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Iron-Deficiency and Dopaminergic Treatment Effects on RLS-Like Behaviors of an Animal Model with The Brain Iron Deficiency Pattern of the Restless Legs Syndrome

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Brain iron deficiency (BID) particularly in the substantia nigra (SN) is a well-documented biological abnormality of the restless legs syndrome (RLS). It has been documented in 24 of 26 reports. These include: 7 Magnetic resonance imaging (MRI) [1–7], 7 midbrain ultrasound [4, 8–13], 4 cerebral spinal fluid (CSF) [14–17] and 5 autopsy studies [18–22]. Only 2 of all 26 studies of brain iron in RLS failed to show BID [23, 24]. These were both MRI studies using the less accurate R2 rather than R2* or R2' measure [6] with small RLS sample sizes (n=11, 12).

Two major features of BID in RLS patients define the pattern of the pathophysiology. First, The BID occurs despite normal peripheral iron stores [5, 14–16]. The critical pathophysiology is the brain and not peripheral iron status. Second, the BID of RLS involves regional more than total brain iron. It occurs mostly for some brain regions, particularly the substantia nigra (SN) in 5 of the 7 MRI studies [1–3, 5, 6], all 7 of the ultrasound studies [4, 8–13] and all 4 of the autopsy studies that evaluated SN iron [18–21]}.

The ubiquity in RLS of BID, particularly of the SN, raises the putative hypothesis that the biology of the RLS BID produces the RLS symptoms. Association, however, does not prove such a direct relation. One approach to study the nature of the BID biology relation to RLS would be an experimental study of an animal model biologically developed to reproduce the RLS BID pattern noted above. This animal model could be experimentally evaluated for effects of factors known to alter clinical RLS, e.g. dopaminergic treatments, increasing iron deficiency. These are both evaluated in the study reported in this paper. The study uses 1) an animal known to have an RLS-like iron biological pattern and 2) an established RLS-like behavioral phenotype appropriate for that animal.

Authors' contributions

All of the authors : Richard P. Allen, Byron Jones, Erica Unger and Christopher Earley contributed to the writing and revision of this paper.

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First, the study uses an animal model based on the RLS iron biology pattern noted above. Forward genetic studies of the BXD inbred strains has identified one strain that has the experimentally induced RLS BID pattern of ventral midbrain (VMB) (containing the substantia nigra) Iron deficiency (ID) without peripheral ID. Twenty-two recombinant BXD strains and their parental strains (C57BL/6J and DBA/2J) upon weaning at post-natal day 21 were placed for the next 100 days on the same pellet diets except these were either iron sufficient (240 ppm) or iron deficient (3–5 ppm) pellets (Teklad, Inc). The VMB iron between mice on sufficient vs. deficient diets varied by 35% between the strains [25]. Moreover, across the 22 strains there were no correlation between the VMB and peripheral iron loss with the iron deficiency diet [26] indicating different genetic regulation of peripheral and brain iron. Strain 40 females were identified as having the RLS iron biological pattern of significant VMB iron loss with little change in Hgb [25, 27, 28]. (Note that in the following the reference to BXD40f is used to indicate BXD40 females. BXD40 males did not show the same iron management profile as the females and thus were not evaluated in the study.)

Second, the study evaluates experimental effects on a previously established RLS-like behavioral phenotype, i.e. increased motor activity in the last part of the active cycle [29]. This RLS-like behavior is based on the RLS clinical diagnostic criterion of a circadian pattern (worse at the end of the day) of an urge or awareness of a drive to move [30].

The study evaluates the RLS-like behaviors in the BXD40f animal model of the RLS iron biology. It tests two important experimental hypotheses linking BXD40f BID to RLS-like behavioral changes, i.e. 1) increasing BXD40f BID will increase the RLS-like behaviors following the clinical pattern seen for increased RLS severity, and 2) Dopamine agonist treatment of the BXD40f will reduce the RLS-like behaviors produced with increased BID. These hypotheses if confirmed would add to the validation of the BXD40f as a useful animal model to study RLS and also support the putative link of the BID biology to RLS.

Methods

Animals

The study follows the animal care guidelines of the National Institutes of Health with Penn State Institutional Animal Care and Use committee approval. The BXD40f were maintained under constant light-dark cycle (05:00– 17:00 hours, lights-off) and housed with 40% relative humidity and 23 ± 2 degrees C ambient temperature. Mice were group housed 3–4 per cage from weaning until the G2 E-Mitter telemetry devices were implanted 10 days prior to the beginning of data collection. After surgery, the mice were housed one per cage.

Dietary treatment

13 BxD40 females upon weaning at post-natal day 21 were randomly assigned 6 to iron-sufficient and 7 to iron-deficient pellet diet (120 ppm or 3–5 ppm) (Teklad, Inc.) given *ad libitum*. Deionized distilled water was also provided *ad libitum*. This followed the procedures previously demonstrated to produce iron deficiency (ID) in the ventral midbrain without significant peripheral iron loss for these BXD40f on the iron-deficient diet [25, 27,

28] . The sample sizes were similar to the 5 –9 mice per condition that showed statistically significant RLS-like behaviors in a prior study [29]. The effect size from this prior study was a mean difference of approximately 1.5 standard deviations giving 80% power for samples sizes of 6 comparing means from 2 samples with a one-sided test [31, 32].

RLS-like behavioral measures

A prior study [29] comparing BXD40f to other BXD strains maintained on iron-sufficient diets identified a significant RLS-like behavior. This was based on essential diagnostic features defining RLS, i.e. a strong circadian increase in the urge or awareness of the drive to move when inactive at the end of the normal waking or active period of the day [30]. This diagnostic feature of RLS leads to a clinical pattern of increased restlessness and motor activity at the end of the waking period [33, 34]. Not only is the RLS restlessness and urge or drive to move expressed more in the evening but also as the condition becomes more severe its expression occurs earlier in the day as most clearly shown by RLS augmentation [35]. This strong circadian feature of RLS was used by one of the first RLS scales which defined severity by how early in the day symptoms usually start [36]. Thus, for animals one major RLS-like behavioral phenotype involves increased motor activity in the end of the active period. A corollary RLS-like behavioral phenotype would be decreased rest periods in the end of active period consistent with the essential RLS diagnostic criteria of rest inducing the RLS drive to move [30]. The RLS-like behavior of increased activity in the last 2 hours of the active cycle was shown in a prior study to be a unique feature of the BXD40f and not other BXD strains examined when fed an iron-sufficient diet [29]. (See figure 1) These BXD40f also had the largest circadian change in VMB iron producing the lowest VMB iron content in the active but not inactive period. Thus, the BXD40f showed both the lowest active-period VMB iron levels and the greatest end-of-active-period activity when on an iron sufficient diet.

Hypothesis testing

It was hypothesized that the BXD40f on an ID diet known to reduce VMB iron but not Hgb would show an RLS-like behavioral pattern similar to the clinical pattern of worsening RLS [36], i.e. onset of the increased activity at the end of the active period occurring earlier than that observed for the iron-sufficient diet condition. Similar changes were hypothesized to occur for decreased resting time. It was also hypothesized that dopamine agonist treatment would reduce ID diet effects on both of these RLS-like behaviors. This study was thus planned assuming the RLS-like behaviors for the BXD40f would occur one hour earlier on the ID diet than the 2-hours before end of the active period previously observed on the iron-sufficient diet shown in figure 1 [29].

Activity and rest measurements

Activity levels were measured using implanted G2 E-Mitter telemetry devices (Respironics/Mini Mitter Company, Bend OR). Mice were anesthetized on postnatal day 92–93 using 3% isoflurane, and telemetry devices were implanted into the peritoneal cavity following the manufacturer's protocol. The mice were then housed one per cage and had a 7-day recovery period. Activity recordings were then obtained by placing home cages on ER4000 energizers/receivers. Gross body movement events were determined by changes in signal

strength as the mouse moved on the receiver board. This counts gross body movements but does not indicate nature or velocity of the movements. The total activity count for body movements was recorded for each 6-minute period for 24 hours of each day in the study using the VitalView program.

The primary outcome measures were the total activity counts for each hour and rest time occurring during each hour. Rest time was defined as any 6-minute period with no activity. A rest epoch was defined as any consecutive sequence of 6-minute rest periods that could be as short as one 6-minute period. The total minutes included in rest periods and the number of rest epochs were evaluated for each hour.

Medication Treatment

It was hypothesized, as noted above, that an ID diet would worsen the RLS-like state with an onset of the RLS-like circadian behavioral phenotype earlier than displayed by BXD40f fed an iron-sufficient diet [29]. The medications were accordingly given IP at 3 rather than 2 hours before the end of the dark (active) period starting at 100 days post-weaning. Five treatments were used: saline (vehicle only), 12.5 mg levodopa, 25 mg levodopa, 0.5 mg quinpirole, and 1.0 mg quinpirole. Each mouse received all 5 treatments in the order given above, but each was started at a different point in this order. Two animals were started at each of 0.5 mg quinpirole and at 25 mg levodopa doses and one animal was started at each of 1.0 mg quinpirole, saline, and 12.5 mg levodopa doses. There were two days between each treatment administration.

Data analyses

The analyses focused on the *a priori* hypothesized times of expected maximal effect sizes. Further analyses explored possible differences during the remaining times studied.

The hypothesized earlier onset of increased activity for the iron-deficient compared to iron-sufficient diet BXD40f mice was tested by a between subjects one-tailed unequal variance t-test of activity levels for the 3rd hour before lights on. It was hypothesized that this would be greater for the iron-deficient (ID) than the iron-sufficient diet animals after saline injection. The differences in the other 2 hours before lights and the differences for each 6-minute period across the 24-hour day were evaluated as exploratory analyses of ID compared to iron-sufficient diet animals. The rest measures were also examined, but floor effects prohibited statistical analyses.

The hypothesized benefits for each of the dopaminergic treatments were similarly evaluated for the first hour after the treatment for total activity, minutes of rest time and number of rest periods using within-subjects unequal variance t-tests. It was hypothesized that the treatment would decrease overall activity and increase resting time and number of rest periods. Exploratory analyses examined dose effects and the persistence of effect over the remaining 2 hours before lights on.

Results

The analyses confirmed the hypothesis of significantly increased activity during the 3rd hour before the end of the active period for animals on the ID compared to control diet (see table 1). There were, however, no significant differences between animals on the ID vs. control diets for the remaining 2 hours of the active period. Both ID and control animals show the same increased activity for these last 2 hours of the active period that is unique for the BXD40f (see figure 1). There were no minutes of resting for either ID or iron-sufficient diet animals in the 3rd hour before end of the active period and only 3 of the 13 animals had any rest activity for the 2nd hour. All but 3 of the animals (2 with ID and 1 with iron-sufficient diet) had some rest time in the last hour before lights-on but there were no significant differences in rest measures between ID and iron-sufficient diet animals (average \pm sd: minutes resting 11.1 ± 9.4 vs. 11.0 ± 12.8 , $p > 0.1$, number rest epochs 1.4 ± 1.1 vs. 1.2 ± 1.0 , $p > 0.1$, respectively).

Exploratory analyses compared the 24-hour pattern of activity levels of animals on ID vs. iron-sufficient (control) diets. The ID compared to iron-sufficient diet animals had generally more activity throughout the active but not the inactive period (see figure 2), but the differences were not persistently statistically significant except for a 90-minute period from 13:06 to 14:36. Figure 2 shows all the times with statistically significant differences ($p < 0.05$, separate unequal variance t-tests for each time period.)

The analyses also confirmed the hypothesized dopaminergic treatment effects for the 1st hour after treatment. Average activity significantly decreased and the rest minutes and number of rest periods significantly increased for all treatments. The animals on the 25 mg but not the 12.5 mg dose of levodopa showed significant persistence of effect for reducing activity in the 2nd hour after treatment but neither dose produced significant differences from saline at 3 hours after treatment. The 1.0 mg quinpirole dose showed persistent significant effect for reducing activity and increasing both rest time and number of rest periods for the 2nd hour after treatment. The 0.5 mg quinpirole dose compared to placebo showed significant increase in number of rest periods but not in the other measures for the 2nd hour after treatment (See table 2 and figure 3).

Discussion

These data provide strong support for the hypothesis that the biology of RLS BID produces RLS symptoms. The BXD40f were selected because they uniquely show the two basic BID features of RLS: i.e. regional BID involving VMB and BID without peripheral ID [25, 27]. A prior study showed that the BXD40f also uniquely showed the circadian RLS-like behaviors that were not shown by other strains without the BID features of RLS [29]. The RLS-like behaviors were present only when the BID features of RLS were present. This current study further demonstrated that dietary ID known to make BID worse in the BXD40f strains [25, 27] also made the RLS-like behaviors occur earlier following the same circadian pattern for effects of increasing RLS severity, i.e. starting earlier in the evening more than persisting longer after starting [36]. Finally, levodopamine and dopamine agonist treatments known to be dramatically effective for reducing RLS symptoms [37–39] similarly reduced

the RLS-like behaviors in these BXD40f. Thus, the BXD40f provide a useful animal model for RLS serving to support the hypothesized relation between RLS BID biology and RLS symptoms. How the BID biology produces these symptoms remains to be further explored, but the BXD40f provide a useful animal model for exploring these relations.

Four features of this BXD40f model deserve special note. First, the model was based on considerations of RLS iron biology attempting to produce the brain iron deficiency without peripheral iron deficiency. The observed behavioral circadian changes cannot be explained by anemia or peripheral iron changes. They reflect an effect of brain iron deficiency on central nervous system (CNS) functioning. It should be noted, however, that concurrent anemia maybe less of a problem for studies in other rodents of BID effects not involving behaviors, e.g. such studies have established BID effects on brain dopamine biology similar to that seen in RLS patients [40–43]. The peripheral anemia seems unlikely to alter these relations within the brain. The genetic differences, however, may have some effect on these relations, but this remains to be evaluated.

Second, the model does not involve any direct manipulation of dopamine or dopaminergic systems. Nonetheless, the dopaminergic treatments reducing RLS circadian urge to move also reduce the RLS-like circadian pattern of excessive activity and lack of resting time in these mice. This may be a non-specific effect of dopaminergic treatment but it is also consistent with the large body of literature relating brain iron deficiency to the dopamine status in RLS [41, 44–47]. Particularly striking are the findings that in rats VMB BID produces RLS-like abnormalities in striatal dopamine [46, 48, 49] that can be reversed by directly infusing iron into the VMB [50]. The RLS-like behavioral response to dopaminergic treatment thus supports viewing the BXD40f as an animal model producing the putative iron-dopamine relations to RLS symptoms.

Third, the behavioral measurements used in this study involve not just changes in activity but also an attempt to measure the duration of the animals' resting periods. This is unfortunately limited by the technology used that cumulated activity over consecutive 6-minute epochs. Mice often have much shorter rest periods, even one-minute periods with sleep. Finer grain analyses of inactive resting periods might, therefore, improve sensitivity of this measurement approach. But the data obtained here are nonetheless interesting. The BXD40f with or without ID diet had in the last part of the wake period almost no 6-minute periods without activity. Six-minute rest periods were, however, seen for all of the animals after levodopa and dopamine agonist treatment indicating somewhat dramatic treatment effects similar to that reported by RLS patients treated with levodopa and dopamine agonist drugs. It deserves note that the RLS diagnosis specifies not only the increased drive/urge to move in the last part of the day but also that it is relieved by movement and brought on by rest [30]. Thus the roles of rest and activity differ in clinical RLS, one engenders and the other decreases the RLS drive to move. While related these measure somewhat different aspects of RLS. In this study of the RLS-like rest and activity behaviors the activity measure accounted for only about 50–55% of the variance in the rest measures. It may be useful to explore both of these RLS-like behaviors in future studies.

Fourth, the 24-hour activity patterns reveal two major effects. First, the data from the animals on iron-sufficient diets replicate the findings previously reported of this RLS-like increased activity in the last two hours of the active period [29]. Second, this RLS-like pattern of increased activity in the last part of the active period starts as expected earlier in the day. This study was designed assuming a one-hour earlier onset, but the data showed about a two-hour earlier onset of the RLS-like behavior, starting at about 13:00. The ID animals' activity remained overall high compared to controls for the remainder of the hours before end of the active period. There was, however, also some indication of an overall increase in activity levels for the ID compared to control animals across the active but not the inactive period. These differences were generally not statistically significant except for the 4th and 3rd hour before the end of the active period and may not replicate in future studies. The ID diet may, however, produce a general increase in activity levels during the active period, but even then, the RLS-like pattern persists with the unique for BXD40f increases in activity during the last hours of the day that does not occur for the other BXD strains. The BXD40f on the ID diet have this increase occurring much earlier in the last part of the active period and persisting possibly even into the very initial part of inactive period. (see fig 2). This pattern matches well the unique clinical feature of increased RLS severity producing earlier onset of symptoms with some less prominent extension later in the day. This feature was used to develop one of the first scales for RLS severity [36].

This study has some significant Limitations. There was only one day of treatment for each condition and no repeated measure for the treatment. The animals had not been pre-conditioned to an IP injection at 14:00 each day. The initial experience and the expectation of this repeating likely altered their behavior. The order of medication dosing was systematically altered to counterbalance these effects across the animals, but this remains a potential problem for these data. These problems, overall, tend to work against finding significant treatment differences as seen in this study.

This study is also limited by the inherent differences between animals and humans, particularly for diseases involving sensory symptoms. Animal RLS-like behaviors were established based on the hypothesis that the animal's response to an RLS-like circadian rest-induced urge/drive to move will at the end of the active period lead to increased activity and also disruption of resting reducing time in resting periods. RLS patients show these behaviors [33, 34]. The assumptions defining the RLS-like behaviors are similar to assumptions accepted for other animal models of sensory symptoms including those defining anxiety-like behaviors in animals, e.g. open field activity [51, 52].

This study was limited to only the BXD40f strain. Future studies evaluating behavioral effects of the ID diet for other strains without the low VMB iron would have been informative. The prior study [29] documented that on the iron-sufficient diet these other strains unlike the BXD40f do not show the RLS-like behavior (See figure 1). Presumably the other strains would not develop the RLS-like behavior even when on the ID diet since they do not have significant VMB iron loss with the ID diet. Similarly, the dopaminergic treatment with these strains would be expected to have little specific effect on the activity patterns late in the active phase (RLS-like behaviors). This remains to be evaluated in future studies.

Finally, the brain and peripheral iron status was not directly tested for these animals. Prior studies, however, have clearly established that the methods used in this study would produce in BXD40f the brain without peripheral iron deficiency [25, 27, 28]. The results in this study, therefore, need to be interpreted in the context of the extensive literature on the BXD40 strains and in particular the BXD40f strain.

Conclusions

Overall the major hypotheses for this study were confirmed. The BXD40f circadian RLS-like behaviors are both exacerbated by iron deficiency and reduced by levodopa and dopamine agonist treatment similar to these effects on RLS symptoms. These results provide support for viewing the BXD40f as a useful animal model for RLS and support the putative role of the RLS brain iron deficiency biology producing RLS symptoms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

Iron deprived BXD40f mice show RLS-like low brain with normal peripheral iron.

RLS-like behaviors occur earlier for BXD40f on iron-deficient vs. sufficient diets.

Dopaminergic treatment reduces RLS-like behaviors of iron-deprived BXD40f

Increasing brain iron deficiency in BXD40f increases RLS-like behaviors

BXD40f provide a useful animal model of RLS

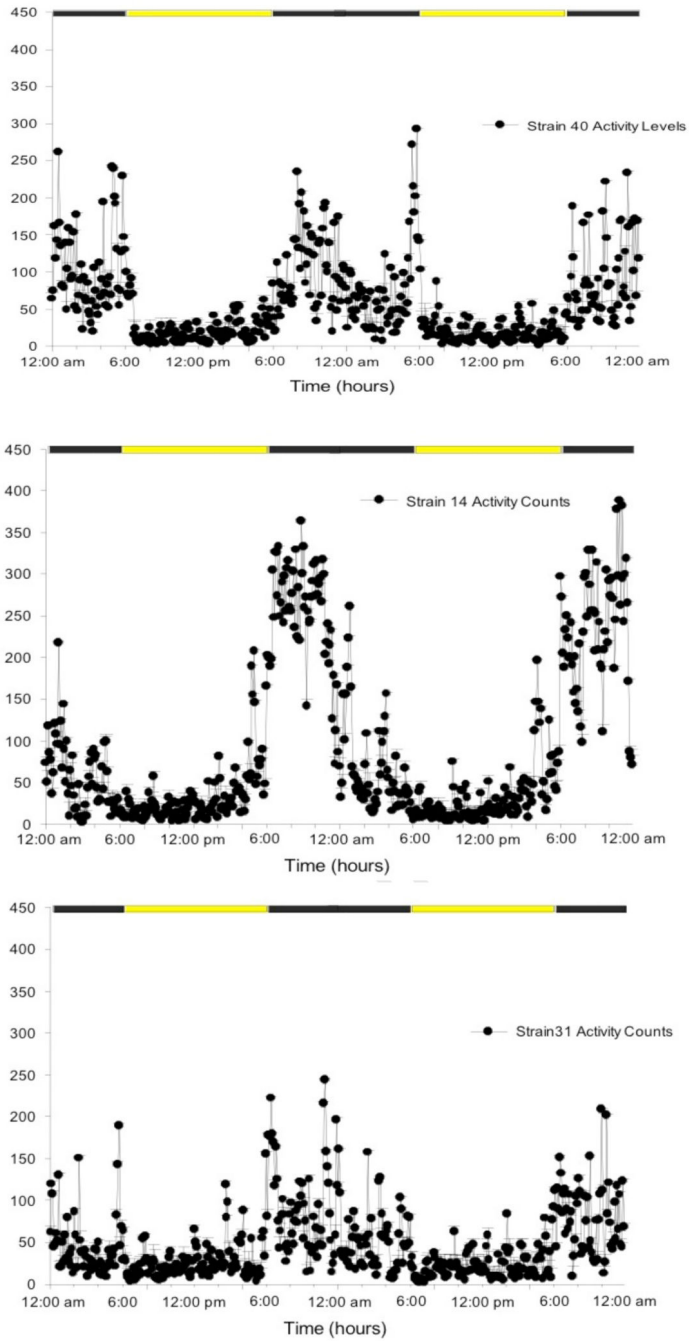


Figure 1: Average total activity counts for every 6-minute period over 2 days for 3 female BXD strains n=8 for strains 40 and 31, n=7 for strain 14). The lights off active period is from 6 PM to 6 AM. Note for BXD40f only (top graph) the repeated peak increases in activity during the last 2 hours of the active cycle from 4 – 6 AM on each day. This one period of time, 2 hours before the end of the active period, has consistently greater activity for the BXD40f compared to all seven other strains evaluated as reported by Earley et. al, Sleep Med, 2019. (Graphs provided by Erica Unger.) The two other

strains shown here for comparison are chosen for overall high (strain 14) or low (strain 31) activity, neither shows increased activity at 4– 6 AM.

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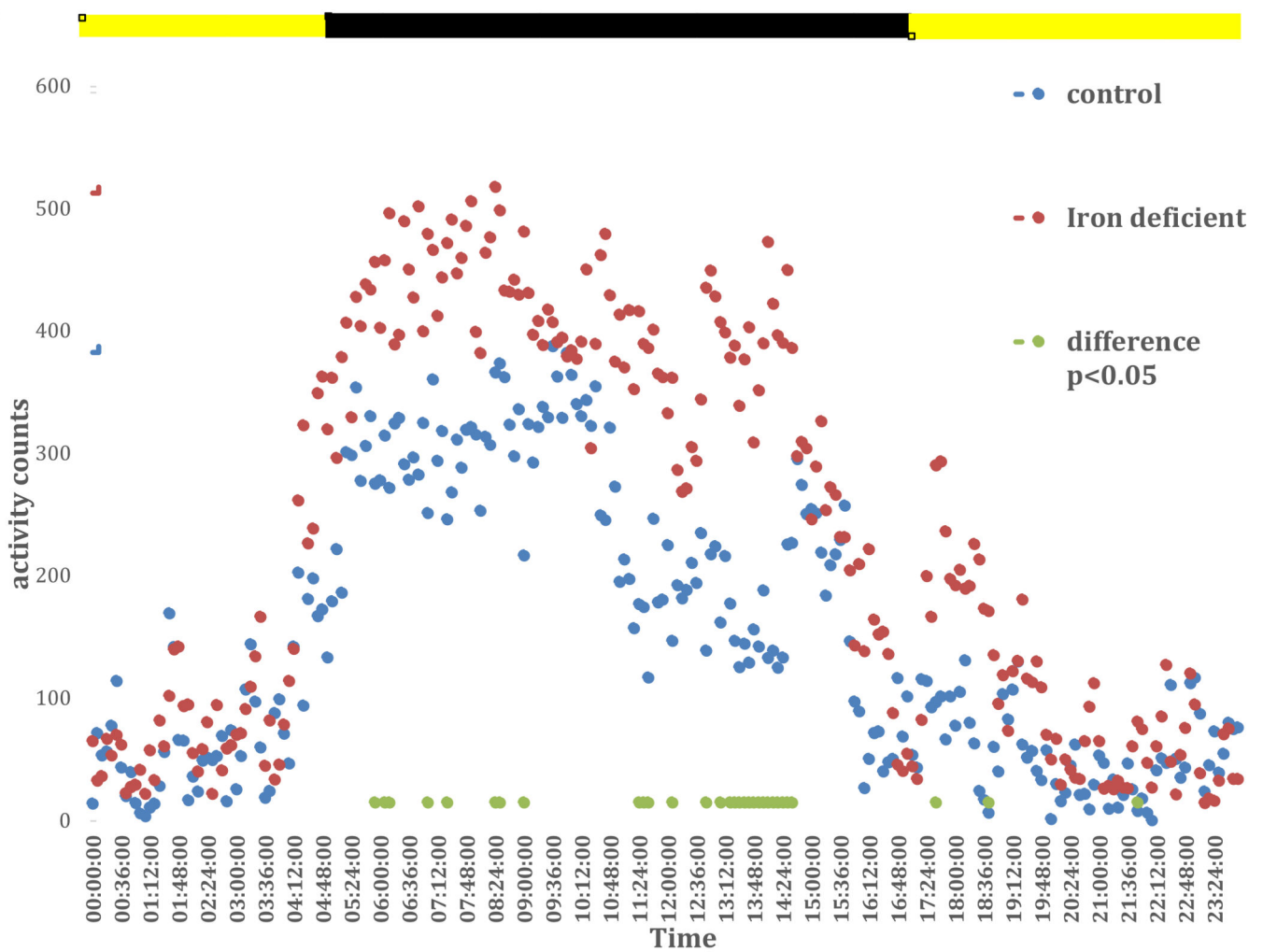


Figure 2:

Average total activity for every 6 minutes for the day with saline injection for BXD40f on Iron-sufficient (control, n=6) and iron-deficient (ID, n=7) diets.

Lights off active period is from 5 AM to 5 PM shown by the dark bar at the top of the figure. Note the control replicates the increased activity to the levels seen earlier in the day for the 2-hour period before lights on (17:00 – 05:00) reported in a prior study (Earley et al, 2019) and shown in figure 1. The ID show the same pattern except it starts about 2 hours earlier and persists even into the very first part of the inactive period. Overall activity during the active period is increased for the ID compared to the control but this is a consistent statistically significant difference only during 13:06 – 14:36, the 4th and 3rd hours before end of the active cycle.

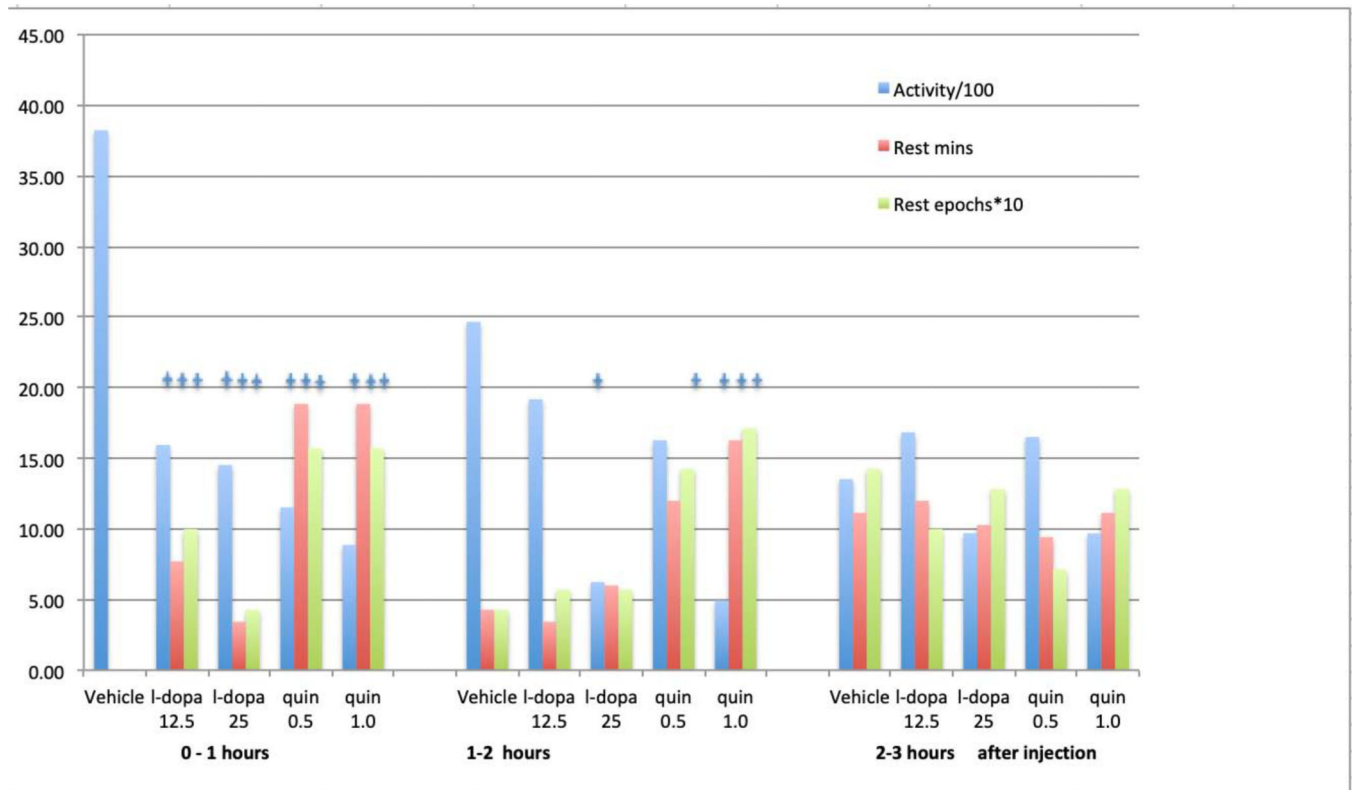


Figure 3:

RLS circadian phenotype behavior for each treatment condition and each of the 3 hours after treatment – ending at onset of the inactive (light) period. (n=7 Iron deprived BxD40f)

The stars over the bars indicate statistically significant differences ($p < 0.05$) compared to vehicle (saline). The same 7 mice (BxD40f) after post-weaning dietary iron deprivation received each of the five treatments in the same order but with rotating starting points in the order. There was one day between each treatment.

Table 1:

Activity counts/1000 average \pm standard deviation during each of the last 3 hours of the active period for mice on iron-deficient or iron-sufficient diets treated with saline.

Hours before end of active period	2– 3 hours	1 – 2 hours	0 – 1 hour
Iron-sufficient diet	2.0 \pm 0.7	2.1 \pm 0.4	0.6 \pm 0.4
Iron-deficient (ID) diet	3.8 \pm 1.1 *	2.5 \pm 2.2	1.4 \pm 0.2

* Difference between iron-deficient and iron-sufficient $p=0.002$, t test, unequal variance

There were no other significant differences between diet groups ($p<0.05$).

Almost all of the animals on both iron-deficient and iron-sufficient diets had no rest times for the 2–3 and 1–2 hours before end of the active period these and showed no difference in rest minutes or epochs for the last hour before end of the active period.

Activity counts: Sum for each animal of number of gross body movements in each 6-minute period detected by the G2 E-Mitter telemetry. Average and SD total activity determined for 6 animals on iron-sufficient diet and 7 on iron-deficient diet.

Table 2

Average \pm SD of total activity counts/1000, minutes resting and number of rest periods for the each of the 3 hours after each treatment – last 3 hours of the active period for the BXD40f on an ID diet.

Treatment	1 st hour			2 nd hour			3 rd hour		
	Activity/ 1000	Mins rest	# rest periods	Activity/ 1000	Mins rest	# rest periods	Activity/ 1000	Mins rest	# rest periods
Saline	3.8 \pm 1.1	0.0 \pm 0	0.0 \pm 0	2.5 \pm 2.2	4.3 \pm 5.7	0.4 \pm 0.5	1.4 \pm 1.6	11.1 \pm 9.4	1.4 \pm 1.1
12.5 mg L- dopa	1.6 \pm 1.1 ****	7.7 \pm 8.3 *	1.0 \pm 1.0 *	1.9 \pm 1.2	3.4 \pm 4.7	0.6 \pm 0.8	1.7 \pm 1.5	12.0 \pm 9.8	1.0 \pm 0.8
25.0 mg L- dopa	1.5 \pm 1.0 ***	3.4 \pm 4.7 *	0.4 \pm 0.5 *	0.6 \pm 0.2 *	6.0 \pm 10.0	0.6 \pm 1.0	0.9 \pm 0.7	10.3 \pm 8.3	1.3 \pm 0.8
0.5 mg Quin	1.2 \pm 0.8 ****	18.9 \pm 16.0 **	1.6 \pm 1.1 ***	1.6 \pm 1.2	12.0 \pm 9.8	1.4 \pm 1.0 *	1.7 \pm 1.2	9.4 \pm 12.0	0.7 \pm 1.0
1.0 mg Quin	0.9 \pm 0.7 ****	18.9 \pm 15.3 **	1.6 \pm 1.1 **	0.5 \pm 0.2 *	16.3 \pm 9.6 *	1.7 \pm 1.0 *	1.0 \pm 0.5	11.1 \pm 4.1	1.3 \pm 0.5

Significance for within subject t test compared to saline

p<0.001

p< 0.005

**
p< 0.01

*
p< 0.05

Activity counts: Sum for each animal of number of gross body movements in each 6-minute period detected by the G2 E-Mitter telemetry. Average and SD total activity determined for 6 animals on iron-sufficient diet and 7 on iron-deficient diet.