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Research report

Behavioral and molecular effects of prenatal continuous light exposure in the adult rat

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ABSTRACT

Disruption of the maternal environment during pregnancy leads to behavioral changes and diseases in the adult offspring. To explore the influence of prenatal continuous light exposure (PCLE) on the adult offspring, we exposed pregnant Wistar rats to constant light during late gestation. Adult PCLE offspring showed an anxiety-like behavior and impairment of short-term memory in different tests. Measurements in the whole brain homogenates from newborn and adult offspring indicated decreased melatonin and serotonin levels and increased reactive oxygen species level in PCLE offspring. Further, we determined melatonin-, serotonin-, oxidative stress-, apoptosis-, and circadian system-related genes expression in different brain areas of adult offspring. The serotonin reuptaker *Slc6a4* displayed a decreased expression in the prefrontal cortex of PCLE group. The circadian rhythm-related gene *Rora* was upregulated in the amygdala of PCLE offspring. Our results point to adverse behavioral effects of PCLE on adult offspring, involving serotonin and melatonin signaling dysregulation, increased chronic oxidative stress, and altered gene expression.

1. Introduction

Continuous light exposure is a strong stressful stimulus, leading to the disruption of circadian rhythms (Fonken and Nelson, 2014) and a subsequent altered melatonin secretion (Wideman and Murphy, 2009). Circadian disruption has been associated with different psychiatric disorders in adult humans like bipolar disorder, depression, schizophrenia, and obsessive compulsive disorder (Pacchierotti et al., 2001; Salgado-Delgado et al., 2011). Maternal circadian rhythm is involved in the programming of fetal and newborn circadian clocks (Irmak et al., 2005; Reiter et al., 2014) and can influence the pineal-defining transcriptome, shown to be established prior to the neonatal period (Hartley et al., 2015). Disturbances of the fetal circadian system have been linked to long-term metabolic and behavioral consequences in the adult offspring (Cisternas et al., 2010; Ferreira et al., 2012; Voiculescu et al., 2015).

Circadian melatonin secretion from the pineal gland exhibits a nocturnal maximum value. Its role during fetal development, when the pineal gland is immature, is supported by its progressively increased concentration in the maternal blood and amniotic fluid during late gestation peaking at term and during delivery (Kivelä, 1991; Nakamura et al., 2001; Okatani et al., 1998; Tamura et al., 2008). Continuous light exposure, known as functional pinealectomy (Briaud et al., 2004; Delibas et al., 2002), is a potent circadian rhythm disruptor suppressing the endogenous circulating melatonin levels (Lewy et al., 1980; Revell and Skene, 2007). This leads to pathophysiological changes, including altered metabolism, endocrine system malfunction, free radical-induced molecular damage, and abnormal behavior in adults (de Matos Cavalcante et al., 2012; Erren and Reiter, 2009; Hardeland et al., 2012; Milczarek et al., 2010; Reiter et al., 2009; Tamura et al., 2013).

Up to date, the impact of prenatal continuous light exposure

Abbreviations: EPM, elevated plus maze; NOR, novel object recognition; OF, open field; PCLE, prenatal continuous light exposure; ROS, reactive oxygen species

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(PCLE) on adult offspring behavior is still poorly understood. Fetal circadian clocks begin to form in the second half of the pregnancy (Sladek et al., 2004), thus in the present study we explored the long-term effects of PCLE during this period of gestation. We assessed the behavior of adult offspring male rats and inquired the related molecular changes. We assessed brain melatonin, serotonin, and reactive oxygen species (ROS) levels and explored expression of genes related to melatonin/serotonin and their respective receptors, oxidative stress balance, apoptosis, and circadian rhythm.

2. Results

2.1. Behavioral changes

2.1.1. Open field test

In the open field (OF) test, PCLE rats showed significantly decreased mobility and exploratory activity, as indicated by an 11.7-fold reduction of the time spent in the central zone (Fig. 1A) and absence of central area crossings (Fig. 1B). The significantly higher number of defecations (3.2-fold) in the PCLE group compared to controls was also suggestive for increased anxiety (Fig. 1C).

2.1.2. Elevated plus maze test

In the elevated plus maze (EPM) test, PCLE rats moved inside the maze over a mean distance of 798 cm, while controls were significantly more mobile ($p=0.007$), with an average distance of 1,288 cm (Fig. 2A). Time spent in the central area was decreased by 5.9-fold (Fig. 2B) and time spent in the open arms of the maze was decreased by 6.5-fold (Fig. 2C) in PCLE group vs. controls. A similar reduction was observed in the number of crossings (2.9-fold) through the central area of the maze (Fig. 2D). Conversely, time spent in the closed arms of the maze was significantly higher (1.5-fold) in the PCLE group compared to controls (Fig. 2E).

2.1.3. Novel object recognition test

In the novel object recognition (NOR) test, during the short-term memory trial, with a retention period of 5 min, PCLE rats took significantly less time (1.5-fold) to explore the novel object (Fig. 3A). However, the time spent with the familiar object did not differ significantly between the two groups. The preference for novel vs. familiar objects, assessed through the discrimination index, was mainly for the novel object in the case of the control group (6 out of 8 rats), while the PCLE rats showed a strong preference ($p=0.027$) for the familiar object (7 out of 8 rats) (Fig. 3B).

The long-term memory trial, with a retention period of 24 h, showed no significant differences between PCLE group and controls (data not shown).

2.2. Molecular evidence for neural alterations

The PCLE model is the equivalent of a functional pinealectomy (Briaud et al., 2004; Delibas et al., 2002). To test the efficacy of the PCLE used, we measured melatonin levels. Since melatonin does not have a specific target in the brain, as it can act for example on the hypothalamus, where it inhibits neuronal firing, or in the midbrain and the result is forebrain dopamine regulation (Smith, 1985), we considered that whole-brain melatonin levels are more informative than the regional ones. Serotonin acetylation is involved in melatonin synthesis (Ganguly et al., 2002) and both melatonin (Cisternas et al., 2010; Ferreira et al., 2012; Voiculescu et al., 2015) and serotonin levels (Gurtman et al., 2002) can influence behavior. We thus measured melatonin and serotonin levels and expression levels of genes related to their metabolism. Moreover, since melatonin influences oxidative stress balance (Reiter et al., 2000) we also inquired the effect of PCLE on ROS and oxidative stress enzyme expression levels. Lastly, we investigated the effect of PCLE on different circadian-related genes.

2.2.1. Melatonin and serotonin levels in the brain

Melatonin levels in offspring brains at the time of birth and in adulthood showed a significant decrease both immediately after birth ($p=0.022$; Fig. 4A) and in adult offspring ($p=0.009$; Fig. 4B). Serotonin levels in offspring brains were decreased ($p=0.01$) in PCLE adults (Fig. 4C), but not in newborn rats.

2.2.2. ROS levels in the brain

Our study showed a significant increase of ROS measured in whole brain homogenates from PCLE offspring immediately after birth ($p=0.006$; Fig. 5A) and from adult PCLE offspring ($p=0.002$; Fig. 5B) compared to controls.

2.2.3. qPCR results

2.2.3.1. Melatonin-related genes. Gene expression levels of the rate-limiting melatonin synthesizing enzyme *Aanat* (arylalkylamine N-acetyltransferase), and melatonin receptors (*Mtr1a, Mtr1b)* were not significantly different between controls and the PCLE group in none of the regions (data not shown).

2.2.3.2. Serotonin related genes. Tryptophan hydroxylase *Tph1* and *Tph2* mRNA levels did not reveal any significant difference between the control and PCLE groups (data not shown). There were no significant differences in the serotonin receptor *Htr1a* gene expression levels between the two groups in either of the investigated brain areas (data not shown). For the serotonin

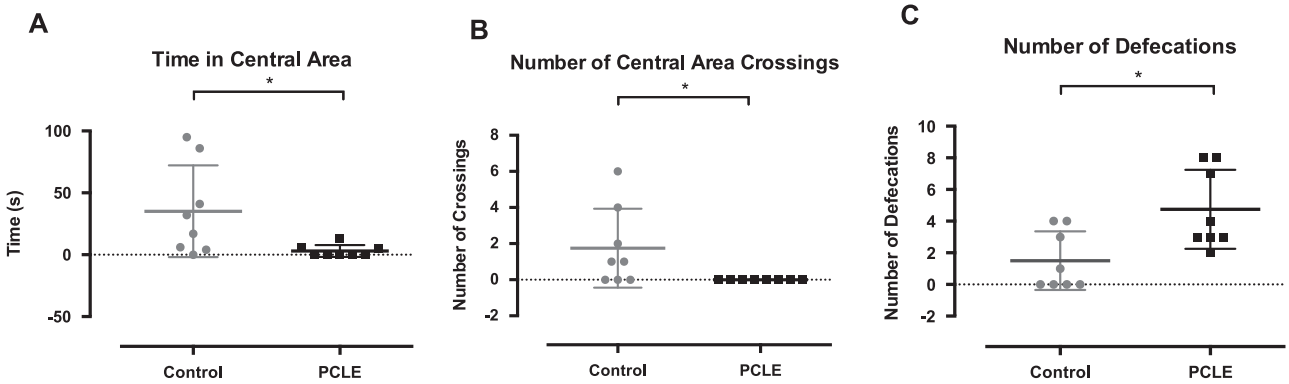

Fig. 1. Anxiety assessment in the OF test. PCLE rats spent shorter time in the central area of the open arena (A) and did not cross the central area (B). The higher number of defecations in the PCLE group is suggestive for increased anxiety (C). Data are expressed as mean ± SD, $n=8$ rats per group, $^{*}p < 0.05$ calculated with a non-parametric Mann-Whitney-Test.

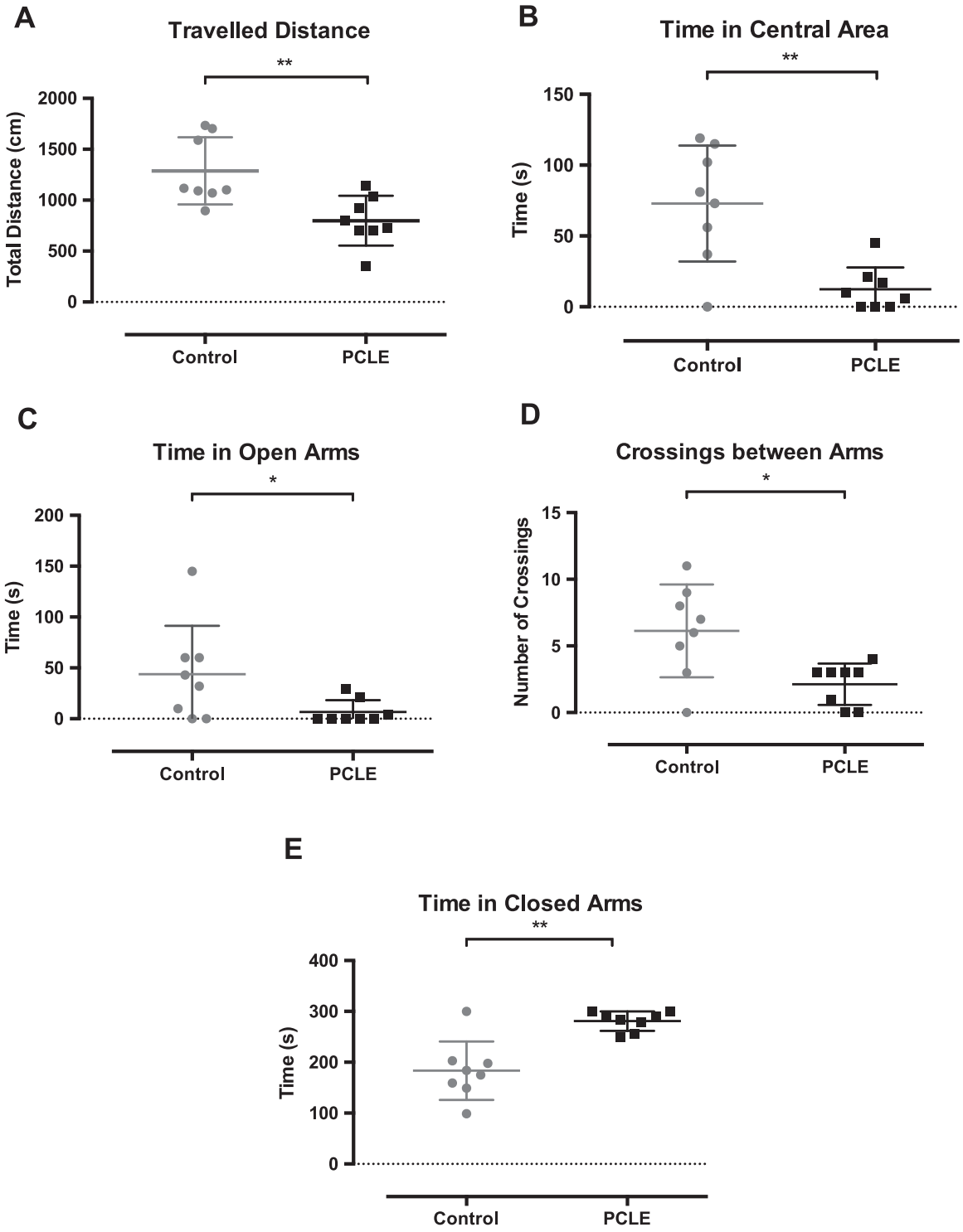

Fig. 2. Anxiety assessment in the EPM test. Total travelled distance was significantly lower in the PCLE group vs. controls (A). Time spent in the central area (B) and open arms (C) of the maze was significantly reduced in the PCLE group. A similar reduction for the PCLE group was observed in the number of crossings through the central area (D), while the time spent in the closed arms was significantly increased (E). Data are expressed as mean ± SD, $n=8$ rats per group, $^{*}p<0.05$, $^{**}p<0.01$ calculated with a non-parametric Mann-Whitney-Test.

reuptaker *Scl6a4*, a significant decrease by 1.93-fold ($p=0.002$) in the mRNA expression levels was detected in the prefrontal cortex of PCLE rats (Fig. 6).

2.2.3.3. Oxidative stress and apoptosis related genes. There were no significant changes between PCLE and control groups regarding the expression of glutathione *syn*thetaze (*Gss*), glutathione peroxidase 1 (*Gpx1*), superoxide dismutase 1 (*Sod1*), and ceruloplasmin (*Cp*) in the investigated brain areas (data not shown).

When investigating apoptosis-related genes, *Bax* showed a 1.64-fold down-regulation ($p=0.07$) in the prefrontal cortex of PCLE vs. control rats, while *Bcl2* had an up-regulation tendency in the adult PCLE rats (1.83-fold) (Fig. 7). However, neither *Bcl2* nor *Bax* reached significance in the changes in expression levels.

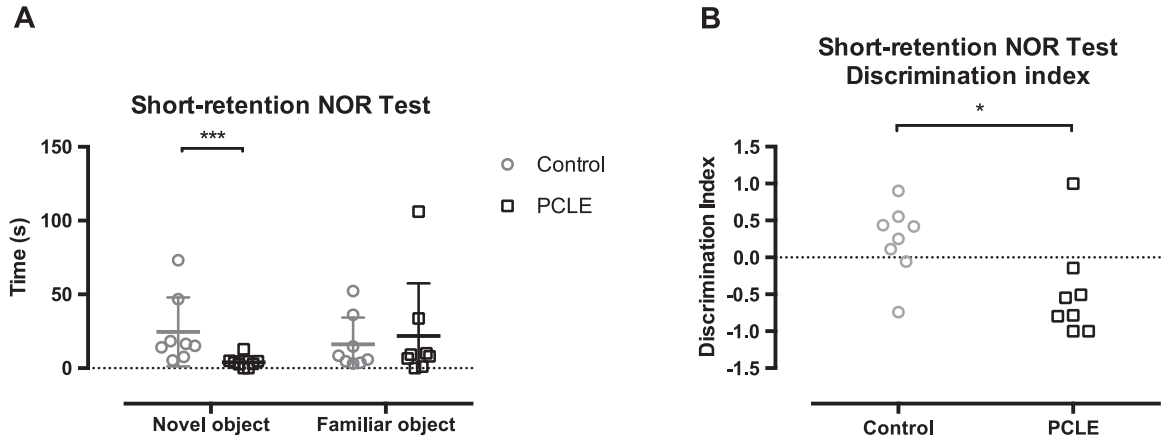

Fig. 3. Short-term memory assessment in the novel NOR test. PCLE rats spent significantly less time than controls exploring the novel object, but no significant difference between groups was registered regarding the time spent exploring the familiar object (A). The positive discrimination index in controls reflects a strong preference for the novel object (6 positive values out of 8 data points), while the negative one in PCLE group indicates a strong preference for the familiar object (7 negative values out of 8 data points) (B). Data are expressed as mean ± SD (A) and scatterplot of the values (B) $n=8$ rats per group, $^{*}p<0.05$, $^{***}p<0.001$ calculated with a non-parametric Mann-Whitney-Test.

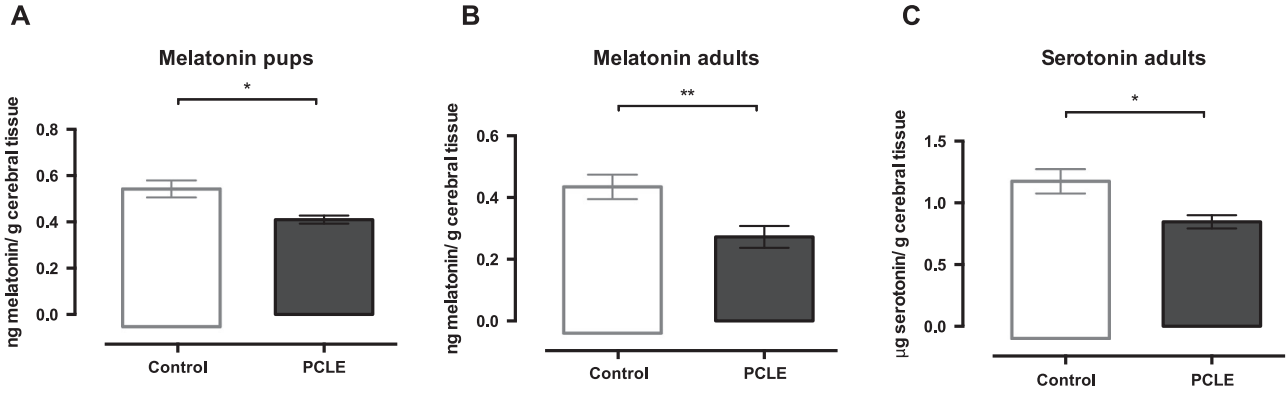

Fig. 4. Melatonin and serotonin concentration in whole brain homogenates. PCLE offspring showed significantly reduced brain melatonin levels immediately after birth (A) and in adulthood (B). Adult PCLE offspring had significantly lower brain serotonin levels (C). Data are expressed as mean ± S.E.M, $n=3$ newborn rats per group and $n=4$ adult rats per group, $^{*}p<0.05$, $^{**}p<0.01$ assessed with a two-sided, unpaired *t*-test with Welch's correction.

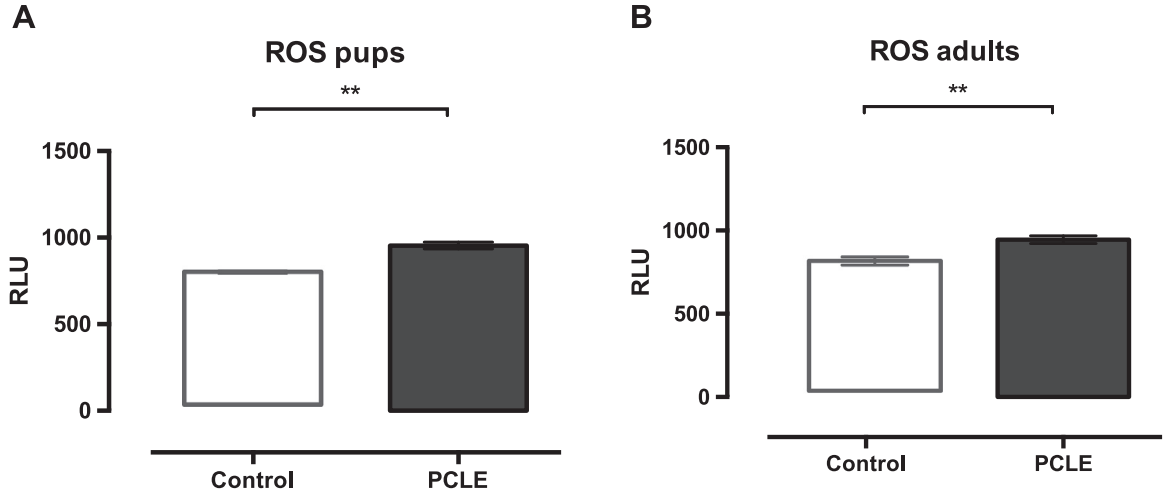

Fig. 5. PCLE leads to increased oxidative stress levels in the brain. ROS levels in the brain were significantly higher in newborn pups (A), and remained higher in the adult offspring (B) in the PCLE group. Data are expressed as mean ± S.E.M, $n=3$ newborn rats per group and $n=4$ adult rats per group, $^{**}p<0.01$ assessed with a two-sided, unpaired *t*-test with Welch's correction.

2.2.3.4. Clock genes. We investigated the expression levels of circadian-related genes (*Clock*, *Arntl1*, and *Rora*). *Rora* was the only one showing a significantly different gene expression, being up-regulated by 1.68-fold ($p=0.024$) in the amygdalae of the PCLE group (Fig. 8).

3. Discussion

Maternal circadian rhythm has been attributed an essential role in fetal maturation (Reiter et al., 2014). The present study reveals an impact of PCLE on offspring behavior and inquires the

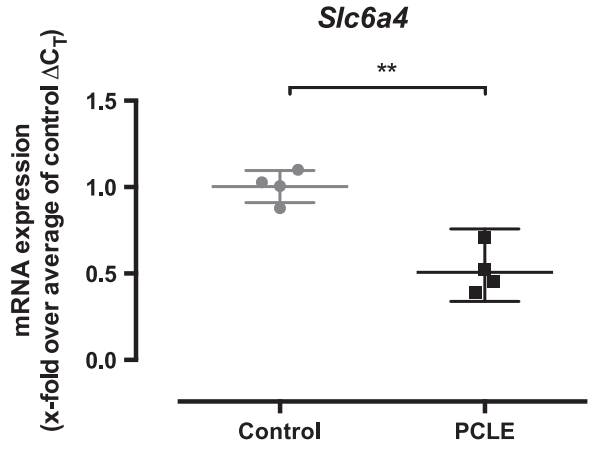

Fig. 6. Serotonin reuptaker expression. *Slc6a4* expression showed a 1.93-fold decrease in the prefrontal cortex of the PCLE group ($p=0.002$). The mRNA levels decrease was calculated as fold change $=2^{-\Delta\Delta CT}$, where $\Delta\Delta CT$ is the difference to the control average ΔCT. Data are expressed as mean $\pm$ SD, $n=4$ rats per group, $^{**}p<0.01$, assessed with a two-sided, unpaired *t*-test with Welch's correction.

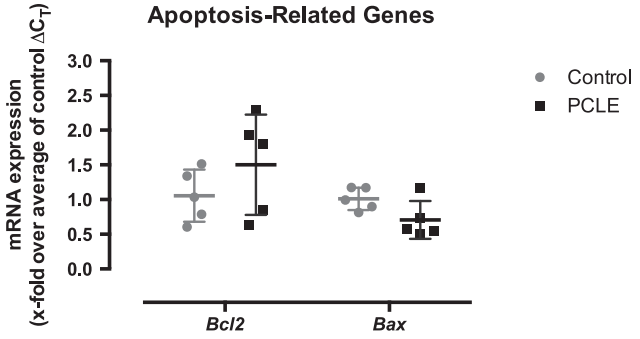

Fig. 7. Apoptosis-related genes expression in the brain. While neither *Bcl2* nor *Bax* showed a significant change in expression levels, a *Bcl2* up-regulation tendency was present in the adult PCLE group (1.83-fold). *Bax* showed a down-regulation trend of 1.64-fold in adult PCLE group vs. controls ($p=0.07$) in the prefrontal cortex. The mRNA levels decrease was calculated as fold change$=2^{-\Delta\Delta CT}$, where $\Delta\Delta CT$ is the difference to the control average ΔCT. Data are expressed as mean $\pm$ SD, $n=5$ rats per group.

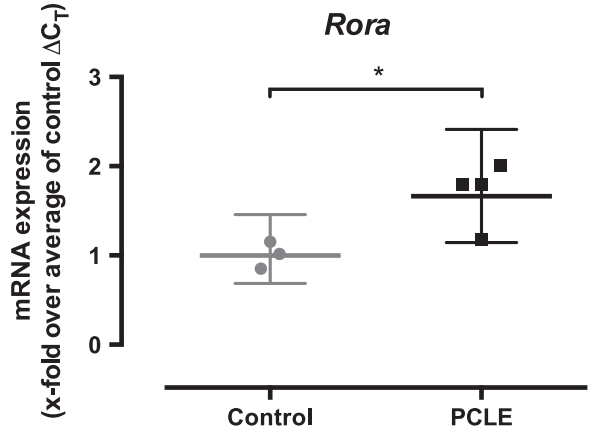

Fig. 8. Rora expression in the brain. *Rora* expression levels in the amygdala showed a 1.68-fold increase in the adult PCLE group ($p=0.024$). The mRNA levels change was calculated as fold change $= 2^{-\Delta\Delta CT}$, where $\Delta\Delta CT$ is the difference to the control average ΔCT. Data are expressed as mean $\pm$ SD, $n_{control}=3$ and $n_{PCLE}=4$ rats, $^{*}p<0.05$ assessed with a two-sided, unpaired *t*-test with Welch's correction.

molecular basis of these changes. We found that PCLE leads to anxiety-like behavior and short-term memory impairment, low melatonin and serotonin, and high ROS levels in both, adult and neonate offspring brains. In the prefrontal cortex of PCLE adult offspring, serotonin reuptaker *Scl6a4* gene expression was down-regulated. None of the measured circadian genes had an altered expression except for *Rora* in the amygdala. As *Rora* has anti-oxidative properties, we have linked this change more to the chronic oxidative stress response than to the long-term effect of PCLE on the circadian clock of the offspring.

3.1. Behavioral changes relationship with melatonin/serotonin system impairment

In the present study OF and EPM behavioral tests results showed that PCLE group displayed an anxiety-like behavior. This is in accordance with the outcome of a previous study using EPM test only, showing the anxiogenic effect of PCLE on adult offspring, which could be prevented by melatonin prenatal administration (Cisternas et al., 2010). The OF test in our study brings the advantage to assess anxiety, but also other behaviors like emotionality, fearfulness, temperament, and locomotor activity (Buccafusco, 2000).

The PCLE model, used as a functional pinealectomy (Briaud et al., 2004; Delibas et al., 2002), resulted in our study in a significant reduction in brain melatonin.

Brain serotonin levels were also low in adult offspring, but close to control levels in P0 pups in the PCLE group (Fig. 4). Timepoint differences between neonatal and adult brain serotonin levels might be related to the immaturity of the serotonergic system in the neonatal population. It is known that *in utero* serotonergic system disruption influences neonatal expression of serotonin receptors (Lauder et al., 2000). Serotonin is involved in neurogenesis (Lauder et al., 1981), but the serotonergic system is not mature at birth. In humans, the synaptic density of the biogenic amine systems in the cortex doubles in the first postnatal year (Huttenlocher and Dabholkar, 1997) and a similar evolution has been observed in rodents, highlighting an equivalent time course for the postnatal development of the serotonergic system (Rubenstein, 1998).

Melatonin deprivation has been pointed to induce behavioral changes (Cisternas et al., 2010; Ferreira et al., 2012; Voiculescu et al., 2015). Moreover, serotonin depletion was shown to lead to anxiety behavior in rats (Gurtman et al., 2002) and serotonin acetylation plays a central role in melatonin synthesis (Ganguly et al., 2002). Hence, the observed behavioral changes in our study might be linked to the reduced level of serotonin and melatonin. However, gene expression of their synthesis enzymes and respective receptors was normal.

Using the same experimental model, we have previously shown that PCLE leads to a depressive-like behavior in adult offspring, suggesting serotonin depletion (Voiculescu et al., 2015). In the present study we show that PCLE induces not only low brain serotonin levels, but also a lower expression of *Slc6a4*, the serotonin reuptaker, in the prefrontal cortex of the adult offspring. Since serotonin depletion is an important cause for depressive disorders (Nutt et al., 1999), serotonin-signaling impairment may constitute a molecular cause for the observed anxiety- and depressive-like behaviors.

Even though NOR is used as a memory exploring task (Ennaceur and Delacour, 1988), the altered short-term memory task response in our study may also be caused by a decreased exploratory activity and increased anxiety, as novelty usually determines fear (Buccafusco, 2000). Nevertheless, melatonin and serotonin have been reported to facilitate short-term memory in adult rats. Argyriou et al. showed that melatonin leads to a short-term memory improvement, while luzindole, a selective MT1 receptor antagonist had no effect (Argyriou et al., 1998). On the other hand, brain serotonin depletion impairs short-term memory, with no effects on long-term memory (Hritcu et al., 2007). Given the

reduced melatonin and serotonin levels in the brain, it cannot be excluded that NOR test results reflect an actual short-term memory impairment.

Different stress models applied to pregnant rats also result in anxiety-like responses and depressive behavior (Richardson et al., 2006; Weinstock, 2001). It is thus hard to disentangle whether the observed behavioral alterations are a direct effect of chronodisruption, or they are rather triggered by the maternal stress induced through continuous light exposure. Considering stress axis involvement in the present study would have been too extensive. It has already been shown that the experimental model we have used increases the activity of the corticosuprarenal gland with a subsequent higher secretion of cortisol (Torres-Farfan et al., 2004). Although we cannot ignore stress axis involvement, the main aim of our study was to find a relationship between serotonin-melatonin axis and behavioral changes in adult offspring.

3.2. PCLE and chronic oxidative stress

Melatonin can act as a scavenger which directly neutralizes reactive oxygen and nitrogen species, or it may influence oxidative enzymes production (Reiter et al., 2000). In our study PCLE induced both a reduction of melatonin levels and a higher load of reactive oxygen species in the brains of newborn and adult offspring. Chronodisruption and melatonin deprivation have been shown to display a direct influence on the mRNA expression of *Sod1, Gpx1*, and *Gss* (Antolín et al., 1996; Pablos et al., 1998; Urata et al., 1999). However, our results showed no significant change in the expression of these genes in the brain of PCLE adult offspring. Melatonin depletion could thus directly trigger increased ROS levels and future studies should be designed to test whether melatonin administration can rescue this effect.

Acute oxidative stress determines a proapoptotic response (Buttke and Sandstrom, 1994), while cells chronically exposed to ROS display an antiapoptotic response, with a high *Bcl2*/*Bax* ratio, phenomenon called apoptosis resistance (Mahalingaiah and Singh, 2014). In the central nervous system *Bcl2* is up-regulated under chronic oxidative stress induced by aging and neurodegenerative diseases (Marshall et al., 1997; Mogi et al., 1996). Although our results do not show significant changes in neither *Bcl2* nor *Bax* expression, a trend for increased *Bcl2* and decreased *Bax* expression in the prefrontal cortex was present in the PCLE group. Additionally, while PCLE offspring did not show signs of postnatal biorhythm alterations, the clock-related molecule *Rora*, shown to protect neurons against oxidative stress (Boukhtouche et al., 2006), was up-regulated in the amygdalae of PCLE rats. Amygdala has an important function in anxiety like behavior modulation, this being related to the normal function of serotonin mediatory system (Forster et al., 2012). Moreover, medication used to treat anxiety disorders is usually influencing monoaminergic system in the amygdala (Forster et al., 2012). We have interpreted that increased *Rora* expression is a sign of chronic oxidative stress which might be involved in the behavioral aspects related to a dysregulation of amygdala functions.

It is thus tempting to speculate that *Rora* up-regulation occurs as an adaptation to chronically increased oxidative stress levels, since the oxidative stress was increased in both newborn and adult PCLE offspring.

Collectively, our results show a major influence of PCLE on the behavior of adult offspring, accompanied by altered serotonin and melatonin levels. Although compensatory mechanisms seem to protect neurons against the chronic oxidative stress triggered in the brain by PCLE, our study raises challenging questions about the potential chronotherapeutic use of melatonin. While we live in an around-the-clock-light environment, the long-term influence of an altered prenatal circadian rhythm remains largely under-studied. The revealed molecular mechanisms could be the starting point for future studies designed to broaden our understanding about chronodisruptive-effects.

4. Experimental procedure

4.1. Animals

Experiments were performed on Wistar rats, which had access to food (standard rat chow) and water ad libitum. All animal procedures were carried out with the approval of the local ethics committee for animal research of Carol Davila University of Medicine and Pharmacy, Bucharest, Romania, in accordance with the European Communities Council Directive 86/609/EEC on the protection of animals used for scientific purposes. Adequate measures were taken to minimize the number of animals used and their suffering. All experiments in adult offspring were performed on male rats to prevent estrus-related variations, while newborn rat measurements were performed regardless of gender.

4.2. Prenatal continuous light exposure

Pregnant Wistar rats were exposed to continuous 500 lx light between days 12–21 of gestation, a model known as functional pinealectomy (Briaud et al., 2004; Delibas et al., 2002). Control rats were kept under a normal light/dark cycle (6 a.m.–6 p.m.). The resulting adult male offspring were divided into 2 groups: the control group from the pregnancies exposed to standard 12:12/light:dark cycle, and the PCLE group, from the dams exposed to the functional pinealectomy. Immediately after birth, all rats were exposed to a standard 12:12/light:dark cycle. After the first postnatal month, when the offspring developed functionally mature pineal glands (Calvo et al., 2004), they were separated from the dams.

4.3. Behavioral tests

Behavioral tests were performed on male offspring from the control and PCLE groups after P60 ($n=8$ for each group, from 3 different litters), using the following sequence: OF, EPM, and NOR tests. A 7-day window was maintained between the OF, EPM, and NOR tests to avoid interest effects (Anchan et al., 2014; Tomihara et al., 2009).

Most of the analyzed studies do not mention if the animals underwent the behavioral tests during the light or dark phases, but as rodents are nocturnal animals they exhibit highest level of activity during the dark phase. We conducted the studies starting at 6 p.m. (the beginning of the dark phase), and the duration depends on the test, lasting differently for each of them, as stated in the specific protocol.

4.3.1. OF test

To assess anxiety-type behavior, rats were tested in the OF test arena for 10 min, following a similar protocol to that used by Prut and Belzung (Prut and Belzung, 2003). At the start of the test rats were always placed in the same corner of the OF arena (100 cm × 100 cm). Performance was tracked according to the time spent in the central zone, the number of central area crossings, and the number of defecations, which were analyzed using the automated video tracking system (EthoVision XT software, Noldus).

4.3.2. EPM test

To acquire further evidence for anxiety-type behavior, the EPM test was conducted as previously described by Walf et al. (Walf

et al., 2007). The maze consists of a cross-sign shaped testing area, which has four 50 cm-long arms by 10 cm side. Two of the arms have no walls (open arms), while the other two are closed, placed at 50 cm above the floor. EPM closed arms have a height of 30 cm. Rats were placed in the center of the maze and allowed to freely explore the apparatus for 5 min. The total distance that they travelled, the time spent in the open or closed arms, as well as in the central area, and the number of crossings from one arm to another, were measured by the automated video tracking system mentioned above. Less time spent in the open arms and/or central area (Lister, 1990) or the number of central crossings represent measures of higher anxiety levels.

4.3.3. NOR test

The NOR test, proposed by Ennanceur and Delacour (Ennaceur and Delacour, 1988), assesses the natural preference for novel objects, while the rat explores the environment, in this case, the OF arena previously used for the OF test. The test consists of three phases: 1) habituation, in which each rat was allowed to freely explore the open-field arena for 5 min in the absence of any objects, after which it was removed from the arena; 2) familiarization, in which one rat at a time was placed back into the arena with two identical sample objects (A+A), and allowed to explore for 5 min; 3) testing, which occurs after a variable retention period, depending on the type of memory to be tested (5 min for short memory and 24 h for long memory assessment). One of the two objects from the previous phase was replaced by a novel object (A+B). Object B was placed in the same place as the familiar one. They were allowed to explore the arena for 5 min.

We used the automated video tracking system mentioned above to record the time spent to explore an object during the familiarization phase (Tf), and the time spent exploring the novel one (Tn) during the testing phase. The discrimination index, DI = (Tn−Tf)/(Tn+Tf) was calculated. DI allows discrimination between the novel and familiar objects using the difference in exploration time of the novel compared to the familiar object; this value is then divided by the total exploration time of both novel and familiar objects (Ennaceur and Delacour, 1988). DI can vary between +1 and −1, a positive score indicates more time spent with the novel object (Tn), while a negative score indicates more time spent with the familiar object (Tf). Zero score indicates a null preference. Since DI compares only three values (positive, negative, and 0), it is more helpful than the effective time to decide the object preference.

4.4. Quantitative determination of melatonin and reactive oxygen species

Brains from newborn and adult offspring were collected after decapitation to measure melatonin and ROS levels in whole brain homogenates ($n=3$ for each group in newborns and $n=4$ for each group in adults, from 3 different litters, for both measurements). Animal euthanasia was performed during daytime (between 2 and 4 p.m.) to avoid the physiological increase in melatonin secretion during the dark phase, and thus an artificial difference between PCLE and control rat pups. First measurement was done in brain collected immediately after birth, in order to validate the efficiency of the functional pinealectomy model we have used. PCLE exposed P0 pups have a low melatonin concentration in the brain, as their mother's pineal gland has been light-inhibited during the second half of the pregnancy. Second, we have measured melatonin in the brain in adulthood, at the same age as the behavioral tests were conducted, as we aimed to explain the molecular basis for the behavioral changes that we have found at that specific timepoint.

After rapid removal, the brains were washed with ice-cold saline solution, weighed, and subjected to homogenization. All of the following steps were performed on ice. The brains were homogenized in 10 volumes of phosphate buffer solution pH 7.4, using a Potter-Elvehjem homogenizer with a Teflon pestle, each sample being subjected to 20 complete strokes. The resultant fraction was treated with 10% trichloroacetic acid, 1:1 (v: v) and was subjected to centrifugation at 3,000 rpm, for 10 min. The supernatant was stored at −20 °C until assayed. Melatonin concentration and the oxidative stress level, in the form of ROS, were determined using chemiluminescent methods in a Perkin Elmer LS 50B luminescence spectrometer, as previously described (Agarwal et al., 2015; Chen et al., 2003).

Melatonin and ROS determination was based on a chemiluminescent reaction, namely the oxidation of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) by sodium hypochlorite. Diazachinone results during the reaction and turns into an aminophthalic molecule (a dicarboxylate dianion) in the presence of reactive oxygen species (ROS). When passing from excited to basal state, aminophthalate emits a photon which is registered as a chemiluminescent signal lasting for 2 s. The intensity of the emitted luminescence signal is directly proportional with the concentration of reactive oxygen species participating in the reaction (Chen et al., 2003; Lu et al., 2002; Martinez et al., 2005; Wei et al., 2007).

4.5. Quantitative determination of serotonin

Brains from adult and newborn offspring were collected after decapitation to measure serotonin levels in whole brain homogenates ($n=4$ for each group in adults and $n=3$ for each group in newborns, from 3 different litters). A rapid and sensitive fluorimetric method for serotonin assay, using o-phthalaldehyde and L-cysteine, was used as described (Curzon and Green, 1970). Brain homogenates were obtained as described above. After centrifugation for 3 min at 1,500 rpm, 2.5 mL of the supernatant was mixed with 0.25 mL ascorbic acid 3 g% and EDTA-Na_2 1% (v:v) solution, 1.25 mL borate buffer 0.5 M, pH 10, and 0.4 g NaCl. The subsequent mixture was further centrifuged for 5 min, at 2,500 rpm. 2 mL of the resulting organic phase was mixed with 5 mL n-heptane and 0.6 mL 0.1N HCl containing 0.1% L-cysteine. The phases were separated by centrifugation as described before. To determine serotonin, 0.2 mL of the aqueous phase was mixed with 0.6 mL of 0.004% o-phthalaldehyde in concentrated HCl. The mixture was heated in a boiling water bath for 15 min and then cooled in water, kept for 24 h in the dark, and fluorescence was measured in micro-cuvettes using a Perkin-Elmer spectrofluorometer. Excitation and fluorescent wavelengths were 365 nm and 470 nm, respectively. Standards were prepared from a stock solution of 50 μg/mL using serotonin creatinine sulfate.

4.6. cDNA preparation and quantification by RT-PCR

Adult rats aged 90 days from PCLE and control groups (n=4 for each group, from 3 different litters) were sacrificed at the same time point (mid-day, on the same day) and various brain regions known to be involved in the previously tested behaviors (frontal cortices, hippocampi, hypothalami, amygdalae) (Bannerman et al., 2004; Eysenck et al., 2007) were dissected on ice. Brain tissue isolation was made freehand, following dissection protocols. The hippocampus was isolated using the method of Mathis et al. (Preparation of Acute Hippocampal Slices from Rats and Transgenic Mice for the Study of Synaptic Alterations during Aging and Amyloid Pathology. Diana M. Mathis, Jennifer L. Furman, Christopher M. Norris, http://www.jove.com/video/2330/preparation-acute-hippocampal-slices-from-rats-transgenic-mice-for).

For amygdala, hypothalamus and prefrontal cortex dissection, we used the protocol of Spijker, 2011 (Spijker, 2011) and a protocol described by Trinity College of Ireland http://www.medicine.tcd.

ie/physiology/assets/docs12_13/lecturenotes/NB/Tissue_Prep_and_Protein_Assay_2012.pdf.

qPCR was performed from cDNA of different brain areas to evaluate levels of circadian rhythm-, oxidative stress enzymes-, serotonin-, and circadian-related genes. The following genes were tested: serotonin pathway synthesis genes (*Tph1, Tph2),* melatonin pathway synthesis gene (*Aanat),* melatonin receptors (*Mtr1a, Mtr1b),* serotonin receptor (*Htr1a),* serotonin reuptaker (*Scl6a4),* oxidative stress related genes (*Gss, Gpx1, Sod, Cp),* apoptosis related genes (*Bax, Bcl2),* and circadian genes (*Clock, Arntl1, Rora).* We considered these particular genes because their expression was shown to be modulated by melatonin and because their related molecules seem to be involved in neurodevelopment of the behavior-related brain areas. Thus, individual frontal cortices, hippocampi, hypothalami, and amygdalae were homogenized in TRIzol (Invitrogen, Carlsbad, CA, USA) and stored at −80 °C. Total RNA was isolated from the different brain areas using TRIREAGENT™ according to the manufacturer's instructions. 550 ng RNA were reverse transcribed (Superscript, Invitrogen™) with oligo (dT) primer in a total reaction volume of 20 μl. 1 μl cDNA was further subjected to RT-PCR using Platinum-SYBR Green RT-PCR Supermix (Invitrogen), forward and reverse primers (0.9 μM), and ROX (5-carboxy-X-rhodamine, passive reference dye) (100 nM). Primers were designed with the Primer3 software (Table S1). qPCR was performed in an Mx3000 P instrument (Stratagene, La Jolla, CA) using the following protocol: 5 min 50 °C, 2 min 95 °C, and 40 cycles of 15 s 95 °C, 30 s 60 °C. A product melting curve confirmed the presence of a single amplicon. The correct amplicon size was checked by agarose gel electrophoresis. Threshold (C_T) values were set within the exponential phase of the PCR. Normalization was performed to β_2-microglobulin, ΔC_T ($C_{T(gene)} - C_{T(\beta 2\text{-}MG)}$). Relative expression levels ($\Delta\Delta C_T$) are the difference between ΔC_T values of controls and PCLE values. The mRNA levels change was calculated as fold change $= 2^{-\Delta\Delta CT}$, where $\Delta\Delta$CT is the difference to the control average ΔCT (Livak and Schmittgen, 2001). To test whether gene expression is determined by PCLE, we performed 1,000 simulations using the function rnorm in R, with the mean parameter set to the mean of the gene expression in the respective group. Out of 1,000 simulations only 22 yielded 2 differentially expressed genes out of a total of 16 ($p=0.022$). This suggests that the differential expression of the two genes observed in the present study is not by chance.

4.7. Statistical Analysis

Nonparametric tests are used in the behavioral sciences when there is no basis for assuming certain types of distributions (Siegel, 1956). While for EPM and OFT time measurements, the data showed normal distribution in intervals, other tests like preference index and defecations had a nominal distribution. Since the number of data points was rather small, and there was no basis for assuming certain types or shapes of distributions, nonparametric tests were most suitable for data analysis (Williamsen, 1974). Additionally non-parametric tests are in general more conservative than parametric tests and make fewer assumptions about the data and the data distribution (Martin and Thompson, 2002). Thus, data from behavioral tests were analyzed using a two-sided, nonparametric Mann-Whitney-Test, assuming non-equal variances. Quantitative determinations of melatonin, reactive oxygen species, serotonin, and fold change of gene expression were subjected to a two-sided, unpaired *t*-test with Welch's correction. Data are presented as mean ± SEM or scatter plots with center lines at the mean and limits at the SD value. Significance was defined as $p < 0.05$.

Conflict of interest statement

The authors declare that they have no competing interests.

Authors' contributions

S.E.V. designed the study, performed the behavioral experiments and wrote the manuscript.

D.L.D. designed and performed gene expression experiments and wrote the manuscript.

A.E.R. contributed to behavioral experiments, data analysis and manuscript writing.

V.Z. contributed to behavioral experiments and data analysis.

D.M.C. performed part of the qPCR experiments and contributed to manuscript writing.

A.L.A., C.M.D., and A.C.N. performed melatonin, serotonin, reactive oxygen species determinations, and respective data analysis.

L.Z. contributed to experimental design and manuscript writing.

T.S. contributed to the design of gene expression experiments and manuscript writing.

A.M.Z. designed and coordinated the study and wrote the manuscript.

All authors have approved the final article manuscript.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.brainres.2016.08.031.

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