freeze in liquid nitrogen. In our experiment, these parts of cortex were included in the control tissue (Figure 3C) for measurement of *Ttr* expression. Next, a cut was performed with the tip of the scalpel along the posterior and anterior edges of the hippocampi to separate them from white matter (Supplementary Figure S1N,O). Finally, the white matter located in front of the hippocampi was completely removed together with the remaining brain tissue (Figures 3D and S1P) and saved for further analysis. In our experiment, this part of brain was included in the control tissue together with cortex removed earlier (Figure 3C,D) for measurement of *Ttr* expression. Next, dorsal parts of hippocampi were separated from tissues located in the third ventricle or its vicinity with angled cuts (Figure 4) that were perpendicular to the longitudinal axis of the hippocampus (Supplementary Figure S1R). At this stage, the dissection exposed dorsal and intermediate parts of both hippocampi (Figure 3D) while the ventral part was still covered with the cortex. To expose the ventral hippocampus, it is necessary to separate the hemispheres (Supplementary Figure S1S) and to roll over the brain to the medial surface created by the cut (Supplementary Figure S1T,U). The border between the ventral hippocampus and the cortex may not be well visible. Therefore, this part of brain was thoroughly rinsed with the stream of ice-cold sterile water to separate the cortex from the hippocampus (Supplementary Figure S1V). Partly detached cortex can be additionally pushed away with a dissecting needle and cut off with scalpel. Final incision was performed along the exposed ventral hippocampus (Supplementary Figure S1W) and between the anterior edge of hippocampus and tissues located beneath (Supplementary Figure S1X). The hippocampus was rolled over with dissecting needle (Supplementary Figure S1Y,Z) and detached from other brain tissues with the stream of ice-cold water (Supplementary Figure S1AA). Partly detached cortex was additionally pushed away with a dissecting needle in case not all connections were disrupted during earlier steps. The hippocampus was picked up with spatula (Supplementary Figure S1AB) and placed on clean paper filter soaked with the ice-cold sterile water for final removal of tissue contamination (Supplementary Figure S1AC–AE). At this stage, all remnants of white matter and cortex were removed with the scalpel. The stream of water was used to remove and visualize remnants of tissues attached to the dorsal and ventral surfaces of the hippocampus (Supplementary Figure S1AC). Wire loop can be used to keep the hippocampus in place during thorough rinsing (Supplementary Figure S1AE). The procedure was performed until there were no strings or pieces of tissue visible at the time of rinsing the hippocampus. Finally, the hippocampus was separated into three equal parts (Supplementary Figure S1AF) corresponding to the dorsal, intermediate and ventral subdivisions (Supplementary Figure S1AF) with the help of the millimeter paper and the scalpel. The dorsal part can be easily recognized by its shape (Supplementary Figure S1AF) resulting from the perpendicular cut performed in earlier step of the procedure (Supplementary Figure S1R). Obtained samples were frozen in liquid nitrogen and stored at −80 °C.

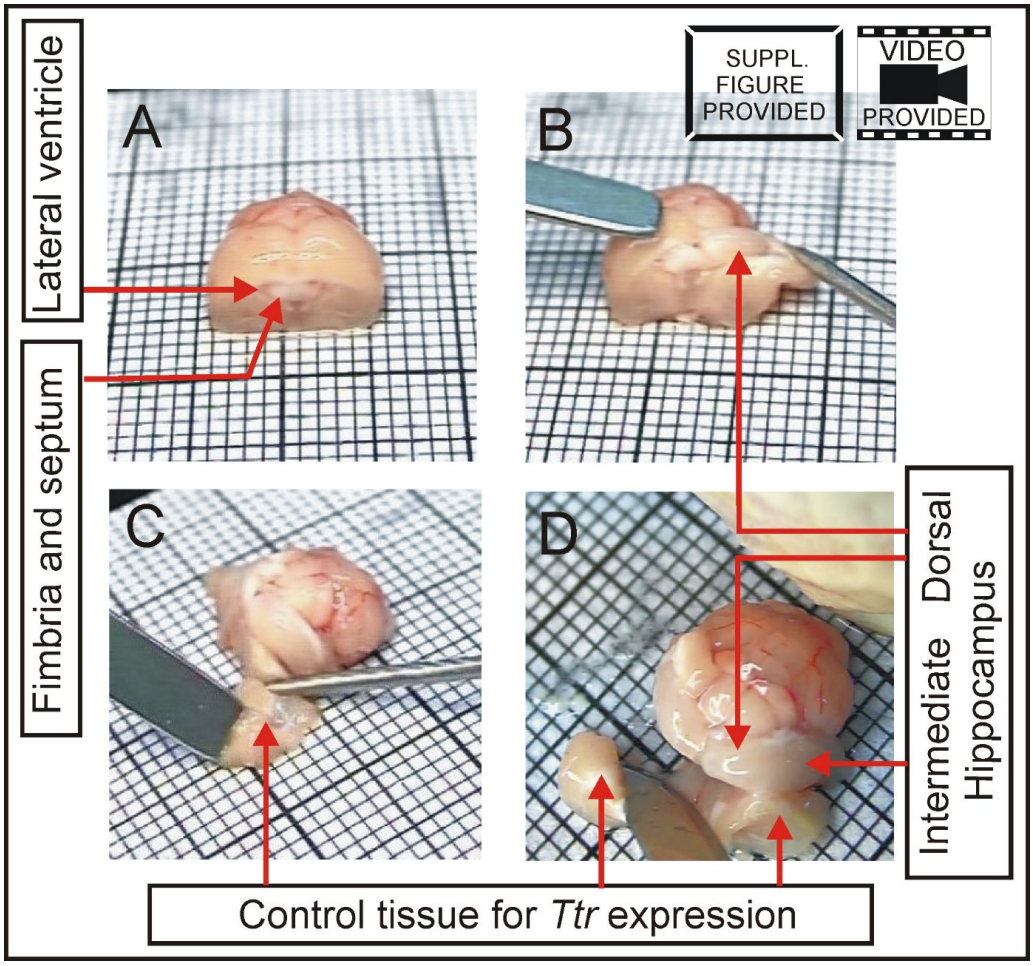

Figure 3. Removal of cortex from dorsal and ventral hippocampus and separation of control tissue colocalized with the choroid plexus to obtain positive control for expression of marker gene *Ttr*. (**A**) Posterior part of the brain after removal of frontal part. (**B**) Removal of cortex from dorsal hippocampus. (**C**) Cutting off the cortex. (**D**) Removal of tissues located in front of the hippocampus. The entire dissection protocol is presented in Supplementary Figure S1 and Supplementary Video S2 File.

2.5. Real-Time PCR (PCR)

Total RNA was extracted from the individual samples using GeneMATRIX universal RNA purification kit (Eurx). The quantity and quality of all RNA samples were assessed by spectrophotometry (ND-1000, Nanodrop). To verify the precision of dissection, we analyzed expression of marker genes *Trhr*, *Lct* and *Ttr* together with reference gene *Hmbs*. The expression was analyzed with SYBR Green-based qPCR performed in 96-well plates on the Roche LightCycler® 96 thermocycler. The primers used in qPCR were designed with the Primer-BLAST tool. The designed primers were located on two different exons and contained all mRNA transcripts of each specific gene. The annealing temperature for individual primers was determined by performing PCR with a set temperature gradient (55°–65°) during 3-step amplification. Primer specifications are presented in Table 1. For retrotranscription into cDNA, 500 ng of total RNA from each sample was used (First Strand cDNA Synthesis Kit, Roche). qPCR was performed on the FastStart Essential DNA Green Master kit (Roche) according to the protocol provided by the manufacturer. All genes were run in three replicates, and each repetition was performed on a separate plate. Each plate contained two negative controls (without cDNA) and a series of 5-fold dilutions of the total cDNA sample to determine PCR efficiency. The reaction volume was 20 µL (*Lct*, *Ttr* and *Hmbs* genes) or 40 µL (*Trhr* gene). The relative expression of marker genes was calculated using Pfaffl method [14].

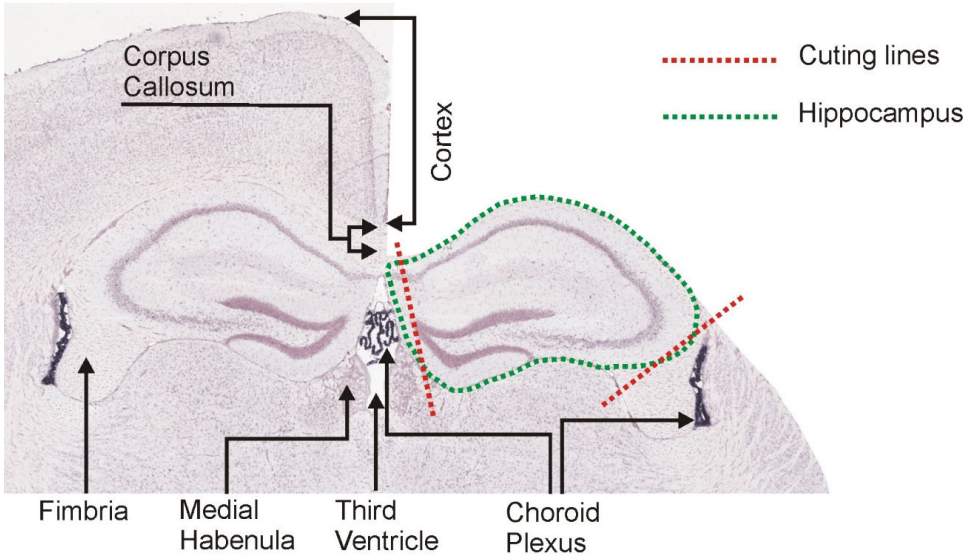

Figure 4. Coronal section of the mouse brain showing spatial relationship between dorsal hippocampus and choroid plexus. The in situ hybridization (ISH) image was derived from the Allen Mouse Brain Atlas (https://mouse.brain-map.org/, accessed on 4 January 2022) [13]. Part of the cortex (right side of the image) has been masked to visualize brain during the dissection process (Figure 3, Supplementary Figure S1 and Supplementary Video S2 File).

Table 1. Primers used for PCR validation of dissection precision.

Gene Name	Forward or Reverse Primer	Primer Sequence	Annealing Temperature	Efficiency
Trhr	F	GAGCCTCTGCTAAGTGATCTTCC	58°	97%
	R	ACGGGGACTCTAAAACATCTTTCC		
Lct	F	CGTCAGCCAAGGTCTACGC	60°	93.7%
	R	GTCTGTGCTTCTGCCGTGC		
Ttr	F	TCGCGGATGTGGTTTTCACAG	60°	106.2%
	R	CTCTCAATTCTGGGGGTTGCT		
Hmbs	F	TCCTGGCTTTACTATTGGAG	60°	95.2%
	R	TGAATTCCAGGTGGGGGAAC		

2.6. Statistics

Raw and square root transformed data [15] were first tested for variance homogeneity with C Cochran, Hartley, Bartlett test. The analysis showed that the data did not meet the requirement of variance homogeneity even after square root transformation. Therefore, we used nonparametric Mann–Whitney U test to compare expression in adjacent parts of the hippocampus (dorsal vs. intermediate and intermediate vs. ventral) in case of molecular markers of hippocampal subdivisions and between hippocampus and control tissue in case of molecular marker of choroid plexus. Data analysis was performed with Statistica software, release 7.1. Values are presented as mean ± SEM (column bar graphs) and scatter plots.

3. Results

3.1. Expression of Choroid Marker Gene Ttr

Real-time PCR analysis revealed very low expression of the Ttr gene in all parts of the hippocampus (dorsal, intermediate and ventral; $n = 9$) in contrast to the control tissue ($n = 5$) composed of brain tissue located in front of the hippocampus and part of the cortex

overlying the hippocampus (Figure 5). Mean expression was about 400 to 700 times higher in the control tissue compared with subdivisions of dissected hippocampi. The differences were significant for all parts of the hippocampus compared with the control ($p = 0.003$).

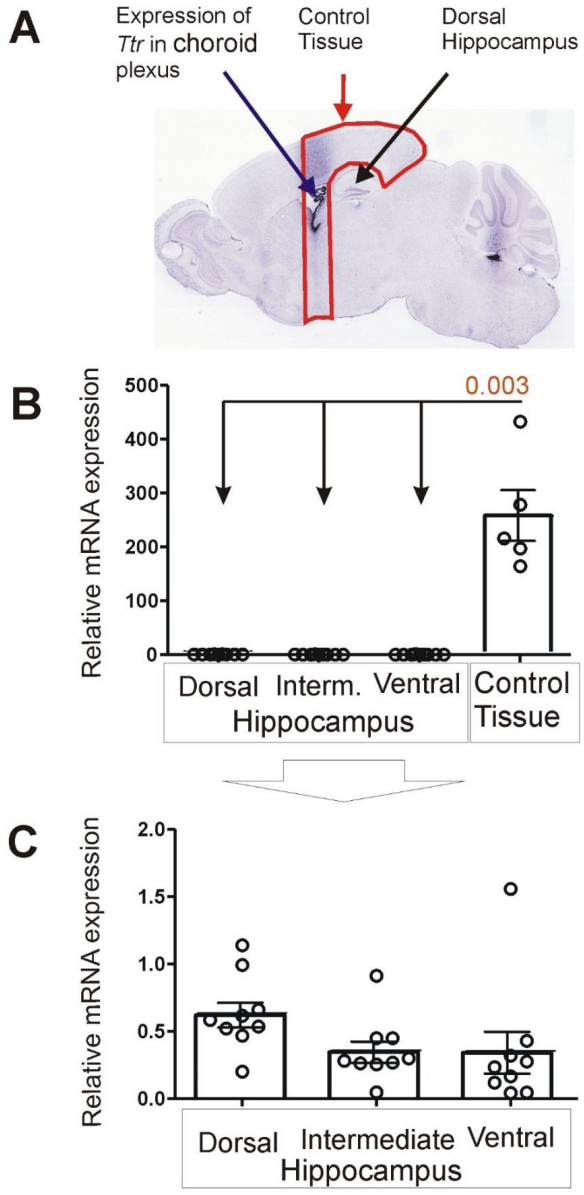

Figure 5. Brain expression of *Ttr*. (**A**) Pattern of expression in choroid plexus and dorsal hippocampus (ISH image) derived from Allen Mouse Brain Atlas (https://mouse.brain-map.org/, accessed on 23 June 2021) [13]. (**B**) Results of PCR analysis performed on control tissue and dissected samples of dorsal, intermediate and ventral hippocampus. (**C**) Details of *Ttr* expression in hippocampal subdivisions presented with altered scale at the Y axis. Data are presented as mean ± SEM (column bar graphs) overlaid on scatter plots.

3.2. Expression of Marker Genes Differentiating between Dorsal and Ventral Hippocampus

Real-time PCR analysis showed that expression of *Lct* was highest in the dorsal hippocampus ($n = 9$) and lowest in the ventral hippocampus ($n = 9$), while the intermediate part ($n = 9$) displayed an intermediate level of expression (Figure 6). The differences between adjacent parts of hippocampus (dorsal vs. intermediate and intermediate vs. ventral) were significant with $p = 0.0003$.

The *Trhr* gene displayed an opposite pattern of expression characterized by the highest level of expression in the ventral hippocampus ($n = 9$) and lowest in the dorsal hippocampus

(n = 7) as indicated by the real-time PCR analysis (Figure 7). In the case of the two samples from the dorsal hippocampus, we have obtained negative results for all repeats of the PCR analysis (triplicate). Because of an uncertainty about the reasons for the negative results (technical error vs. lack of expression), we omitted these two samples from the statistical analysis, reducing the total number of samples to seven. The intermediate part (n = 9) displayed an intermediate level of expression of *Trhr*. The differences were significant, with p = 0.0009 (dorsal vs. intermediate part) and p = 0.0003 (intermediate vs. ventral part).

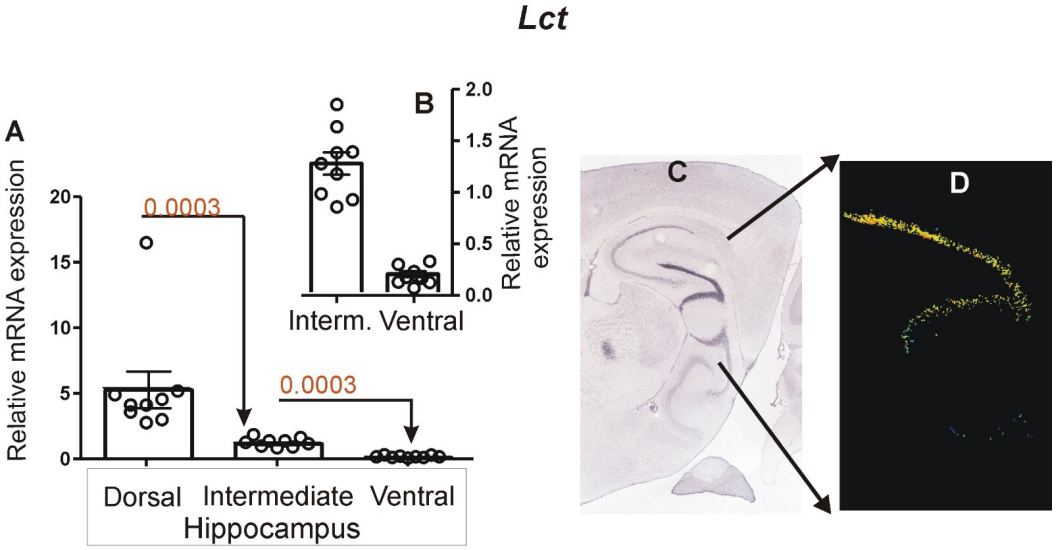

Figure 6. Hippocampal expression of *Lct*. (**A**) Results of PCR analysis performed on dissected samples of dorsal, intermediate and ventral hippocampus. (**B**) Details of *Lct* expression in intermediate and ventral parts presented with altered scale at the Y axis. (**C**) Pattern of expression in brain slice (ISH image) containing all hippocampal subdivisions derived from Allen Mouse Brain Atlas (https://mouse.brain-map.org/, accessed on 24 June 2021) [13]. (**D**) Expression detection mask retrieved from Allen Mouse Brain Atlas showing dorsal hippocampus with highest *Lct* expression. Data are presented as mean ± SEM (column bar graphs) overlaid on scatter plots.

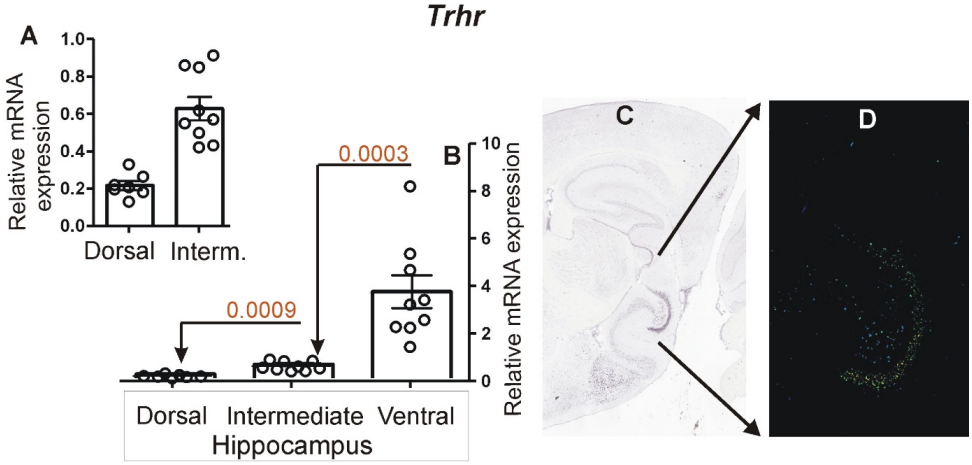

Figure 7. Hippocampal expression of *Trhr*. (**A**) Details of *Trhr* expression (PCR) in dorsal and intermediate parts presented with an altered scale at the Y axis. (**B**) Results of PCR analysis performed on dissected samples of dorsal, intermediate and ventral hippocampus. (**C**) Pattern of expression in a brain slice (ISH image) containing all hippocampal subdivisions derived from Allen Mouse Brain Atlas (https://mouse.brain-map.org/, accessed on 24 June 2021) [13]. (**D**) Expression detection mask retrieved from Allen Mouse Brain Atlas showing mediodorsal hippocampus with highest *Trhr* expression. Data are presented as mean ± SEM (column bar graphs) overlaid on scatter plots.

4. Discussion

Our dissection method is different than most of the other gross dissection protocols that require initial separation of hemispheres before exposition of the hippocampus [16–21]. Most of these protocols also require complete removal or displacement of the brainstem and diencephalon (thalamus and hypothalamus) to access the hippocampus from the inside of the mouse or rat brain (Table 2). As a result, dissection of the hippocampus is preceded by disintegration of the brain, which makes such dissection methods less intuitive and obscures the spatial relationships between the hippocampus and the rest of brain. In contrast, our method relies on removal of the cortex starting from the dorsal part of the hippocampus before the separation of the hemispheres and therefore makes it easy to observe spatial relationships in brain anatomy (Figure 3, Supplementary Figure S1 and Supplementary Video S2 File). In general principle, our protocol resembles the approach used previously by Spijker [22], although the details of these protocols are different in many respects. The unique feature of our protocol is the angled cuts used to separate the right and left hippocampus (Figure 4) without collection of the choroid plexus located between them in the third ventricle (Figure 4). Other protocols apply a single cut along the midline of the brain [16–21] or disrupt this part with forceps [22] without removal of tissues located between hippocampi. The second distinctive feature of our protocol is the usage of a stream of water to minimize damage or distortion of hippocampi at the time of the tissue separation and to remove potential tissue contaminations. Finally, we put a special emphasis on the removal of all remnants of white matter along the edges of hippocampi as a part of an effort to remove tissue contamination that can be contributed to, for example, by fimbria with the adjacent choroid plexus (Figure 4). To verify the effectiveness of our protocol, we used the transcriptomic marker of the choroid plexus [8,9]. The PCR analysis showed that the transthyretin gene (*Ttr*) is expressed at a residual level in hippocampal samples that display an mRNA level several hundred lower than adjacent control tissue colocalized with the choroid plexus (Figure 5) consistently with our previous assessment based on analysis of brain slices [9]. This indicates that the applied method for dissecting hippocampus from fresh brain allows for replicable removal of majority of choroid plexus from hippocampal samples. Therefore, the presented dissection method is especially suitable for molecular studies performed on homogenized tissues that are sensitive to contamination [9–11]. Such tissue contamination can be responsible not only for false positive findings that are present in many published datasets but may also obscure genuine changes in expression of some genes shared between tissues [11]. Importantly, no other gross dissection protocol for mice or rats (Table 2) has tested the presence of contaminations in collected samples, and most of these papers [16–20,22] do not even mention the fact that the choroid plexus is in dissected brain tissue. This issue is also neglected in protocols describing free-hand dissection of rat and mouse hippocampi from brain slices [23,24]. The only available alternative that was proved to be effective in the removal of the choroid plexus is laser microdissection [8].

Table 2. Summary of available protocols for gross dissection of hippocampus or its parts in rodents.

Author	Entire Hippoc.	Subparts	Species	General Dissection Strategy	Video	Application
[16]	Yes	No	Rats	Separation of hemispheres and removal of the brainstem/diencephalon to expose lateral ventricle and medial side of the hippocampus	Yes	General
[18]	Yes	No	Rats	Separation of hemispheres and displacement of the brainstem/diencephalon preceding the exposition of lateral ventricle and medial side of the hippocampus	Yes	Electro-physiol

Table 2. *Cont.*

Author	Entire Hippoc.	Subparts	Species	General Dissection Strategy	Video	Application
[17]	No	Dentate gyrus	Mice	Separation of hemispheres and removal of the brainstem/diencephalon to expose the lateral ventricle and medial side of the hippocampus	Yes	General
[20]	Yes	No	Mice	Separation of hemispheres and removal of the brainstem/diencephalon to expose the lateral ventricle and medial side of the hippocampus	Yes	Electro-physiol
[21]	Yes	No	Mice	Separation of hemispheres and eversion of the lateral ventricle	Yes	General
[19]	Yes	CA1, CA3 and Dentate gyrus	Mice	Separation of hemispheres and removal of occipital cortex starting from lateral side of the brain	No	General
[22]	Yes	No	Mice/ Rats	Removal of occipital cortex starting from the dorsal part of the brain	No	General

It should be noted that some research methods may not be affected by contamination. An example is electrophysiology performed on slices, which is associated with the precise localization of electrodes under microscopic control together with the characterization of the electrical properties of recorded cells. In fact, precise removal of the choroid plexus before electrophysiological experiments is a waste of time. Instead, crucial factors for such studies are the speed of dissection determining the viability of cells and the preservation of specific neural circuits. Therefore, the selection of dissection approach depends on the aim of the experiment, and some available protocols were designed specifically for electrophysiology performed in mouse and rat hippocampal slices with different planes of cutting [18,20,25,26]. Some of these protocols use larger parts of the mouse [18,25] or rat [26] brain to cut slices containing both the hippocampus and surrounding tissues.

We also showed that our dissection method is convenient for separating dorsal, intermediate and ventral hippocampus. This is important because of functional and transcriptomic differences between these subdivisions [1,12,27,28]. Some special aspects of our protocol make it especially suitable for this purpose. Gross dissections applied previously relied on cutting the hippocampus into three equal parts corresponding to dorsal, intermediate and ventral subdivisions [27] or just into two parts without precise specification of borders between them [28]. Importantly, a precise separation of these subdivisions depends on dissection of the entire hippocampus and preservation of its size. This is crucial because fresh brain tissue is soft and malleable and therefore can be easily damaged or distorted during dissection, leading to serious errors at a time when different parts of hippocampus are attributed to subdivisions observed in an intact brain. Therefore, we are not using forceps or tweezers to prevent changes in the shape and length of the hippocampus. Instead, we rely on using a stream of water to separate hippocampus from adjacent brain areas. Second, the dorsal and ventral part can be easily mistaken when the hippocampus is removed from the rest of the brain and manipulated for removal of remnants of other tissues. This problem is avoided in our protocol because we apply cuts that are perpendicular to the longitudinal axis of the hippocampus (Supplementary Figure S1R) to separate its dorsal part from tissues located in the third ventricle (Figure 4). Additionally, these cuts differentiate between the dorsal and ventral ends of dissected hippocampi (Supplementary Figure S1AF) and therefore facilitate dissection of hippocampal subdivisions. The sufficient precision of this approach has been confirmed by the expression of *Lct* and *Trhr* genes (Figures 6 and 7) that are known as molecular markers of the dorsal and ventral hippocampus identified previously in brain slices [1,12]. It means that our gross dissection of fresh brain recapitulated observations of differential gene expression detected in brain slices [1,12]

It should be noted that our protocol can be easily combined with dissection methods relying on cutting slices from fresh brain with tissue choppers or special forms to obtain various brain regions [23,29]. Alternatively, some gross dissection protocols for collection of multiple brain areas can also be included [16,21,22]. Such a combination of methods will allow for dissection of additional brain areas located between the frontal pole and the hippocampus. A special emphasis on the removal of tissue contamination and avoidance of tissue distortions makes our protocol especially suitable for molecular experiments performed either on the entire hippocampus or its subdivisions. Therefore, this provides an alternative to laser microdissection from frozen slices [8], which requires expensive equipment and is time consuming.

Supplementary Materials: The following supporting information can be downloaded at: https://zenodo.org/record/6515868#.YnJCb4fP2DJ (Doi:10.5281/zenodo.6515868); Video S1—procedure for removal of mouse brain; Video S2—procedure for collecting hippocampus; Figure S1—procedure for collecting hippocampus.

Author Contributions: Conceptualization, G.R.J. and A.J.; methodology, G.R.J., A.M.S. and A.J; formal analysis, A.M.S., G.R.J. and A.J.; investigation, A.J.; writing—original draft preparation, G.R.J.; funding acquisition, G.R.J. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Science Centre (grant number 2017/27/B/NZ2/02796).

Institutional Review Board Statement: The study was performed on spare tissues collected during project 2017/27/B/NZ2/02796 that was performed with the permission of the Second Local Ethical Committee in Warsaw (permit number: WAW2/090/2018) and in accordance with the Polish Act of 15 January 2015 on the protection of animals used for scientific and educational purposes and the 3Rr principle.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data are provided in the form of scatter plots in the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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International Journal of
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Overnight Corticosterone and Gene Expression in Mouse Hippocampus: Time Course during Resting Period

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Abstract: The aim of the experiment was to test the effect of an elevated level of glucocorticoids on the mouse hippocampal transcriptome after 12 h of treatment with corticosterone that was administered during an active phase of the circadian cycle. Additionally, we also tested the circadian changes in gene expression and the decay time of transcriptomic response to corticosterone. Gene expression was analyzed using microarrays. Obtained results show that transcriptomic responses to glucocorticoids are heterogeneous in terms of the decay time with some genes displaying persistent changes in expression during 9 h of rest. We have also found a considerable overlap between genes regulated by corticosterone and genes implicated previously in stress response. The examples of such genes are *Acer2*, *Agt*, *Apod*, *Aqp4*, *Etnppl*, *Fabp7*, *Fam107a*, *Fjx1*, *Fmo2*, *Galnt15*, *Gjc2*, *Heph*, *Hes5*, *Htra1*, *Jdp2*, *Kif5a*, *Lfng*, *Lrg1*, *Mgp*, *Mt1*, *Pglyrp1*, *Pla2g3*, *Plin4*, *Pllp*, *Ptgds*, *Ptn*, *Slc2a1*, *Slco1c1*, *Sult1a1*, *Thbd* and *Txnip*. This indicates that the applied model is a useful tool for the investigation of mechanisms underlying the stress response.

Keywords: glucocorticoids; corticosterone; brain; hippocampus; mice; transcriptomics; microarrays; gene expression; circadian cycle

Citation: Jaszczyk, A.; Stankiewicz, A.M.; Goscik, J.; Majewska, A.; Jezierski, T.; Juszczak, G.R. Overnight Corticosterone and Gene Expression in Mouse Hippocampus: Time Course during Resting Period. *Int. J. Mol. Sci.* **2023**, *24*, 2828. https://doi.org/10.3390/ijms24032828

Academic Editors: Cristoforo Comi, Benoit Gauthier, Dimitrios H. Roukos and Alfredo Fusco

Received: 22 December 2022
Revised: 17 January 2023
Accepted: 18 January 2023
Published: 1 February 2023

1. Introduction

Glucocorticoids (GCs) are important in medicine for many different reasons. First, they constitute a crucial component of the stress response system [1] and are implicated in mechanisms underlying stress-related disorders such as posttraumatic stress disorder [2–4] and depression [5–7]. Second, glucocorticoids are commonly used in medicine due to their potent anti-inflammatory properties [8]. Finally, excessive levels of endogenous or exogenous glucocorticoids lead to Cushing's syndrome, characterized by a set of metabolic, physiological, cognitive and psychiatric symptoms [9–11]. However, despite their importance in medicine, there are considerable gaps in our understanding of the mechanism mediating the effect of glucocorticoids on brain metabolism and physiology [12,13]. One of the most important problems is that previous experiments were focused on responses to glucocorticoids during the first 2 to 3 h after acute treatments while longer latencies are commonly neglected [12,13]. However, few available studies testing responses at multiple time points after treatment in astrocytic cell cultures [14] and liver [15] show that most of the transcriptomic responses to glucocorticoids appear at least 4 h–6 h or later after the treatment, which is consistent with delayed proteomic [15,16] and metabolic effects [17] peaking at about 7 h–12 h after the treatment. This delayed buildup

of transcriptomic responses results from the fact that glucocorticoid receptors regulate the expression of numerous transcription factors and other regulatory molecules that lead to secondary effects [13]. The second issue is that the bulk of available transcriptomic data was obtained in in vitro cell cultures derived from perinatal brain tissue [13]. Such data are difficult to interpret because of profound physiological and metabolic differences between a developing and mature brain. The developing brain undergoes fast growth of cells governed by complex transcriptomic programs and specialized between-cell communication guiding the elongation of cellular protrusions and shaping connections between cells. In contrast, growth is severely restricted in the mature intact brain that specializes at this stage in the processing and integration of information and the complex regulation of transport between vascular and neuronal compartments. Additionally, the cell cultures are devoid of structure and components typical for the brain, including neurovascular units and distinct layers of cells having highly specialized local and long-distance connections with cells releasing various neurotransmitters. Finally, cell cultures are devoid of the contexts created by multiple hormonal systems scattered across the body and sleep-waking cycles orchestrating the functions of the entire organism. These methodological issues are further complicated by the problems inherent to past transcriptomic studies that are based on a small number of samples that were commonly pooled to decrease the costs of analyses [13,18]. As a result, available transcriptomic data suffer from low statistical power, leading to a large proportion of false positive and negative findings [13,18].

To fill the existing gaps in knowledge and to overcome limitations associated with past experiments, we designed an in vivo experiment to test the effect of corticosterone administered for 12 h during the period of circadian activity associated with the light–dark cycle. Furthermore, we also tested the dynamics of transcriptomic effects during the resting period when the level of corticosterone in mice returns to the baseline. As a result, we gained an insight into processes taking place in the brain after day-long stress or medication with glucocorticoids and during a subsequent resting period associated with the light–dark cycle. As far as we know, there are no other comparable studies. We also used relatively large groups (n = 8) that in combination with multiple time points and lack of pooled samples provide a large transcriptomic dataset (total n = 48) which is rarely encountered in transcriptomic studies.

2. Results

2.1. Blood Corticosterone and Glucose

Animals that received corticosterone in drinking water during the active period (dark phase) displayed an increased level of corticosterone at the beginning of the light phase (first hour/Figure 1A). The corticosterone returned to the baseline during the fifth hour of the resting period and remained at this level during the ninth hour in corticosterone-treated mice (Figure 1A,B). In contrast, control animals displayed a slight increase in corticosterone level during the last tested time point (ninth hour/Figure 1A,B). The data did not meet the requirement of variance homogeneity and, therefore, were analyzed with a nonparametric Mann–Whitney *U* test that showed significant differences between corticosterone-treated and control mice during the first (U = 2, n1 = n2 = 10, $p = 0.0003$) and ninth hour (U = 20, n1 = 9, n2 = 10, $p = 0.04$) and a lack of differences during the fifth hour of the resting period (U = 45, n1 = n2 = 10, $p = 0.7$). Differences between groups in the level of blood corticosterone were not associated with differences in water usage that includes both amounts of ingested water and spillage during the course of the experiment. The Mann–Whitney *U* test showed the lack of significant differences in water usage between control and corticosterone-treated mice during the first (U = 28, n1 = n2 = 10, $p = 0.096$), fifth (U = 27.5, n1 = n2 = 10, $p = 0.089$) and ninth (U = 42, n1 = 9, n2 = 10, $p = 0.81$) hour.

The blood level of glucose in corticosterone-treated and control mice (Figure 1C) was similar during the first hour of the resting period but displayed a gradual decrease in

corticosterone-treated mice during subsequent time points (fifth and ninth hour). ANOVA revealed a significant effect of treatment [F(1,53) = 31.59, $p < 0.0001$] and a significant interaction between treatment and time of sample collection [F(2,53) = 5.49, $p = 0.007$] with significant differences between corticosterone and control groups during the fifth ($p = 0.002$) and ninth hour ($p < 0.0001$) of the resting period as indicated by the post hoc Fisher's LSD test.

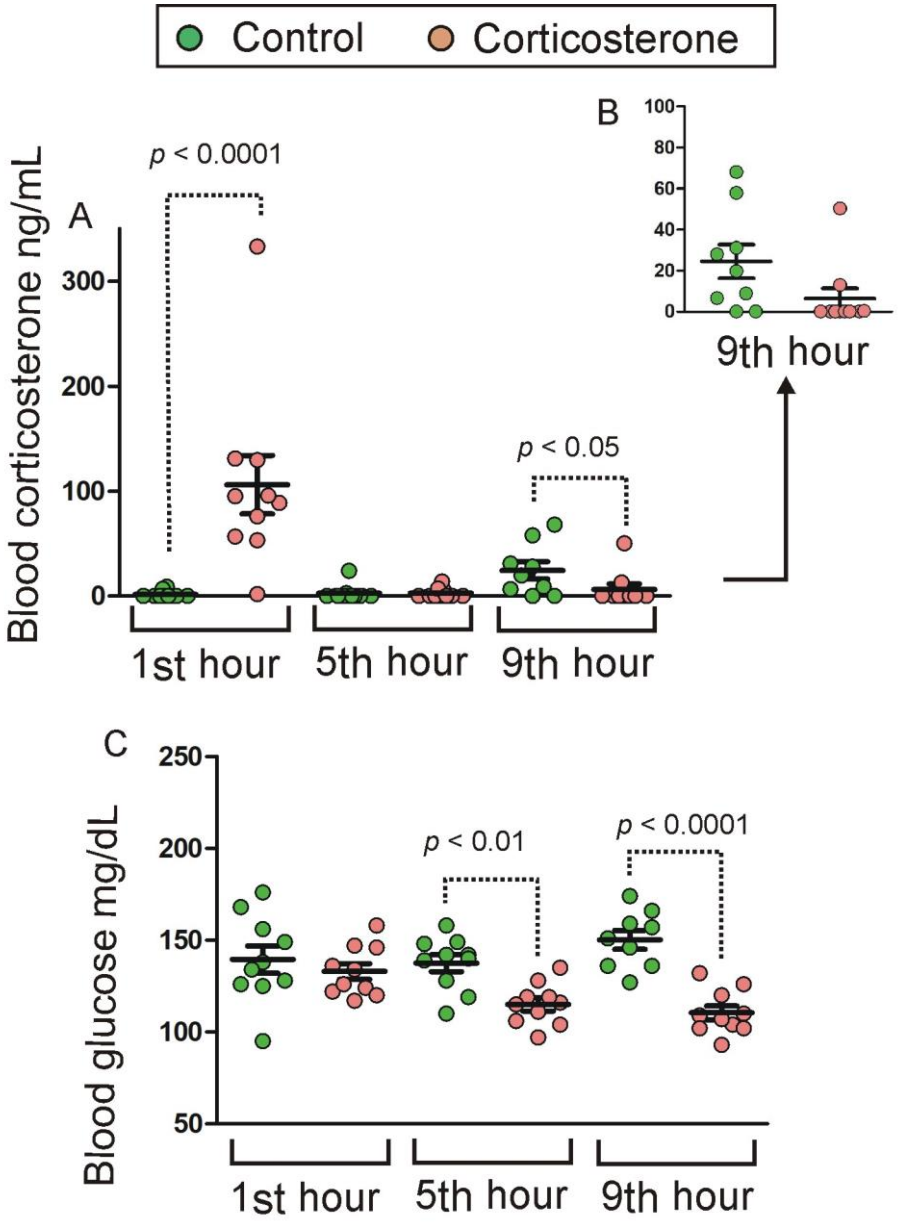

Figure 1. Effect of overnight corticosterone administered in drinking water on the blood level of corticosterone (**A**,**B**) and glucose (**C**) during the resting period occurring during the light phase. Data are presented as mean ± SEM overlayed on scatter plot of individual values. (**B**) part of data from panel (**A**) (ninth hour) shown with altered scale of Y axis.

2.2. PCR Validation of Microarray Results

Initially, we selected six genes (*Sult1a1*, *Lao1*, *Etnppl*, *Apoc3*, *Plin4* and *Pla2g3*) for validation but for two of them (*Plin4* and *Pla2g3*) we were not able to design proper starters because they yielded additional products. Therefore, the final validation was performed for *Sult1a1*, *Lao1*, *Etnppl* and *Apoc3*. While the selection of genes was based on significant effects observed for specific probes: A 55 P2117155 (*Apoc3*), A 51 P391616

(*Etnppl*), A 55 P2101021 (*Lao1*), A 55 P2005475 (*Sult1a1*) and A 51 P321341 (*Sult1a1*), the results of PCR analysis were compared with all microarray probes annotated to selected genes (Figure 2). The calculation of correlations shows high congruence between PCR results and initially selected microarray probes (Figure 2A–D,G) indicating that microarrays reliably detected a level of validated genes. Similar conclusions are drawn from between-group comparisons. The PCR analysis showed increased expression of *Sult1a1* and *Lao1* in all tested time points (Figure 3) with a *p*-value < 0.001 as indicated by the Mann–Whitney *U* test (U = 1, n1 = n2 = 8) and the same pattern of expression has been found in microarray data (File S1).

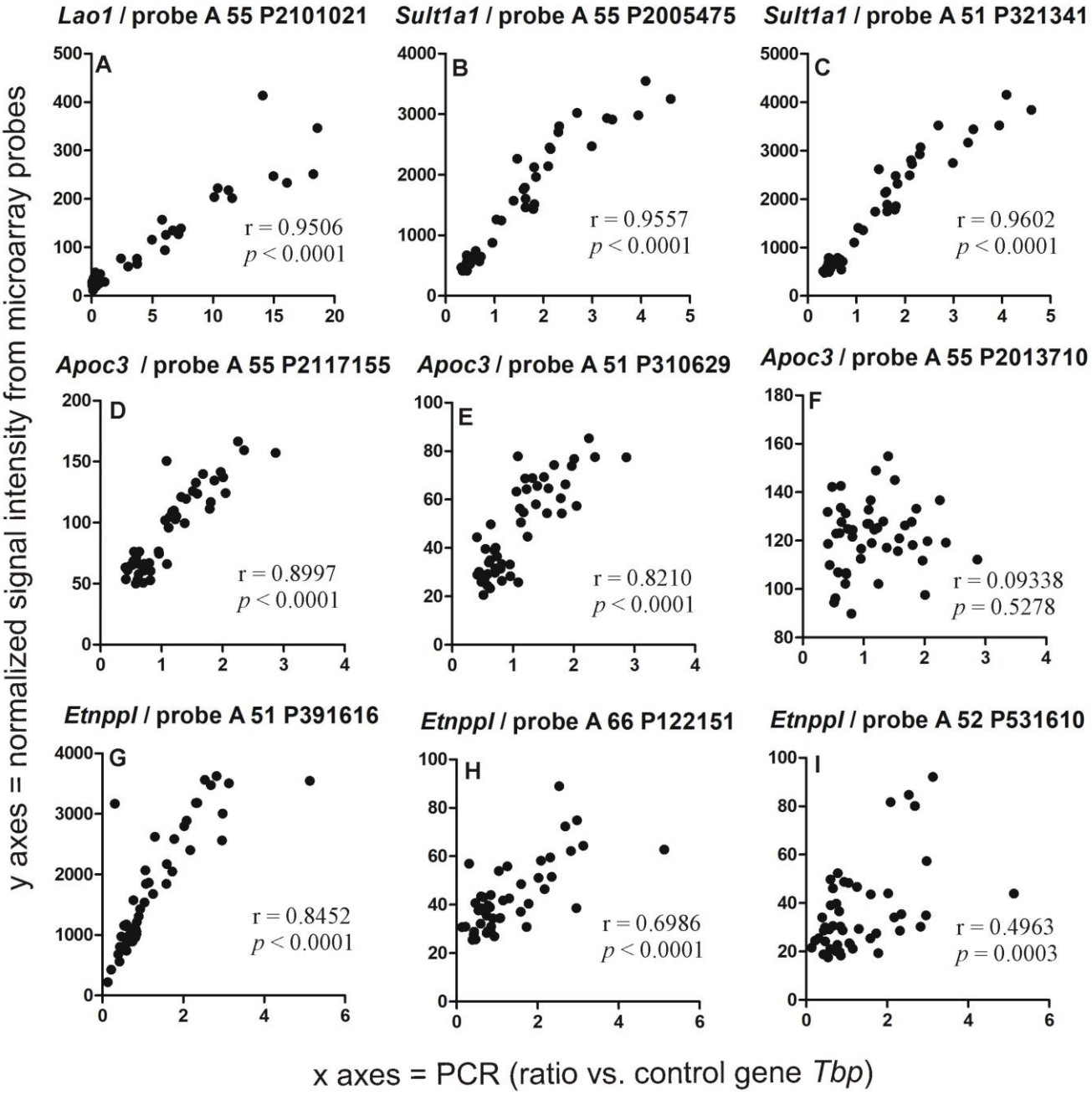

Figure 2. Correlation between results obtained with PCR and microarrays calculated separately for each probe annotated to tested genes *Lao1* (**A**), *Sult1a1* (**B**,**C**), *Apoc3* (**D**–**F**) and *Etnppl* (**G**–**I**). The microarray signal was normalized and decomposed into single channels as described in the methods section. r—Pearson's correlation coefficient.

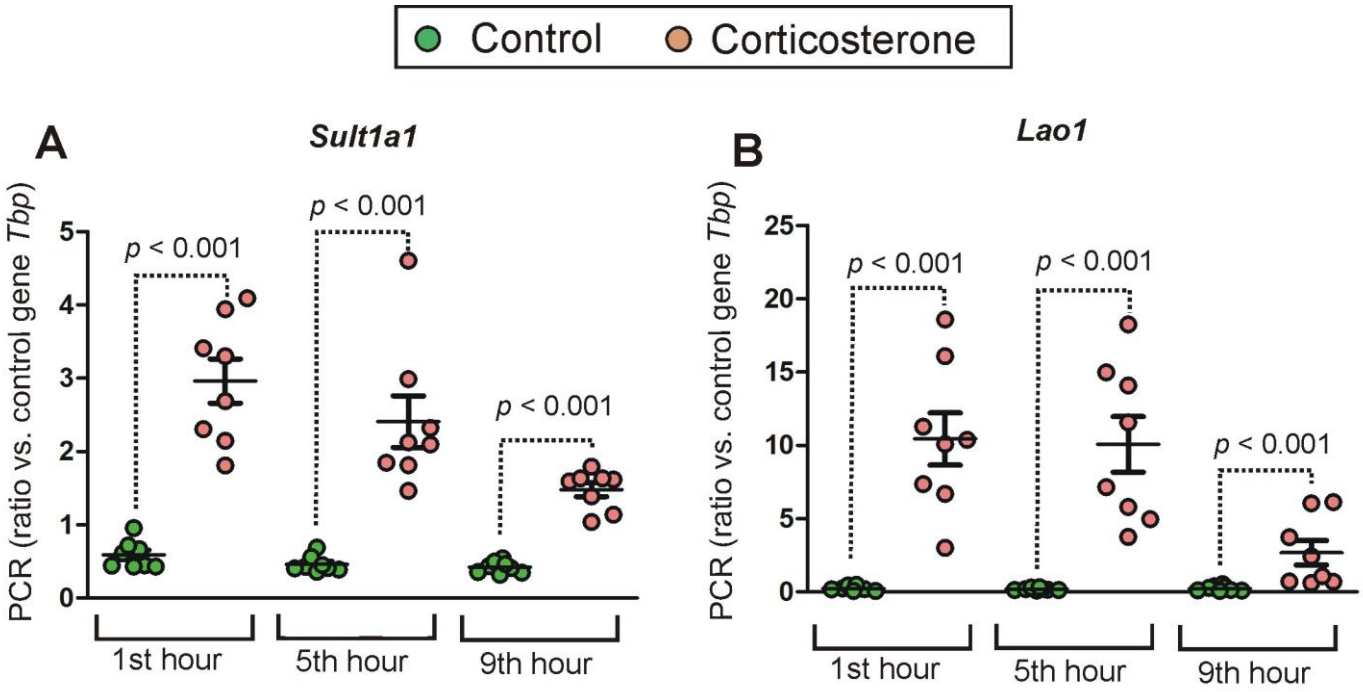

Figure 3. Expression pattern of *Sult1a1* (**A**) and *Lao1* (**B**) revealed by PCR. Data are presented as mean ± SEM overlayed on scatter plot of individual values. *p* values were determined based on Mann–Whitney U test.

Additionally, the analysis shows variability between individual probes in their ability to detect the expression of annotated genes. These differences were most striking in the case of the gene *Apoc3* (Figure 2D–F). Comparisons between groups (File S1) showed that the two best probes for detecting *Apoc3* (determined on the basis of correlation with PCR results/Figure 2D,E) detected similar changes between control and corticosterone-treated animals (Figure 4B,C). This finding is consistent with PCR results (Figure 4A) that revealed a significant effect of treatment [$F(1,42) = 129.78.49$, $p < 0.0001$] with significant differences between corticosterone and treatment groups during all tested time points ($p < 0.0001$, Fisher's LSD test). In contrast, the third probe that was not correlated with the PCR results (Figure 2F) failed to detect the effect of treatment (Figure 4D). To understand better the differences between probes that were initially annotated to the same gene, we retrieved additional information on detected transcripts from the Ensembl/BioMart database. These data revealed that the probes detect various variants of the *Apoc3* transcript and that the best-correlated probes (Figure 2D,E) share the ability to detect the Ensembl canonical transcript (Figure 4B,C) defined as a variant having the highest coverage of conserved exons, highest expression, longest coding sequence and represented in other key resources.

Even more complex patterns emerged in the case of the second gene (*Etnppl*) detected by multiple probes that provided highly discrepant results in terms of correlation with PCR (Figure 2G–I). Between-group comparisons of PCR data (Mann–Whitney *U* test) showed significant differences during the first ($U = 1$, $n1 = n2 = 8$, $p = 0.001$), fifth ($U = 9$, $n1 = n2 = 8$, $p = 0.016$) and ninth hour of the resting period ($U = 1$, $n1 = n2 = 8$, $p = 0.001$) (Figure 5A). Between-group comparisons of microarray data (File S1) showed that one probe detected an increased expression in all three time points (Figure 5B), consistent with PCR data. The second probe detected increases in two time points (Figure 5C). Finally, the last one detected increased expression in the first time point and an opposite effect in the last time point (Figure 5D) following, in fact, changes in the level of the blood corticosterone level (Figure 1A). Additionally, these probes displayed considerable differences in signal intensity (Figure 5B–D). Data retrieved from the Ensembl/BioMart

database showed that the probe detecting the Ensembl canonical transcript (Figure 5B) provided results displaying the highest correlation with the PCR while the lowest correlation was obtained in the case of the probe detecting only alternatively spliced transcripts considered to contain intronic sequences (Figure 5D). These results prompted us to retrieve Ensembl/BioMart data for all other probes available in this database.

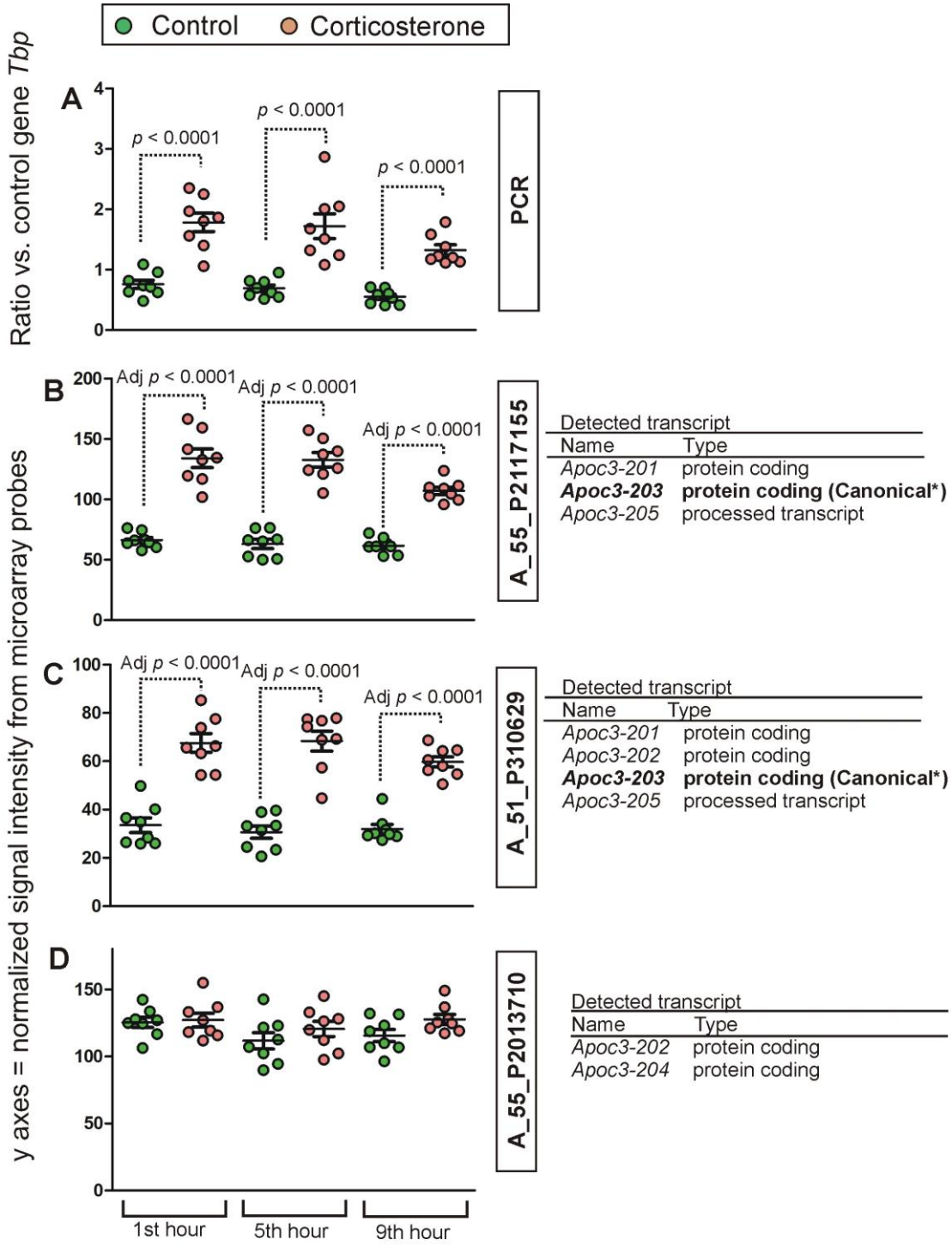

Figure 4. Between-group comparison of *Apoc3* expression detected with PCR (**A**) and different microarray probes (**B**–**D**). *p* values were determined based on LSD test of PCR results (**A**) and separate channel test applied for microarray data (**B**,**C**). The right panel provides information about transcript variants (Ensembl/BioMart database) that can be detected by the probes. *—Ensembl canonical transcript having the highest coverage of conserved exons, highest expression, longest

coding sequence and represented in other key resources, such as NCBI and UniProt. Definitions of transcript types are provided in File S2.

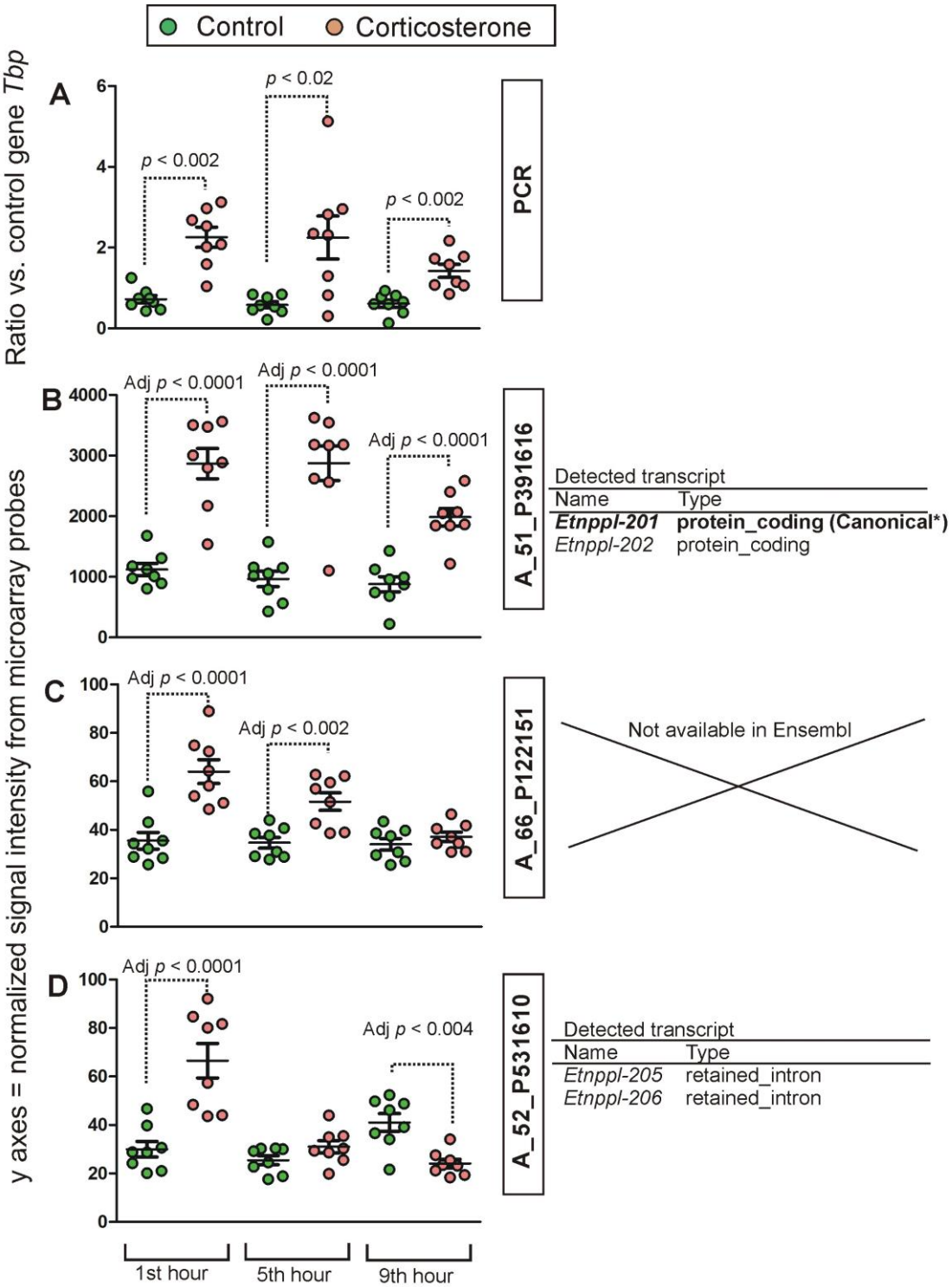

Figure 5. Between-group comparison of *Etnppl* expression detected with PCR (**A**) and different microarray probes (**B**–**D**). *p* values were determined based on Mann–Whitney U test applied to microarray PCR results (**A**) and separate channel test applied to microarray data (**B**,**C**). The right panel provides information about transcript variants (Ensembl/BioMart database) that can be detected by the probes. *—Ensembl canonical transcript having the highest coverage of conserved exons, highest expression, longest coding sequence and represented in other key resources, such as NCBI and UniProt. Definitions of transcript types are provided in File S2.

2.3. Transcriptomic Changes in Control Animals during the Resting Period

Circadian rhythms in gene expression are not the main objective of this study but provide a crucial context for changes in the expression of GC-responsive genes during the resting period (Figure 6). Therefore, we compared the transcriptome in control animals during consecutive time points of the light phase. The largest effect was found in the comparison between the first and the last time points (first hour vs. ninth hour) because 312 unique microarray probes displayed significant differences with an adjusted *p*-value < 0.05, and this comparison detected most of the significant findings (File S3). In contrast, only three microarray probes displayed significant differences in the comparison between the first and the second time points (first hour vs. fifth hour) while 11 probes differed between the second and the third time points (fifth hour vs. ninth hour). These additional comparisons revealed a few additional genes (adjusted $p < 0.05$) that were only specific for the comparison between the first and fifth hour (*Slc15a3*) or between the fifth and ninth hour (*Hrk* and *Cables1*).

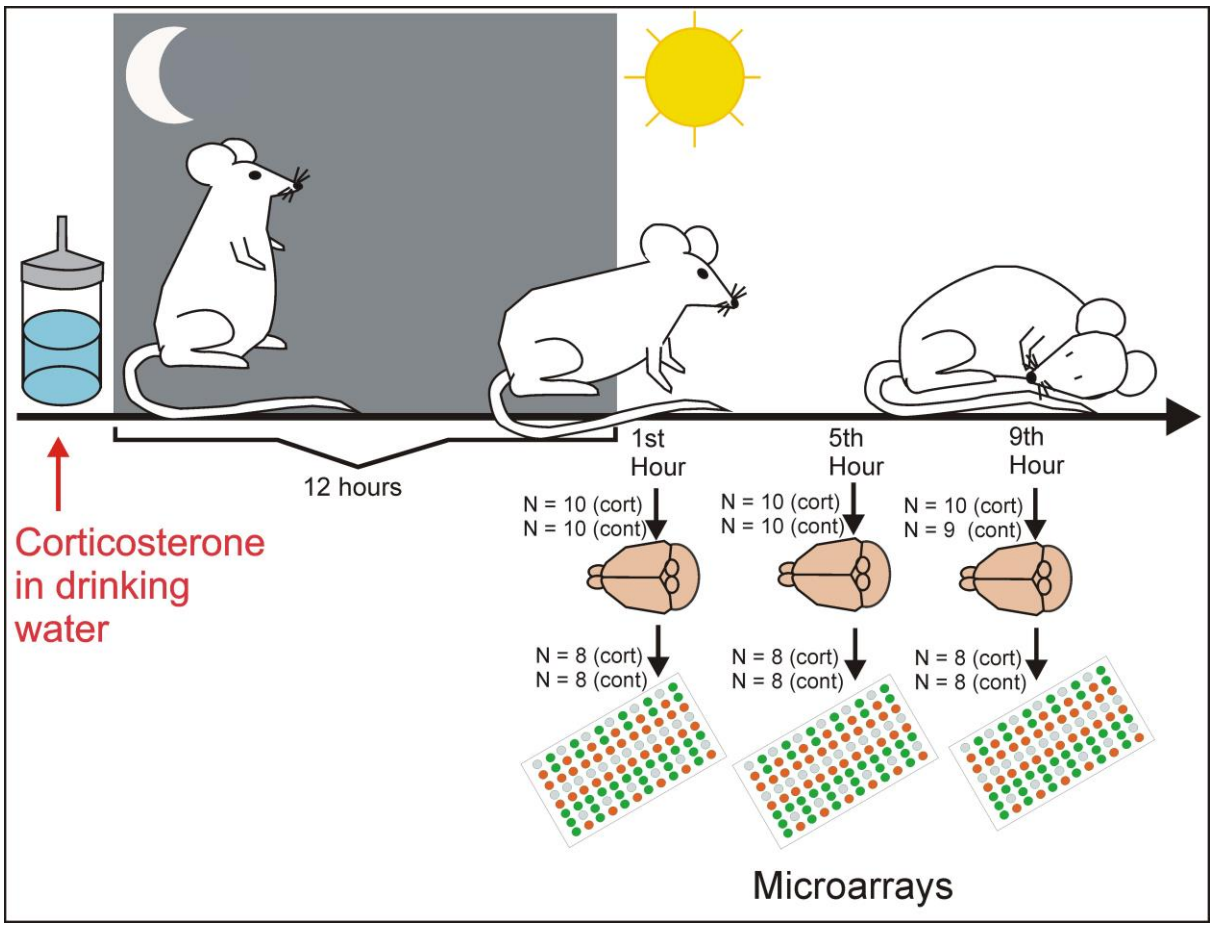

Figure 6. The design of the experiment. Mice received either corticosterone solution or vehicle at the end of the light phase, followed by 12 h of dark phase which is a period of mouse activity. The next day, the animals were sacrificed at three time points to collect brain samples for transcriptomic analysis performed with microarrays. For more details please see Section 4.2.

The 312 microarray probes displaying significant differences during the longest testing interval (first hour vs. ninth hour) included 11 probes that failed the annotation, two probes that were inconsistently annotated depending on the applied method, and 10 probes that can detect more than one gene. Data available in BioMart indicate that according to currently applied models almost 90% of probes displaying a time-dependent effect detect protein-coding transcripts, while the remaining 10% of probes detect lncRNAs (5.8%) or transcripts considered as dysfunctional (nonsense-mediated decay,

retained intron, protein coding loss-of-function variants, processed transcript and processed pseudogene). Importantly, the time-dependent changes in gene expression included genes known to be involved in the regulation of circadian rhythms such as *Nr1d1*, *Dbp*, *Ciart* (*Gm129*), *Arc* and *Fos* [19–22] confirming a pattern typical for the resting period in rodents. Examples of genes that changed the expression in control animals during the resting period are shown in Figure 7.

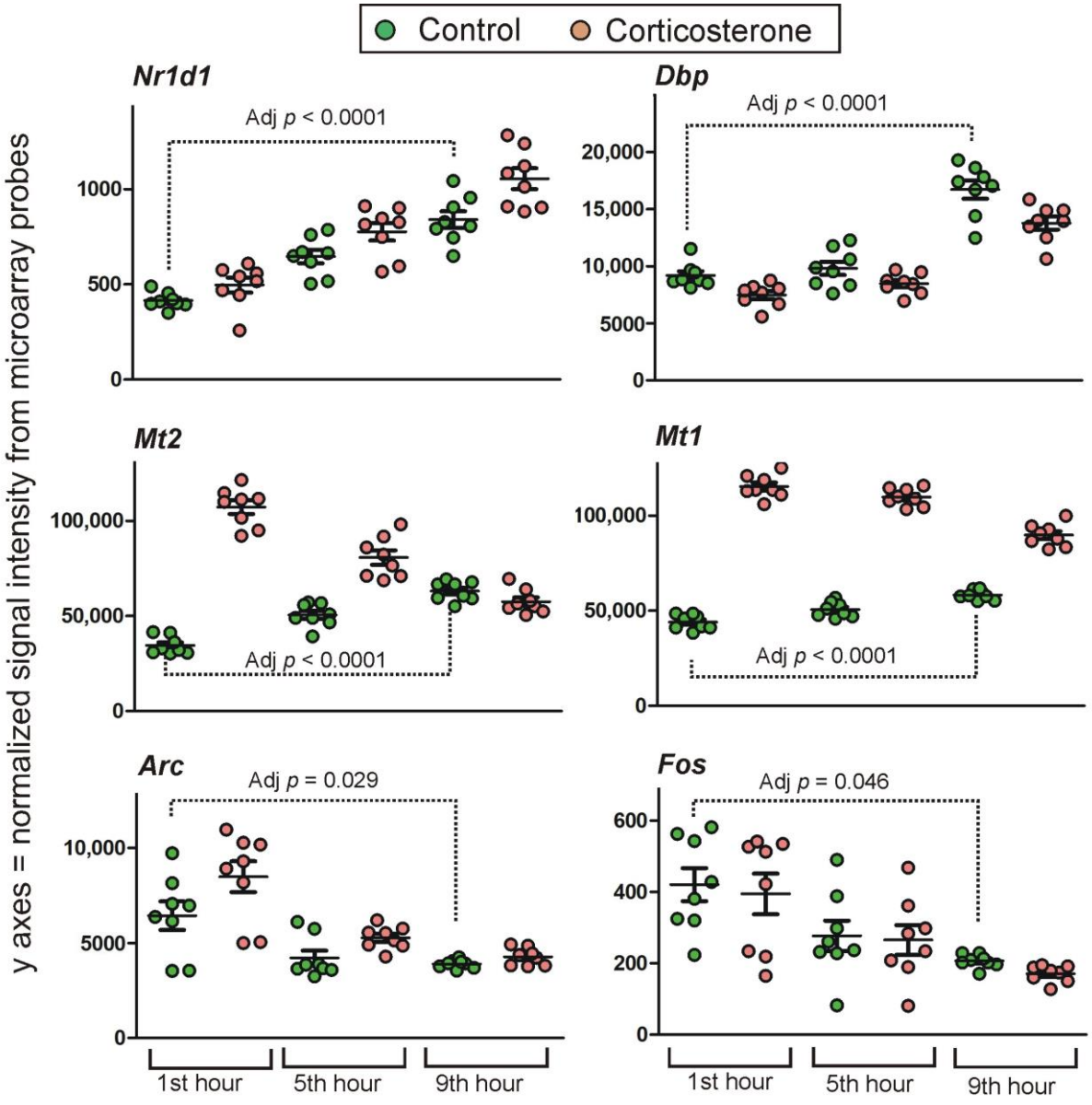

Figure 7. Selected genes displaying significant changes in control animals during the resting period. Y axis indicates probe signal intensity decomposed into green and red channel after final background correction, within-array and between-array normalization. All data are available in File S3.

2.4. Effect of Corticosterone—General Characteristics of Microarray Results

The statistical analysis revealed significant changes between corticosterone-treated and control animals in all three tested time points (File S1). In total, 17,444 unique probes indicated significant differences between groups during at least one tested time point while the remaining 39,161 probes provided only insignificant results. To differentiate gross regulatory mechanisms, we divided the microarray results into primary effects

(10,969 unique probes/Figure 8) that were already significant at the time of an elevated level of corticosterone (the first hour of the light phase) and secondary effects (6475 unique probes/Figure 9) that became significant during the fifth and seventh hour when the level of corticosterone returned to the baseline in corticosterone-treated animals.

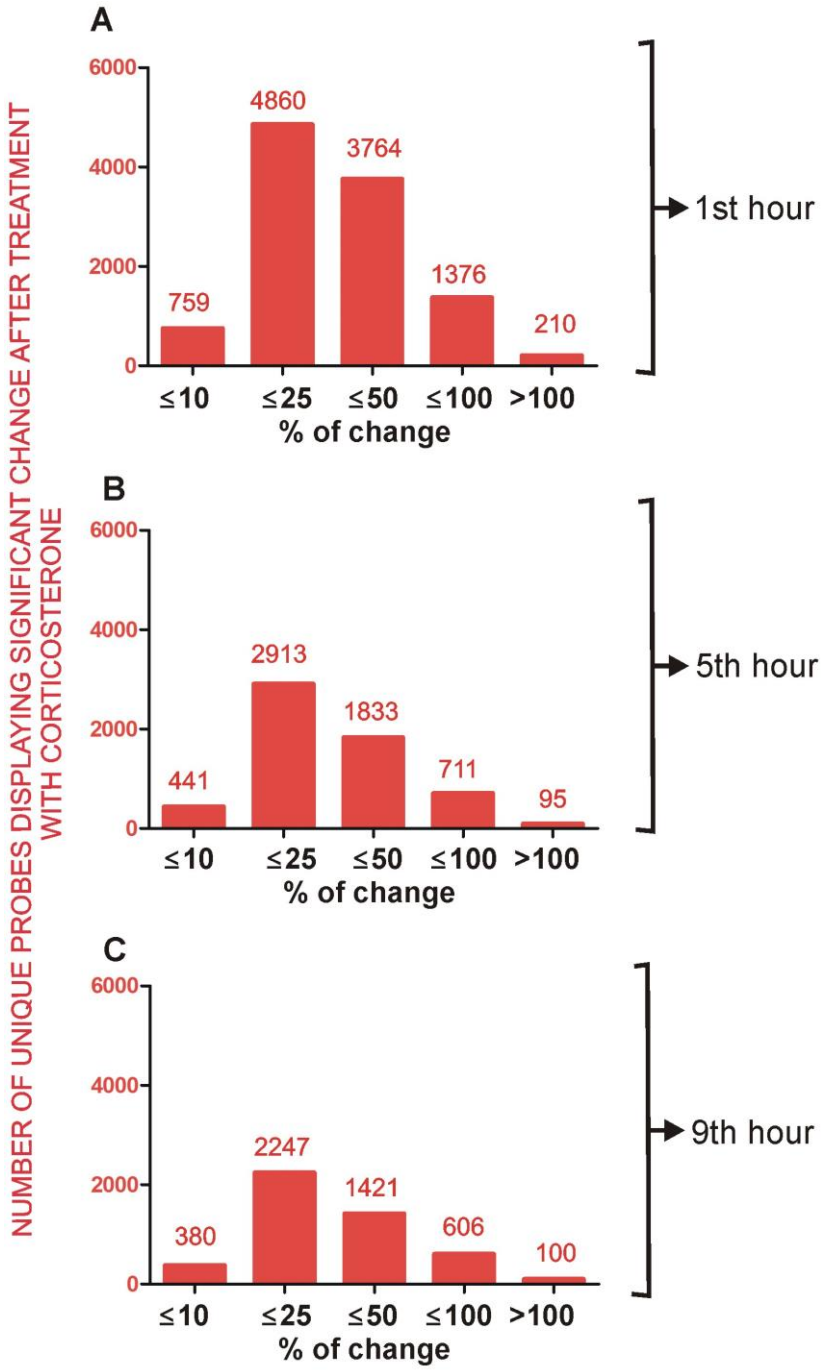

Figure 8. General characteristics of primary effects of corticosterone that are significant at least during the first hour of the resting period. (**A**)—number of unique probes that indicated significant differences during the first hour of the resting period. (**B**)—number of unique probes that indicated significant differences during the first and the fifth hour of the resting period. (**C**)—number of unique probes that indicated significant differences during the first, fifth and ninth hour of the resting period. Numbers above the bars indicate an exact number of unique probes showing significant differences between groups separately for categories based on the magnitude of altered expression. All data are available in File S1.

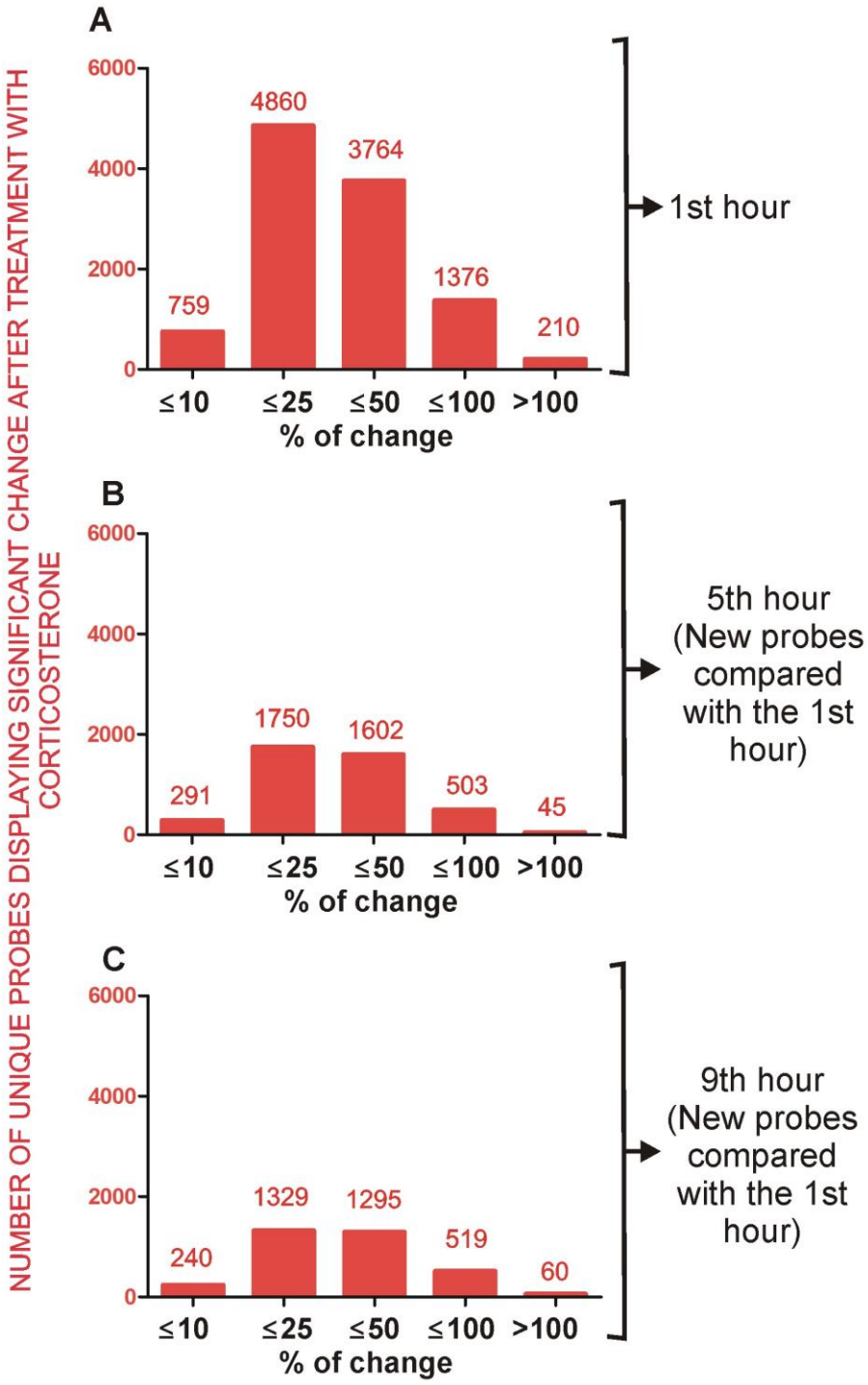

Figure 9. General characteristics of secondary effects of corticosterone characterized by significant differences occurring only during the fifth and/or the ninth hour of the resting period (**B**,**C**) in comparison with primary effects shown in panel (**A**). (**A**)—number of unique probes that indicated significant differences during the first hour of the resting period representing primary effects. (**B**)—number of unique probes that indicated the lack of effect of corticosterone during the first hour and significant differences during the fifth hour of the resting period. (**C**)—number of unique probes that indicated the lack of effect of corticosterone during the first hour and significant differences during the ninth hour of the resting period. Numbers above the bars indicate an exact number of unique probes showing significant differences between groups separately for categories based on the magnitude of altered expression. All data are available in File S1.

2.4.1. Primary Effects

The comparison between corticosterone-treated and control animals sacrificed during the first hour (Figure 8A) revealed almost 11,000 unique probes that differed between groups although half of them (51.2%) displayed small changes that were not larger than 25% (absolute value of log2 fold change ≤ 0.32). In contrast, there were 3764 probes differing within the range of 25% and 50% (absolute value of log2 fold change > 0.32 and ≤0.58), 1376 probes differing within the range of 50% and 100% (absolute value of log2 fold change > 0.58 and ≤1) and only 210 probes displaying differences larger than 100% (absolute value of log2 fold change > 1). The primary effects decreased over time. After 9 h of rest, the number of probes displaying a significant effect of corticosterone decreased to 43% of all significant effects observed during the first hour (Figure 8C).

Long-Lasting Primary Effects

Some primary effects were maintained for the entire testing period (first, fifth and ninth hour) despite the fact that elevated levels of glucocorticoids were observed only during the first hour in corticosterone-treated mice compared with the control group. In total, 3670 probes displayed significant differences during all three tested time points with the same direction of changes between groups. Some microarray probes were not specific to single genes according to the BioMart/Ensembl database (145 probes) or were inconsistently annotated by different databases (74 probes). After the removal of such probes, there were 3451 probes specifically annotated to 3144 genes. Most of the genes code proteins although there were also lncRNAs (154), miRNA (1) lincRNA (2), rRNA (1), TEC (To be Experimentally Confirmed) genes (14), 169 pseudogenes (4.9%) and 96 dysfunctional transcripts (2.8%) classified in the Ensembl database as processed transcripts, transcripts retaining introns, nonsense mediated decay and antisense transcripts (definitions are provided in File S2).

The majority of probes (2991) indicated small (<25%) and medium (<50%) differences between groups. Differences larger than 100% (log2 fold change > 1) after 9 h of rest were indicated by only 77 probes annotated to 71 genes. Furthermore, the majority of them displayed very low signal intensity (mean < 50). After the rejection of these probes, the group was restricted to seven protein-coding genes (*Etnppl*, *Sult1a1*, *Heph*, *Pygm*, *Pla2g3*, *Clcnka* and *Lao1*). It should be noted that in the case of *Etnppl* (Figure 5), *Pygm*, *Pla2g3* and *Heph* (File S1) the effect was specific for some variants of the transcripts. Importantly, a prolonged expression of *Etnppl*, *Pygm*, *Pla2g3* and *Heph* was detected by probes binding canonical variants of transcripts. Smaller but still considerable differences (the range between 50 and 100% after 9 h of rest) were indicated by 383 probes annotated to 327 genes (File S1). This group was also dominated by probes with small signal intensity (mean < 50). After their rejection, the group was restricted to only 22 genes (*Mt1*, *Ptgds*, *Apod*, *Fam107a*, *Timp4*, *Phyhd1*, *Aqp4*, *Pxmp2*, *Hmgcs2*, *Agt*, *Pygm*, *Plin4*, *Vmn1r48*, *Kansl3*, *Rgs12*, *Opalin*, *Smim4*, *Col5a3*, *Apoc3*, *Ugt1a6b*, *Olfr145*, *Gm10447*). In the case of *Opalin*, the effect was detected by a probe-binding transcript variant with retained intronic sequences. The examples of genes displaying the most persistent changes in expression during all tested time points are shown in Figure 10.

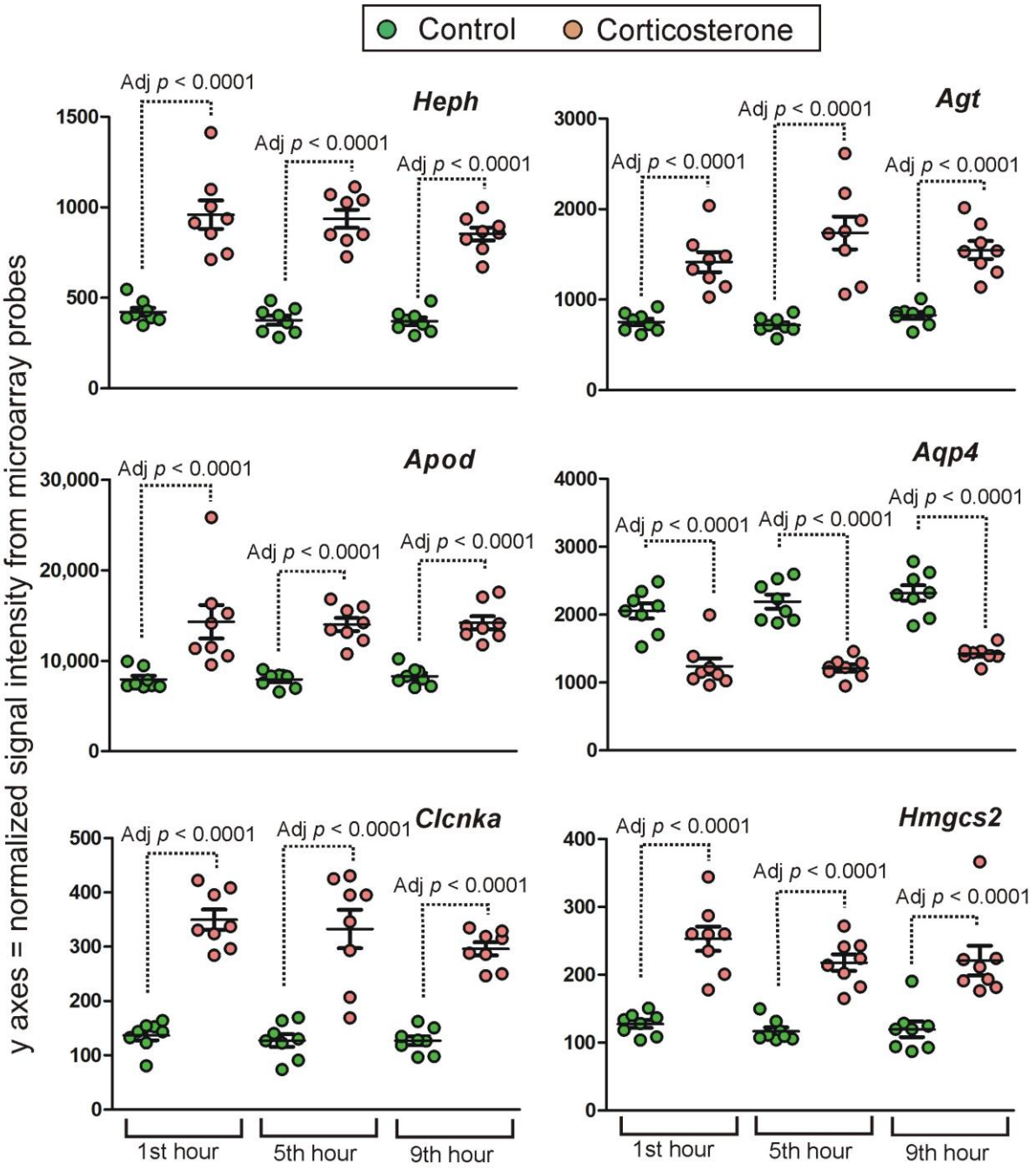

Figure 10. Examples of genes displaying long-lasting primary effects. Y axis indicates probe signal intensity decomposed into green and red channel after final background correction, within-array and between-array normalization. All data are available in File S1.

Intermediate Primary Effects

A number of 2301 unique probes displayed significant differences during the first and the fifth hour with the same direction of changes during both tested time points but returned to the basal level during the ninth hour of the resting period. Examples of this pattern of expression are shown in Figure 11. As already noted (Figure 5), there is variability in the pattern of expression detected by probes binding different variants of transcript derived from the same gene. To restrict the data to genes that display altered expression exclusively up to the fifth hour, we excluded 248 probes indicating primary effects that were significant at a longer interval (ninth hour). Some remaining microarray probes were inconsistently annotated by different databases (34 probes) or were not

specific to single genes according to the BioMart/Ensembl database (63 probes). After their removal, there were 1956 probes specifically annotated to 1781 genes. Most of them code proteins although there are also lncRNAs (77), miRNA (1) lincRNA (1), 12 TEC genes (To be Experimentally Confirmed), 2 unknown but likely coding genes, 41 (2.1%) pseudogenes and 52 (2.7%) dysfunctional transcripts, classified in the Ensembl database as processed transcripts, transcripts retaining introns, antisense, nonsense-mediated decay and LoF transcripts (definitions are provided in File S2). The majority of probes (1737) indicated small (<25%) and medium (<50%) differences between groups. Differences larger than 100% (log2 fold change > 1) after 5 h of rest were indicated by only 11 probes annotated to 11 genes but most of them displayed very low signal intensity (mean < 50). After the rejection of these probes, the group was restricted to two protein-coding genes (*Cdkn1a* and *Maff*). In the case of *Cdkn1a* (File S1), the effect was specific for some variants of the transcripts. Importantly, highly significant changes occurring during the first and fifth hour were detected by a probe binding canonical variant of *Cdkn1a* transcripts. Smaller but still considerable differences (the range between 50 and 100% after 5 h of rest) were indicated by 208 probes annotated to 181 genes (File S1). This group was also dominated by probes with small signal intensity (mean < 50). After their rejection, the group was restricted to only 26 genes (*8430426J06Rik*, *Alpl*, *Atp2c2*, *Ccl12*, *Cmtm2a*, *Cntfr*, *Ecscr*, *Fmo2*, *Gbp2*, *Gbp3*, *Gjb6*, *Glp2r*, *Gm12022*, *Gm4285*, *Lrg1*, *Map3k6*, *Mt2*, *Ninj2*, *P2ry12*, *Pdpn*, *Sdc4*, *Slc38a5*, *Stab2*, *Tekt4*, *Tmem52* and *Tmprss6*).

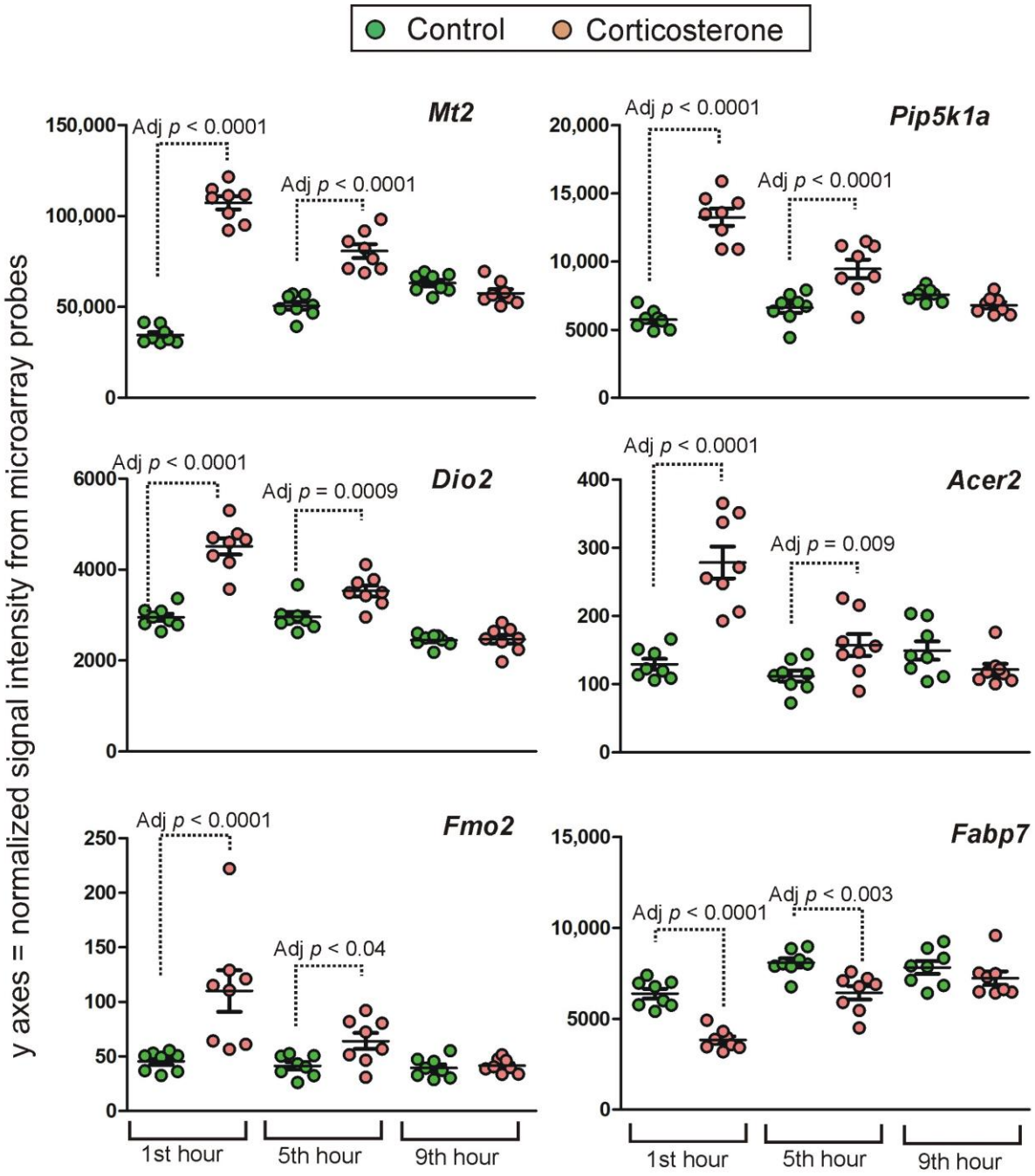

Figure 11. Examples of genes displaying intermediate primary effects. Y axis indicates probe signal intensity decomposed into green and red channel after final background correction, within-array and between-array normalization. All data are available in File S1.

Short-Lasting Primary Effects

A number of 3911 probes indicated differences between groups only during the first hour of the resting period which was associated with an increased level of glucocorticoids in animals receiving exogenous corticosterone. Examples of this expression pattern are shown in Figure 12. Transcription leads to the occurrence of different variants of transcripts derived from the same gene and thus probes annotated to the same gene may provide a discrepant result (Figure 5). Therefore, we have removed 916 probes to restrict the data to genes that displayed differences only during the first hour of the resting period. Additionally, some microarray probes were not specific to single genes according to the BioMart database (75 probes) or were inconsistently annotated by different databases (27

probes). After the removal of these probes, there were 2893 probes specifically annotated to 2490 genes. Most of them code proteins although there are also lncRNAs (198), miRNAs (2) lincRNAs (2), snRNA (1), 26 TEC genes (To be Experimentally Confirmed), 71 (2.5%) pseudogenes and 74 (2.6%) dysfunctional transcripts classified in the Ensembl database as processed transcripts, transcripts retaining introns, antisense, nonsense-mediated decay and LoF transcripts (definitions are provided in File S2).

The majority of probes (2490) indicated small (<25%) and medium (<50%) differences between groups. Differences larger than 100% (log2 fold change > 1) were indicated by only 33 probes but most of them displayed very low signal intensity (mean < 50). After the rejection of these probes, the group was restricted to six genes (*Kcnq2*, *Depp1*, *Galnt15*, *Plekhf1*, *Cxcl10* and *Phactr3*). In the case of *Kcnq2*, *Cxcl10* and *Phactr3* (File S1) the effect was specific for some variants of the transcripts but only in the case of *Cxcl10* the significant effect was detected by a probe binding canonical variants of the transcripts. The most perplexing case is the *Kcnq2* gene because a significant effect was detected by a probe binding only dysfunctional variants of the *Kcnq2* transcripts (retained intron and processed transcript lacking an open reading frame) while five other probes indicated a lack of differences between groups. Smaller but still considerable differences (the range between 50 and 100% after 5 h of rest) were indicated by 370 probes (File S1). This group was also dominated by probes with small signal intensity (mean < 50). After their rejection, the group was restricted to only 20 genes (*Hes5*, *Sgk1*, *Mgp*, *Fzd2*, *Arrdc2*, *Pdk4*, *Vgll3*, *Thbs4*, *Rtp4*, *Gata2*, *Ifit3b*, *Tnfsf10*, *Cytl1*, *Tcim*, *BC018473*, *A330032P22Rik*, *Phf11d*, *Lhx3*, *BC053393* and *Acss3*).

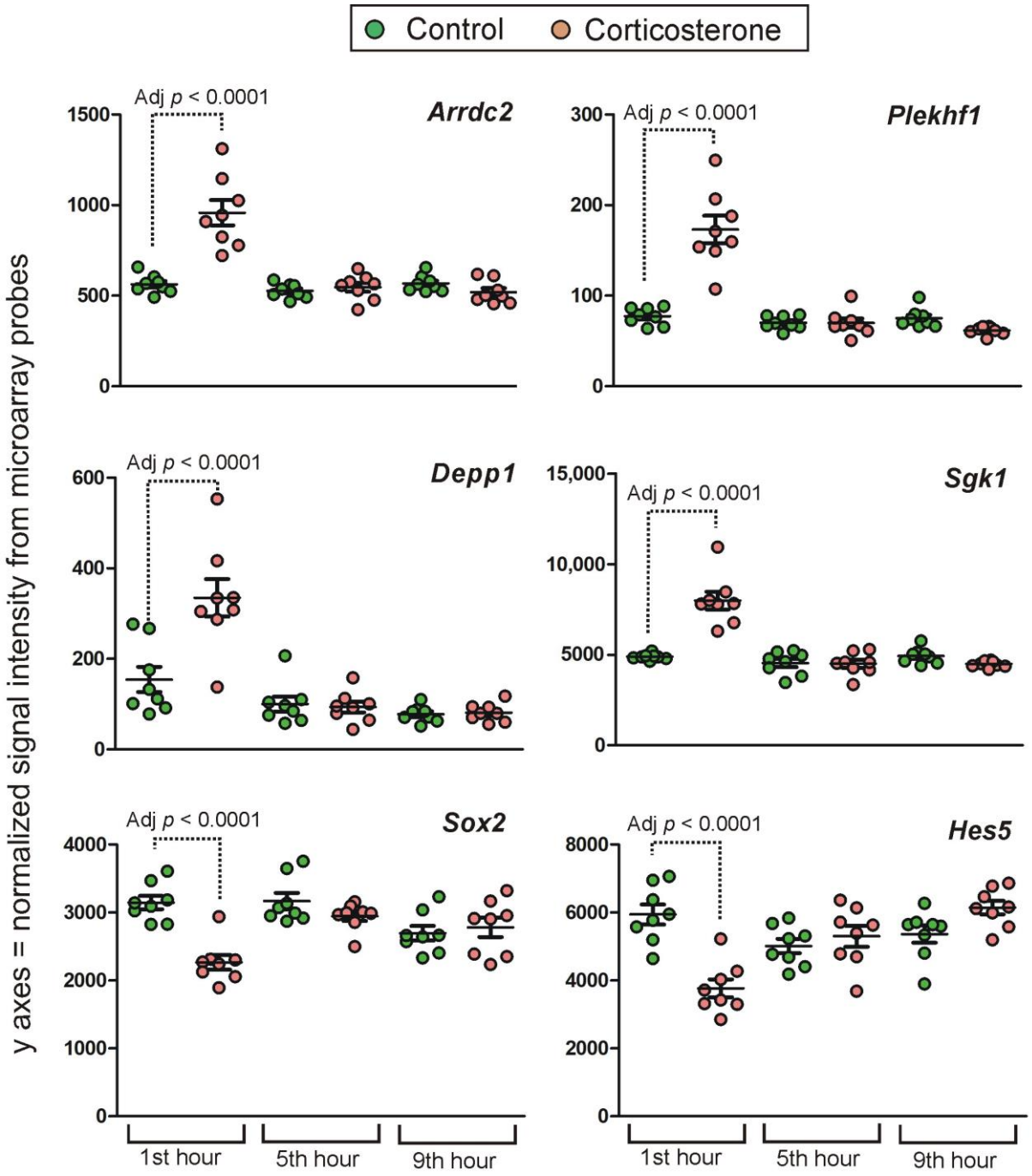

Figure 12. Examples of genes displaying short-lasting primary effects. Y axis indicates probe signal intensity decomposed into green and red channel after final background correction, within-array and between-array normalization. All data are available in File S1.

Time-Dependent Reversal of Primary Effects

A relatively small number of corticosterone-responsive genes significantly reversed the direction of expression during the resting period. Examples of this expression pattern are shown in Figure 13. There were 75 probes indicating a reversal in expression and most of them detect protein-coding genes with exception of one lncRNA, two processed pseudogenes and one transcript containing intron. Additionally, some probes are inconsistently annotated in different databases (two probes) or are annotated to more than one gene in the Ensembl/BioMart database (two probes). After the removal of probes that are lacking specificity, we found 71 genes that reversed the expression including 39 genes

that were unique for the category of primary transcriptomic responses with the time-dependent reversal. The remaining 32 genes also displayed other expression patterns detected by additional probes. All unique 39 genes displayed signal intensity larger than 80 and the largest differences between groups (of more than 50%) were found in the case of seven protein-coding genes (*Paqr5*, *Fas*, *Nfkbia*, *Fkbp5*, *Fgfrl1*, *Mc4r*, *Smim3*).

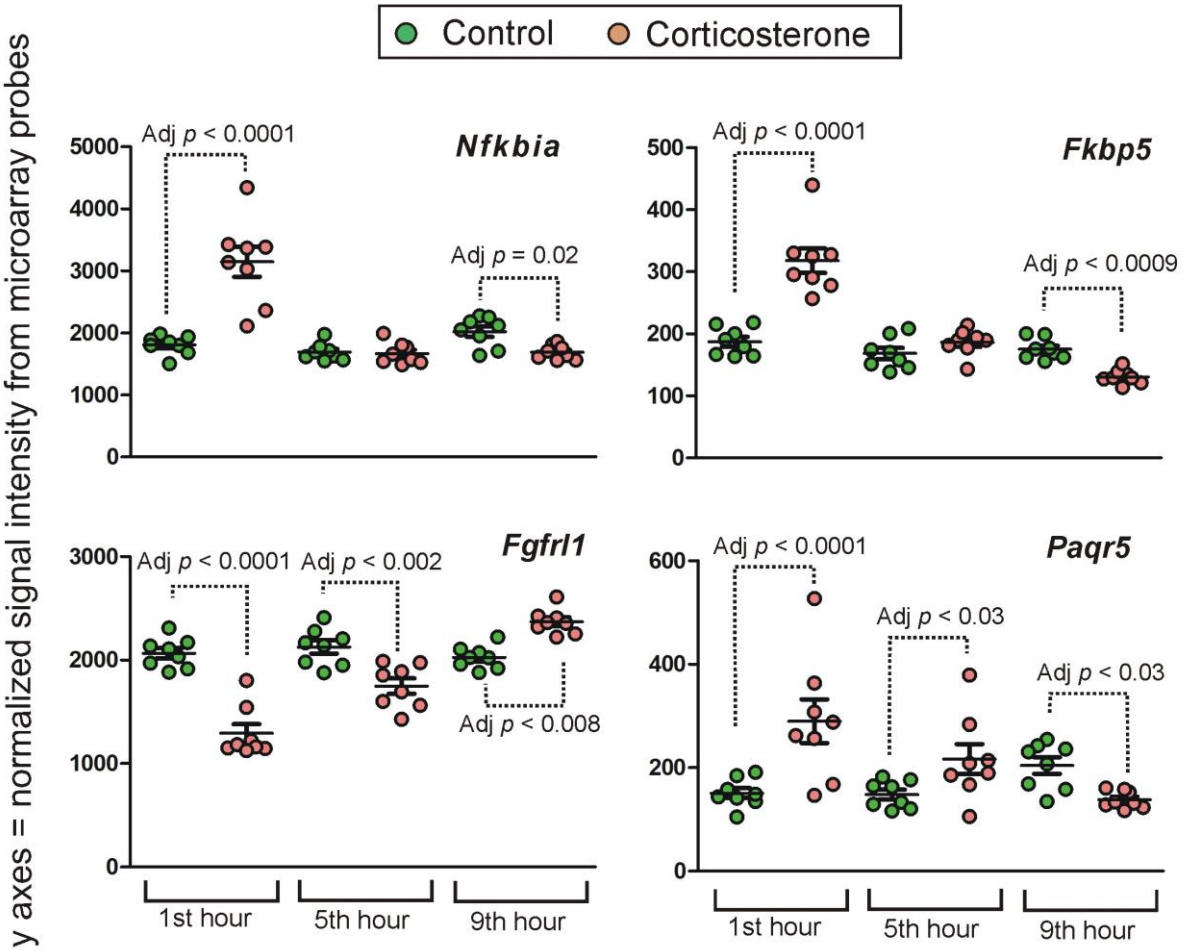

Figure 13. Examples of genes displaying time-dependent reversal of primary effects. Y axis indicates probe signal intensity decomposed into green and red channel after final background correction, within-array and between-array normalization. All data are available in File S1.

2.4.2. Secondary Effects

One of our assumptions was that the transcriptomic changes induced during an elevated level of corticosterone (first hour) will trigger a second wave of transcriptomic effects that will develop when corticosterone returns to the baseline. However, the secondary effects were smaller in terms of the number of probes, indicating significant differences only during the fifth and ninth hour of the resting period (Figure 9B,C) compared with the first hour (Figure 9A). Furthermore, although there were 60 probes indicating changes larger than 100% after 9 h of rest, all of them were characterized by small signal intensity (mean < 50). Very low signal intensity was also found in the case of probes indicating differences in the range between 50 and 100% after 9 h of rest because most of them (99%) were characterized by very low signal intensity (mean < 50). After the rejection of probes with the lowest signal intensity, the group was restricted to only five genes (*Zbtb16*, *Sh3pxd2b*, *Rhcg*, *Asb4*, *LOC102635912*, *Gjb3* and *Gipc2*).

3. Discussion

3.1. Characteristics of Experimental Model

From a methodological point of view, the most important findings are corticosterone data (Figure 1A) because they confirm the effectiveness of our experimental procedure. The experimental design allowed both for a significant increase in blood corticosterone level and its normalization during the resting period. Therefore, this procedure enabled us not only to study transcriptomic responses to an elevated level of glucocorticoids but also to determine the time course of their decay and to identify persistent effects that remain despite the return of glucocorticoids to the baseline. This information is important to better understand the prolonged effects of glucocorticoids during circadian rhythms of activity and rest. The corticosterone data also show that exogenous glucocorticoids administered in drinking water inhibited the release of endogenous corticosterone consistently with the well-known regulatory mechanisms [23,24]. This effect is visible in corticosterone-treated mice as a decrease in blood corticosterone in the afternoon compared with control mice that display a small increase in blood corticosterone (Figure 1A,B) consistently with physiological fluctuations of glucocorticoid release [25]. It should be noted, however, that we could observe only an early stage of the rising level of endogenous corticosterone because the last tested time point in the afternoon (ninth hour) was about 3–4 h before the peak of endogenous corticosterone secretion. The afternoon increase in the blood level of corticosterone in resting control animals is associated with the increase in the level of glucose (Figure 1C) which is in accordance with the role of glucocorticoids in the regulation of glucose homeostasis [12]. However, we have not observed such an effect during the first hour of the resting period that was associated with a highly elevated level of glucocorticoids in corticosterone-treated mice. Such a finding may seem to be surprising but in fact is consistent with experiments showing that an acute effect of glucocorticoids on blood glucose in mice is present in fasted animals but not in animals having free access to food [26]. Therefore, the detection of an altered level of glucose was most likely in our experiment after several hours of rest when animals were sleeping but not immediately after the end of the active phase associated with food consumption. Our observations indicate that during the first hour after turning on the lights (the first hour of the resting period), mice are still awake but their activity is declining compared with the dark period. In contrast, during later periods of the light phase mice are laying motionless with closed eyes indicating sleeping. We were not directly monitoring sleep in this experiment because of technical limitations (EEG) and because the most important factor for us was the return of blood corticosterone level to the baseline. This was sufficient for drawing conclusions about the persistency of transcriptomic responses to exogenously applied glucocorticoids. However, some insights can be provided by a pattern of expression of genes associated with the regulation of the circadian rhythms (Figure 7) such as *Nr1d1*, *Dbp*, *Ciart* (*Gm129*), *Arc* and *Fos* [19–22]. These data indicate an undisturbed circadian rhythm and provide a context for the interpretation of transcriptomic responses to corticosterone.

3.2. General Characteristics of Transcriptomic Response to Corticosterone

The transcriptomic response to overnight corticosterone treatment was dominated by primary effects (Figure 8) that were present at the time of an elevated level of blood corticosterone during the first hour of the resting period (Figure 1A). These effects involved much more probes (Figure 9A) than secondary effects that occurred later during the resting period (Figure 9B,C). The primary effects waned over time although some of them persisted even after 9 h of rest (Figure 8C) despite the fact that corticosterone returned to the baseline. This indicated that transcriptomic responses to corticosterone are heterogeneous in terms of the decay latency and that some of them may contribute to the long-lasting effects of glucocorticoids. It is also worth noting that the time course of decay depends on the probe used for the detection of individual genes (Figures 4 and 5). Some

probes annotated to the same gene provide almost identical results (Figure 2B,C) while other probes give discrepant results (Figure 2D–I). Some light is shed by the data retrieved from the BioMart/Ensembl database showing that probes annotated to the same gene are frequently detecting different variants of transcripts (Figures 4 and 5) including both the most representative canonical variants and a number of dysfunctional variants. This indicates that each unique probe should be considered separately even if several probes are annotated to the same gene. This approach provides opportunities to better understand patterns of the expression of individual genes but also creates challenges in the interpretation of large-scale datasets. Unfortunately, not all microarray probes are available in the BioMart/Ensembl database leading to numerous gaps in data concerning transcript variants.

The second problem with the interpretation of transcriptomic data is a large number of significant results with small changes between groups (Figures 8 and 9). Therefore, it is important to understand both biological and methodological mechanisms leading to the occurrence of such results. A small magnitude of detected changes may result from genuine differences between groups. For example, transcriptomic changes restricted to a small population of highly specialized cells are diluted in the total pool of transcripts isolated from homogenized tissue [18] and such a scenario is especially likely to occur in the brain that is highly heterogeneous in terms of cells [27]. Furthermore, the collection of samples may be performed at the early or late stages of gene regulation when the observed changes are small. Our results support such a hypothesis because some well-known GC-responsive genes such as *Errfi1*, *Klf9*, *Bcl6* that are responsive to the acute administration of glucocorticoids [13] display significant but small differences (<30%) after the overnight administration of corticosterone. On the other hand, small changes in detected expression may constitute systematic errors generated by background correction and array normalization. Therefore, we should assume that the lower the magnitude of detected effects, the higher probability of false positive findings in transcriptomic data. Nonetheless, there is no perfect method allowing for the separation of genuine effects from technical errors in a single study. This problem can be overcome, however, by a meta-analytic approach that allows for the identification of replicable findings and their separation from random effects in pooled datasets derived from different studies [18]. This approach depends, however, on the availability of data and is negatively affected by the selective publishing of transcriptomic results [18]. Therefore, we publish all significant results in supplementary data although the main focus of the paper is genes displaying the most conspicuous differences between groups.

3.3. Time-Course of Transcriptomic Responses to Corticosterone during the Resting Period

Our study shows that transcriptomic responses to glucocorticoids are heterogeneous in terms of the decay time (Figures 10–13). Importantly, the number of transcriptomic responses that display short-term duration or even time-dependent reversal during the resting period (*Errfi1*, *Cdkn1a*/*p21*, *Ddit4*/*Redd1*, *Ndrg2*, *Sesn1*, *Wnt7a*) are involved in the negative control of cell growth and proliferation [13,28–35]. It is known that acute stress triggers widespread activation affecting 96% of the brain [36]. Therefore, stress-induced inhibition of cell growth and proliferation is considered an adaptive mechanism protecting the brain from the adverse effects of excessive excitation including the genotoxic action of reactive oxygen species and the redundant tropic effect of glutamate [18]. However, prolonged inhibition of trophic processes can adversely affect cognitive processes that depend on neurogenesis and neuronal plasticity. Our results suggest that GC-induced impairment in cell growth and proliferation in mice is prone to recovery during resting periods associated with low levels of glucocorticoids. This is especially important in the case of patients suffering from post-traumatic stress disorder (PTSD) that display a decreased brain volume [37] and typically suffer from sleep disturbances such as insomnia and nightmares [38–40]. Furthermore, our findings support the therapeutic approach of applying a treatment of sleep impairments as a crucial step in the treatment

of PTSD [40]. It should be noted, however, that molecular processes taking place in the human brain are very poorly investigated and, therefore, we do not know whether processes observed in rodent brains are comparable with human biology.

On the other hand, the number of GC-responsive genes including some core genes displayed persistent changes in expression during the entire resting period, despite a quickly normalized level of blood corticosterone. Such prolonged effects may result from persistent changes in methylation leading to altered accessibility of chromatin or from very slow degradation of some transcripts. The case of *Etnppl* gene (Figure 5) suggests that some prolonged effects may indeed result from the slow rate of transcript degradation. This is because there was a prolonged increase in the level of the dominant variant of the *Etnppl* transcript (Figure 5B), while variants with retained intronic sequences displayed a short-term increase in expression that was followed by a reversal of differences (Figure 5D) corresponding to the level of the blood corticosterone (Figure 1). In this case, the immature or aberrant transcripts with intronic sequences are likely to indicate the rate of transcription while a dominant variant may represent the rate of transcript degradation. Obviously, this is only a hypothesis and the precise mechanism underlying prolonged changes in transcript levels should be verified experimentally. It should also be noted that not all data concerning the variants of transcripts are easy to interpret. For example, prolonged changes in the expression of *Opalin* during all tested time points were only detected by a probe binding transcript variant with retained intron but not by a probe expected to bind canonical variant of this gene according to the BioMart/Ensembl database. This indicates that despite considerable progress, there is still uncertainty about the properties of some probes and/or bioinformatic models used to predict the properties of different variants of transcripts.

Importantly, persistent transcriptomic responses occurring during the resting period indicate long-lasting processes affected by glucocorticoids. Inspection of the most affected genes that differed more than 50% after 9 h of rest indicates that GCs can induce long-lasting effects including the metabolism of lipids (*Etnppl*, *Apod* and *Pla2g3* [41–43]), ketones (*Hmgcs2* [44]) and glycogen (*Pygm* [45]), homeostasis of iron (*Heph* [46]), water and potassium (*Aqp4* [47]), blood pressure (*Agt*), peroxisomal transport (*Pxmp2* [48]), actin dynamics (*Fam107a* [49]), inhibition of tissue remodeling (*Timp4* [50]), epigenetic regulation (*Kansl3* [51]), voltage-sensitive chloride channels (*Clcnka* [52]) and, finally, removal of toxins and signaling molecules (*Ugt1a6b*, *Sult1a1* and *Mt1* [53–57]). Some of these genes also induce pleiotropic effects. For example, *Ptgds* (*L-PGDS*) is responsible for the synthesis of prostaglandin D2 regulating a wide range of processes such as vasodilation, immune responses and sleep homeostasis [58]. The functions of affected genes are consistent with a broad range of effects induced by glucocorticoids including the metabolism of lipids, glycogen and iron [12,13], the immune response [59] and cardiovascular system [60,61].

Our study suggests that persistent changes in gene expression constitute an important mechanism of the delayed effects of glucocorticoids. The second mechanism of delayed effect is the reversal of expression during the resting period due to the inhibition of the endogenous release of corticosterone. In our study, there were relatively few genes reversing expression but it should be noted that we tested expression at an early stage of the rising level of corticosterone. However, endogenous corticosterone achieves the highest level at the beginning of the active period [25]. Therefore, differences between control and corticosterone-treated mice can increase over time leading to a larger number of genes reversing expression due to inhibition of the release of endogenous glucocorticoids in corticosterone-treated animals. Finally, the third mechanism of delayed effects of glucocorticoids may involve changes in the expression of genes that are indirectly regulated by glucocorticoids due to changes in the expression of various transcription factors [13]. Our study suggests that these secondary responses play a minor role during the resting period. Importantly, although a considerable number of probes indicated secondary changes larger than 50% (Figure 9B,C), the signal intensity obtained

from the majority of these probes is very low. Such signal can be provided by a small number of cells with distinct patterns of gene expression compared with the majority of cells associated with the central nervous system. Potentially, the source of such genes can be various blood cells trapped in the dissected tissue. Therefore, the significance of these findings for brain physiology is uncertain.

3.4. Comparison with the List of Established GC-Responsive Genes

The comparison between transcriptomic data derived from different studies constitutes a special challenge because of frequent changes in gene nomenclature and inconsistencies between different databases used for the annotation of microarray probes [18,62]. Additionally, a common problem encountered in transcriptomic studies is a low statistical power due to small groups and pooling of samples leading to a large number of false positive and negative findings in individual studies [18,63–65]. Finally, the comparability between studies is decreased by the selective publishing of transcriptomic data [18]. As a result, individual studies available in the literature contain a mixture of true positive, false positive and false negative findings. To avoid this problem, we compared our current results with a referential list of GC-responsive genes that is based on the meta-analysis of standardized data retrieved from 17 studies [13] and includes a most recent update of gene nomenclature [18]. The referential dataset [13] is based both on in vivo [66–73] and in vitro experiments [14,74–81] and is dominated by acute data obtained during the period ranging from 1 to 6 h after administration of glucocorticoids. Based on these literature data, we created a list of the most frequently and consistently reported genes that were additionally divided into core and extended parts differing in the number of supporting studies. The core list contains 88 most frequently and consistently regulated genes that displayed the same direction of change in at least four papers [13,18] while an extended list contains 251 genes that displayed the same direction of change in three independent studies in response to glucocorticoids [18].

In our present experiment, we found significant changes in the expression of 69 (78%) core genes and 188 (75%) GC-responsive genes from an extended list (assignment to already established GC-responsive genes is provided in File S1). Therefore, we have found most of the expected genes indicated already in the literature data [13]. The changes in expression of the core GC-responsive genes displayed several patterns. Some of these genes (*Cdo1*, *Ddit4*, *Ehd3*, *Fzd1*, *Lyve1*, *Mtmr2*, *Nedd9*, *Pdk4*, *Plekhf1*, *Rhou*, *Sesn1*, *Sgk1*, *Sox2*, *Tle4*, *Tmem109*, *Wnt7a*, *Zfp36l1*) displayed significant differences only during the first hour of rest, which was associated with an elevated level of corticosterone, and returned to baseline during the rest of the experimental period. The second group was altered during the first hour, maintained significant differences during the fifth hour of rest and returned to the baseline during the ninth hour of rest (*Gjb6*, *Klf15*, *Mertk*, *Mt2*, *Ndrg2*, *Pim3*, *Prr5*, *Rasl11b*, *Rhob*, *Sdc4*). Additionally, a few genes (*Cdkn1a* and *Svil*) could be classified either to the first or second group depending on the probe used for their detection. The third group of genes (*Fkbp5* and *Nfkbia*) reversed the direction of expression at the end of the experiment following a similar effect on the level of blood corticosterone. The fifth group (*Arl4d*, *Azin1*, *Calm2*, *Chst1*, *Lhfp*, *Ppp5c*, *Rdx*, *Sult1a1*) displayed significant differences during all three-time points with the same direction of expression. Finally, the remaining core genes displayed a more complex pattern of expression that was frequently probe-specific. While the involvement of these genes in the response to GC is well established [13], the time-course of their expression during the resting period was not previously reported. Our study, which is based on a large number of independent samples, also indicates that the previous list of most replicable glucocorticoid-responsive genes which was based mostly on acute effects [13] should be extended with a special emphasis on genes that are regulated at longer intervals such as 8–12 h. Especially striking findings are genes displaying a replicable pattern of expression during two and three independent time points with a high magnitude of detected changes after 12 h of treatment that were not previously implicated in the glucocorticoid response [13]. Genes such as *Pip5k1a*,

Pmaip1, *Gbp3*, *Tekt4*, *Gm11627*, *Maff*, *Ddc*, *Pnpla2*, *Pglyrp1*, *Alpl*, *Slc38a5*, *Lao1*, *Etnppl*, *Clank*, *Heph*, *Phyhd1*, *Timp4*, *Agt*, *Timp4*, *Vmn1r48*, *Pdzd2*, *Pygm*, *Apod*, *Serpinb1a*, *Crybb1*, and *Tfcp2l1* belong to this group. Therefore, these data will constitute an important contribution to an update of our previous meta-analysis [13] that is scheduled after the publication of the remaining data from our ongoing glucocorticoid project.

3.5. Comparison with Transcriptomic Response to Stress

An important question is to what extent the effects induced by exogenous corticosterone overlap with effects observed during the stress response that are much more complex than just a release of glucocorticoids and involves other components such as vascular effects [18,82,83] and the release of neurotransmitters [84,85]. In addition, neurotransmitters and glucocorticoids not only induce their specific effects on gene expression [13,84,85] but also interact with each other [12,86,87]. Therefore, not all effects observed after the administration of corticosterone may be relevant to the stress response. Therefore, we compared the corticosterone results with the referential list of stress-responsive genes [18]. The comparison showed that 1702 GC-responsive genes are also reliably detected in experiments testing the effect of stress on brain transcriptome (File S1). This indicates that GCs can contribute up to 63.7% percent of transcriptomic responses observed during the stress response and this estimate is much higher than the previous one based predominantly on acute responses obtained during the period ranging from 1 to 6 h after administration of glucocorticoids [18]. In the group of transcriptomic responses common for GCs and the stress response are genes displaying the most persistent changes during the resting period such as *Etnppl*, *Heph*, *Fam107a*, *Apod*, *Aqp4*, *Agt*, *Ptgds*, *Mt1*, *Plin4*, *Sult1a1* and *Pla2g3*. Importantly, we have also found some genes that were not previously implicated in the glucocorticoid response [13] but were found to be top genes in the stress response [18] such as *Depp1*, *Galnt15*, *Mgp*, *Hes5*, *Txnip*, *Il1r1* and *Elovl7*, and in the case of short-term primary effects, *Slc2a1*, *Acer2*, *Fabp7*, *Pglyrp1*, *Lrg1*, *Htra1*, *Fmo2*, *Htra1*, *Gjc2*, *Lfng*, *Thbd*, *Jdp2*, *Slco1c1*, *Fjx1*, *Pllp* in the case of intermediate primary effects and *Opalin*, *Mobp*, *Slc4a4*, *Tmem88b*, *Trf*, *Ptn*, *Actb*, *Qk*, *Homer1*, *Junb*, *Ptn*, *Creb5* and *Kif5a* (long-lasting primary effects). This indicates that the applied model of overnight corticosterone treatment is a useful tool for studying mechanisms underlying the stress response.

4. Materials and Methods

4.1. Animals

Sixty Swiss-Webster male mice (weighing 39.0 ± 3.8 g (mean ± SD) and 12 weeks of age) were used in the experiment. Mice were obtained from a breeding colony located at the Institute of Genetics and Animal Biotechnology (Jastrzebiec, Poland). Animals were housed in cages with fine sawdust bedding (4–5 mice per cage) under standard conditions (12/12 h light cycle, 22 ± 2 °C, and 55 ± 5% humidity). The animals had an enriched environment and free access to dry food (Labofeed H, Kcynia, Poland) and tap water. The experiment was performed with the permission of the Second Local Ethical Committee in Warsaw (permission number: WAW2/090/2018) in accordance with the Polish Animal Protection Law of 15 January 2015 on the protection of animals used for scientific and educational purposes.

4.2. Experimental Procedure

Three-month-old mice were relocated from family cages to individual cages and, next, were moved to the experimental room dedicated only to this experiment in order to limit the human presence and activity that could disturb animals. The separation into individual cages was performed for two major reasons. First, to enable a selection of individual mice for tissue collection without disturbing other animals. Second, to avoid antagonistic behaviors that frequently occur in group-housed male mice that develop a social hierarchy with dominant and subordinate littermates [88,89]. After the separation,

the mice were divided into control and corticosterone-treated groups. The mice assigned to the corticosterone-treated group were divided randomly into three subgroups (n = 10). For each corticosterone group a separate control group (n = 10) of siblings was assigned so that the obtained results could be compared between brothers from both groups. Each group contained animals from five different litters. Although the initial number of animals was 10 in each group, the final number of animals decreased to nine in one of the control groups (Figure 6) because we noticed a tumor of salivary glands in one of the mice during the later stage of the experiment. Single-housed animals were left undisturbed for 21 days to habituate them to the new conditions following the procedure used previously in our laboratory [82,83]. The habituation is performed because mice separated into individual cages display an increased reactivity to environmental stimuli that returns to the baseline after about 3 weeks [83] together with a lowered level of corticosterone [90].

The main part of the experiment started on day 22 when half of the animals received corticosterone dissolved in drinking water (100 μg/mL) with the addition of hydroxypropyl-β-cyclodextrin (0.45%) which is a cyclic oligosaccharide used to dissolve steroid hormones in water [91,92]. Corticosterone was initially dissolved in a 30% solution of hydroxypropyl-β-cyclodextrin with the help of a vortex/magnetic stirring bar and was diluted to obtain the final concentration. The dose of corticosterone was set on the basis of previous studies [93,94] and additional pilot experiments. Both literature data [93] and our results confirmed that detected levels of corticosterone are within the range observed under physiological conditions after stress in mice [95,96]. Control mice received only water with the addition of hydroxypropyl-β-cyclodextrin (0.45%). Mice received new bottles with either corticosterone solution (22 mL) or vehicle (22 mL) at the end of the light phase, followed by 12 h of dark phase which is a period of mouse activity. Additionally, the bottles were weighed before and after the experiment to control water utilization including the amount of water ingested by animals and spillage caused by the manipulation of bottles by experimenters and the activity of animals. These control measurements showed that all animals had a sufficient amount of available water. The next day, the animals were sacrificed at three time points to collect samples for analysis (Figure 6). The first group of corticosterone-treated mice and assigned control subjects were sacrificed during the first hour of the light phase when animals are still awake although their activity was declining. The remaining corticosterone-treated and control mice were sacrificed during the fifth and ninith hour, i.e., during the resting phase. Cages with animals intended for sample collection were quietly transferred from the experimental room to the adjacent dissection room immediately before the sacrifice was performed by cervical dislocation followed by decapitation. Animals from the control and corticosterone groups were sacrificed in alternating order. Trunk blood was collected for corticosterone and glucose assessment while brains were removed for hippocampal dissection performed according to the protocol described previously [97]. Dissected whole hippocampi were placed in freezing vials, then frozen in liquid nitrogen and stored at a temperature of −80 °C.

4.3. Analysis of Blood Samples

Blood was collected in Eppendorf tubes containing 20 μL of 0.4 mM Na_2EDTA. Next, 1 μL of blood was used to assess the level of glucose with a Microdot glucometer (Cambridge Sensor USA, Plainfield, USA) and dedicated test strips (9–10 biological replicates per group and two technical replicates per mouse). The remaining blood was centrifuged (10 min/5000 RPM at +4 °C) to collect plasma that was stored at −20 °C. The plasma corticosterone level was checked by an enzyme-linked immunosorbent assay (Demeditec Corticosterone rat/mouse ELISA kit). One sample was replicated twice on the plate. It means that there were 9–10 biological replicates per group and two technical replicates per mouse. The test was performed according to the protocol provided by the manufacturer, and the absorbance for each well was read at 450 nm.

4.4. RNA Isolation

The total RNA was extracted from the individual hippocampal samples using GeneMATRIX universal RNA purification kit (EURx Ltd., Gdansk, Poland) following the protocol provided by the manufacturer. The quantity and quality of all RNA samples were assessed by Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and Bioanalyzer 2000 microcapillary electrophoresis (Agilent Technologies, Santa Clara, CA, USA). High-quality samples (260/280~2.1, RIN > 9) were next selected for the microarray analysis (n = 8 in each group).

4.5. Microarrays

The analysis of the gene-expression profile was performed using SurePrint G3 Mouse Gene Expression v2 8 × 60 K Microarray, 8 × 60 K (Agilent Technologies, Santa Clara, CA, USA) and Agilent Technologies Reagent Set according to the manufacturer's instructions. The characteristics of microarrays are provided in Table 1. RNA Spike In Kit (Agilent Technologies, USA) was used as an internal control, the Low Input Quick Amp Labeling Kit was applied to amplify and label (Cy3 or Cy5) target RNA to generate complementary RNA (cRNA) for oligo-microarrays; 300 ng of cRNA from control (Cy3-labelled) and corticosterone-treated (Cy5-labelled) mice were hybridized together on two-color microarrays without pooling samples from the same groups. In total, we used 24 microarrays printed on three slides, with eight microarrays applied for each time point. It means that there were eight biological replicates per group and one technical replicate per mouse. Both control and corticosterone-treated animals from all analyzed time points were assigned to each slide in a pseudo-random way. The Gene Expression Hybridization Kit was used for fragmentation and hybridization and the Gene Expression Wash Buffer Kit was used for washing slides after hybridization. The acquisition and analysis of hybridization intensities were performed using an Agilent DNA Microarray Scanner G2505C. Data were extracted and the background subtracted using the standard procedures included in the Agilent Feature Extraction Software version 10.7.3.1. Data extraction included Lowess normalization. The data were deposited in the GEO database (accession number GSE218508).

Table 1. The characteristics of microarrays (SurePrint G3 Mouse Gene Expression v2 8 × 60 K Microarray, 8 × 60 K).

Total number of microarray probes	62,976
Number of technical/control probes	3671
Number of unique probes detecting mouse genes and printed in 1 copy on the microarray	56,305
Number of unique probes detecting mouse genes and printed in 10 copies on the microarray	300
Total number of unique probes detecting mouse genes (without copies of probes)	56,605
Total number of probes detecting mouse genes (including copies of probes)	59,305

4.6. Annotation of Microarray Data

Due to the variability between different genomic databases [62,98], we have applied consensus annotation [98] combining two different annotation approaches. The first annotation consisted of the following steps. Each probe was annotated with a gene symbol list using biomaRt R package with "agilent sureprint g3 ge 8 × 60 k" attribute [99], GPL21163-3202.txt annotation file from the GEO database [100] and GPL21163_noParents.an.txt annotation file from the gemma database [101]. If no gene symbol existed, the probe sequence was annotated with Ensembl identifiers using the rBLAST R package [Basic local alignment search tool, https://github.com/mhahsler/rBLAST, accessed on 1 April 2022] followed by the translation of these identifiers to gene symbols using BioMart. The second annotation was based on BioMart/Enseble database and combined data from mouse and mouse strain

databases (version 107) since some probes are included only in the mouse strain databases. BioMart/Ensembl does not contain the most recent version of the Agilent mouse microarrays that were used in our experiments (v2 8 × 60 K). Therefore, we combined data retrieved for 8 × 60 K and WholeGenome agilent microarrays. In the retrieved BioMart/Ensembl annotation dataset, we included information about the gene name and type, gene description, transcript name and type and assignment to the Ensembl canonical category of transcripts having the highest coverage of conserved exons, highest expression, longest coding sequence and represented in other key resources (https://www.ensembl.org/info/genome/genebuild/canonical.html, accessed on 19 July 2022). Finally, we compared the first and the second annotation to identify consistently annotated probes, including gene synonyms retrieved from BioMart/Ensembl with the term "gene name" selected in the filter panel and the terms "gene name" and "synonyms" selected in the attributes panel.

4.7. Microarray Data Analysis

The raw data files were analyzed with the Limma package from the Bioconductor project using the same criteria for all files [102]. The 'normexp' background correction method [103] has been applied. The background correction was followed by within-array normalization carried out with the loess procedure and between-array normalization was conducted with the quantile method [104,105]. Normalized data without offset were used for the calculation of fold changes and retrieval of separate channel intensities from M (binary logarithm of red/green intensity ratio) and A (average $\log_2$ intensity of the microarray spot) values with the following formulas:

$$Red\ channel = \sqrt{2^{2 \cdot A\ value} \cdot 2^{M\ value}}$$

$$Green\ channel = \frac{Red\ channel}{2^{M\ value}}$$

Data with offset 50 (variance stabilizing transformation) were used for the calculation of p values following previous guidelines [103,106]. The statistical analysis was performed with separate channel tests which take into consideration the intra-spot correlation [107]. p-values were corrected using the Benjamini and Hochberg procedure controlling False Discovery Rate [108]. Genes showing adjusted p-values < 0.05 were considered differentially expressed.

4.8. PCR Validation

We have selected six genes (*Sult1a1*, *Lao1*, *Etnppl*, *Apoc3*, *Plin4* and *Pla2g3*) for validation with RealTime qPCR based on the statistical analysis of microarray data and our interests in the function of individual genes. Primers were designed using the Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/, accessed on 14 June 2021). Primers included all mRNA transcripts of each gene and were located on two different exons. The verification of primers was performed by temperature gradient PCR (55 °C–65 °C), followed by gel electrophoresis. We were not able to design proper starters for two genes (*Plin4* and *Pla2g3)* because they yielded additional products. Therefore, these genes were excluded after we failed to design two sets of primers for each of these two genes. The reference gene *Tbp* was selected using NormFinder v0.953 software (https://moma.dk/normfinder-software [109], accessed on 14 June 2021) from a group of four candidate genes (*Hmbs*, *Ywhz*, *Tbp* and *Gapdh*). The specification of primers used for the validation of microarray data is shown in Table 2. Reverse transcription was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland); 1 μg of total RNA was used for the process. The quantitative real-time PCR analysis was performed in a Light Cycler 96 (Roche, Basel, Switzerland). SYBER Green I (Roche) was used for the detection of amplified products. All genes were tested in triplicate, and each replicate was on a separate plate (eight biological replicates per group

and three technical replicates per mouse). Additionally, each plate contained a series of five-fold dilutions of the cDNA sample to determine the efficiency of the reaction. There were also three negative controls (without cDNA) on the plate. The final reaction volume for each gene was 20 μL. PCR products were subjected to the melting curve analysis using dedicated software for the Light Cycler 96 (Roche, Basel, Switzerland) to confirm amplification specificity. Relative expression was calculated using the Pfaffl method [110].

Table 2. PCR primers.

Gene Name	Forward or Reverse Primer	Primer Sequence	Annealing Temperature	Efficiency
Sult1a1	F	GATGGGAAAGTGTCCTATGGGT	60 °C	98.8%
	R	TGAAGGATGTGTGGTGAACAATTA		
Lao1	F	ACAACGCTATCGTGCCTCAG	60 °C	95%
	R	CATCAGGTAAGCCTTGGTGGA		
Etnppl	F	TTGGTGAAGGACCGTGAGAAA	60 °C	108.6%
	R	AACTTTGCATCGTCTTCCGTG		
Apoc3	F	ATGGAACAAGCCTCCAAGACG	60 °C	111.7%
	R	TTGCTCCAGTAGCCTTTCAGG		
Tbp	F	GCAGTGCCCAGCATCACTATT	60 °C	108.3%
	R	AAGCCCTGAGCATAAGGTGG		

4.9. Statistics

The statistical analysis of microarray data is described in Section 4.7 while statistical procedures applied to the remaining data are provided in this section. Corticosterone, glucose and PCR data were first tested for variance homogeneity with C Cochran, Hartley, Bartlett and Levene's tests. Data that did not meet the requirement of variance homogeneity were first subjected to the square root transformation [111] and next were tested again with C Cochran, Hartley, Bartlett and Levene's tests. Data with homogenous variance were analyzed with ANOVA followed by the Fisher's least significance difference (LSD) test. The data that did not meet the requirement of variance homogeneity even after SQRT transformation (corticosterone, water usage, *Sult1a1*, *Lao1* and *Etnppl*) were analyzed with the nonparametric Mann–Whitney U test. Pearson's coefficient was used to assess the correlation between microarray and PCR results. The data analysis was performed with Statistica software, release 7.1 (StatSoft Inc., Tulsa, USA). Values are presented as mean ± SEM (column bar graphs) and scatter plots.

5. Conclusions

Our experiment showed that transcriptomic responses to corticosterone are heterogeneous in terms of the decay latency and that some of them persist for at least 9 h despite a quickly normalized level of blood corticosterone. This indicates that persistent changes in gene expression constitute an important mechanism of the delayed effects of glucocorticoids. Genes that differed more than 50% after 9 h of rest suggest that the affected long-term processes include the metabolism of lipids, ketones and glycogen, homeostasis of iron, water and potassium, regulation of blood pressure, voltage-sensitive chloride channels, actin dynamics, epigenetic modifications, inhibition of tissue remodeling, peroxisomal transport and, finally, the removal of toxins and signaling molecules. On the other hand, a number of transcriptomic responses that display a short-term duration or even time-dependent reversal during the resting period are involved in the negative control of cell growth and proliferation. Therefore, the obtained results suggest that GC-induced impairment in cell growth and proliferation is prone to recovery during resting periods associated with the low level of glucocorticoids. Finally, the

obtained results indicate that GCs can contribute up to 63.7% percent of transcriptomic responses observed during the stress response.

Supplementary Materials: The supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms24032828/s1.

Author Contributions: A.J. participated in the planning of the experiment, performed experiment and molecular analyses, prepared results for publication, and participated in the preparation of the manuscript; A.M.S. performed statistical and bioinformatic analysis of microarray data; J.G. performed statistical analysis of microarray data; A.M. performed microarray hybridization; T.J. reviewed and edited the manuscript. G.R.J. provided a general concept for the study and acquired funding, participated in the experiment, contributed to bioinformatic analysis, performed statistical analysis (PCR, corticosterone and glucose) and participated in the preparation of the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Science Centre [grant number 2017/27/B/NZ2/02796].

Institutional Review Board Statement: The experiment was performed with the permission of the Second Local Ethical Committee in Warsaw (permission number: WAW2/090/2018) in accordance with the Polish Animal Protection Law of 15 January 2015 on the protection of animals used for scientific and educational purposes.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data are available in GEO database (accession number GSE218508) and Supplementary Files.

Acknowledgments: The editorial assistance of Ewa Pajak is highly appreciated.

Conflicts of Interest: The authors declare no conflict of interest.

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