A novel approach for studying brain iron homeostasis in animal models using stable isotope tracers

Metallomics

ARTICLE

Imbalance of iron influx and efflux causes brain iron accumulation over time in the healthy adult rat

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Brain iron accumulation is supposed to play a central role in neurodegeneration by inducing oxidative stress. Currently it is unknown to which extent iron entering brain over lifetime exchanges with body iron or if uptake of iron is unidirectional without significant efflux from brain. To study brain iron dynamics *in vivo*, up to three stable isotope tracers were fed continuously with a standard rodent diet up to 5 months to healthy adult male Wistar rats (n=8) in a staggered design. Brain iron uptake was found to be bi-directional but iron influx and efflux were unbalanced leading inevitably to brain iron accumulation over time. Brain iron turnover was found to be very low at a half-life of ca. 9 months for tracer iron entering brain. Observed tracer accumulation in brain iron can be extrapolated to an increase of brain iron by ca. 30% in the healthy rat from early adulthood to the end of their lives. In contrast to current beliefs that brain uptake of dietary iron is negligible during adulthood following short-term radiotracer studies, our long-term feeding experiments point to a possible role of diet in brain iron accumulation and, subsequently, neurodegeneration.

Introduction

Iron is the most abundant trace element in brain. The ease in donating and accepting electrons renders iron an indispensable cofactor for a plethora of enzymatic reactions involved in neurogenesis, brain development and cognition sustenance [1]. However, iron is potentially toxic if not bound to iron binding proteins such as transferrin, ferritin or iron containing enzymes. Unbound iron can induce oxidative stress via catalyzing the formation of ROS, i.e. highly reactive oxygen species [2, 3].

Age-related brain iron deposition has been observed in all species examined, including mice, rats, monkeys and humans [4-9]. Progressively elevated brain iron with age may pose a threat to the brain's oxygen-rich environment by inducing ROS formation [10-12]. Many lines of evidence support now the hypothesis that brain iron accumulation with age might be implicated in the pathogenesis of age-related neurodegeneration with iron induced oxidative stress as the underlying mechanism [13, 14]. It has been speculated that iron deposition in brain could be reflective of dietary iron exposure and an overall accumulation of iron in the body.

In an earlier study we have pioneered the use of stable iron isotopes for tracing brain iron uptake from diet in adult rats [15]. In agreement with earlier radiotracers studies [16-18] we found that absolute brain iron uptake was very low (< 0.001% of dietary iron). At the same time we could show that this small amount constituted about 9% of total brain after four months of tracer/iron feeding. This observation became only possible by using stable isotope tracers which can be fed continuously in

contrast to radiotracers which were commonly given once by injection in earlier studies. The key question that remains now is whether iron is imported in brain without export or if more iron is imported than exported from brain and at which rate. The first scenario would not affect brain iron content in the long-term and tracer influx would reflect brain iron turnover. The second scenario would point to gradual accumulation of iron over time.

In our first study we fed a single tracer continuously to mimic dietary iron influx into brain. In the present study, we fed up to three stable isotope tracers to rats in a staggered design to assess iron influx/efflux and the half-life of dietary iron in brain. As in our first study, we conducted experiments in adult rats since the rate of whole brain iron accumulation slows down after the first three decades of life [4] and it is possibly the amount of iron accumulated after that contributes mainly to the development of the disease.

Experimental section

Study Design

The fate of dietary iron was investigated by feeding up to three different stable isotope tracers (^{54}Fe, ^{57}Fe and ^{58}Fe) with the diet. While the dual-isotope feeding scheme provides a qualitative assessment of brain iron balance, the triple-isotope feeding scheme permits to study iron dynamics quantitatively by comparing rates of iron influx and iron efflux and by

estimation of the half-life of iron taken up by the brain, respectively.

The dual isotope feeding scheme involves the staggered administration of two different stable iron isotopes, Tracer 1 (T_1; ^{57}Fe) and Tracer 2 (T_2; ^{54}Fe) which are fed continuously over an equal duration of time. Two possible scenarios are illustrated in Fig. 1. If there is no export of iron after its deposition into brain, tracer amount ratios in brain must equal that of the feed at the time of sacrifice (see Fig. 1a). The more of imported iron is exported from brain, the larger is the difference in the amount ratio of both tracers in brain and feed at the end of the study (see Fig. 1b).
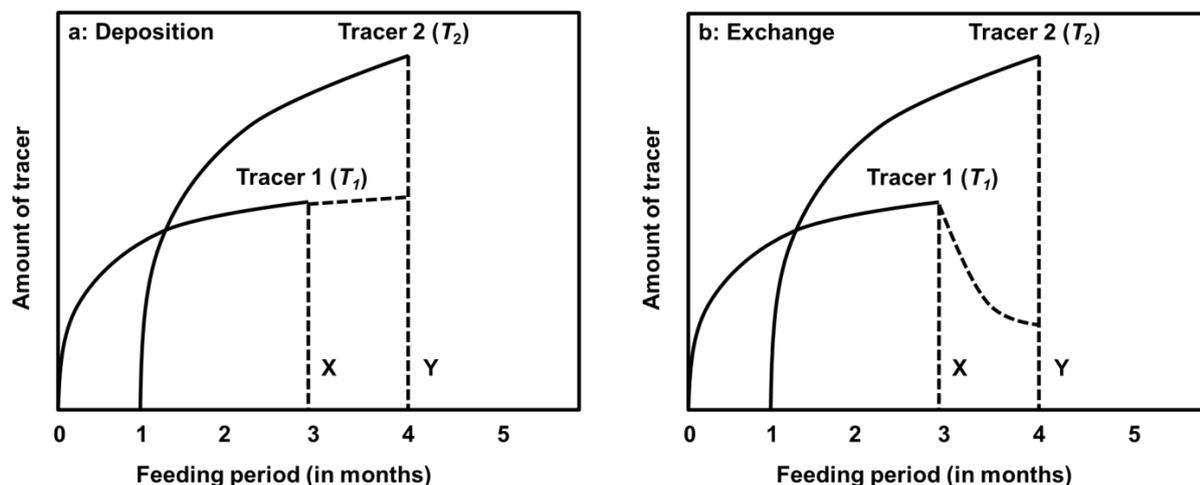

Fig. 1 Dual-isotope tracing for studying iron import and export from organs/tissues. Tracer 1 (T_1) is administered from month 1 to 3 and Tracer 2 (T_2) from month 2 to 4 together with the feed. Vertical lines X and Y indicate the time points when tracer administration is terminated. Dashed curve lines represent the fate of the tracer thereafter. In Fig. 1a, iron is deposited in the brain in an uni-directional manner, resulting in the accumulation of both tracers in the brain over time. Without iron export from brain, the amount ratio of both tracers in brain should be equal to the amount ratio in the consumed feed. In Fig. 1b, iron enters the brain in a bi-directional manner. If iron is exported from brain, the amount of the first tracer in brain will decrease when tracer administration is terminated. The amount ratio of both tracers in the brain is then lower than the ratio in the consumed feed.
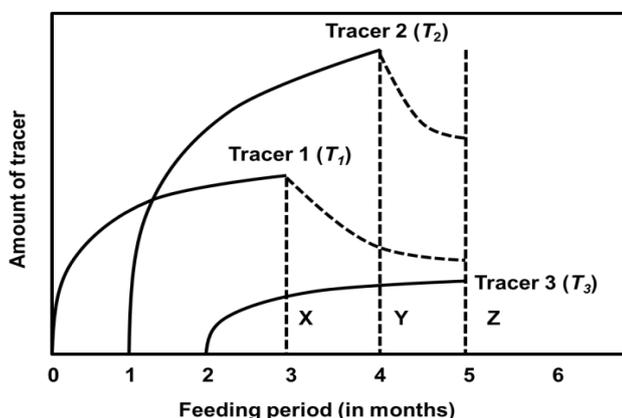

Fig. 2 Triple-isotope tracing for modeling kinetics of iron release from organs/tissues. The first tracer (T_1) is administered from month 1 to 3, the second tracer (T_2) from month 2 to 4 and the third tracer (T_3) from month 3 to 5. Vertical lines X and Y indicate time points when tracer administration is terminated. Dashed curve lines represent the fate of the tracer thereafter. At the time of sacrifice (Z), amount ratios of tracers in brain differ from that of the feed when tracer has been exported from brain. Differences in amount ratios T_1/T_3 and T_2/T_3 in brain will be proportional to the rate by which deposited iron tracer is released from brain.

The triple-isotope feeding scheme involves the staggered administration of three different iron isotopes, Tracer 1 (T_1; ^{57}Fe), Tracer 2 (T_2; ^{54}Fe) and Tracer 3 (T_3; ^{58}Fe), respectively, see Fig. 2. If iron taken up from diet is exported from brain, tracer amount ratios in brain should be lower than that of the feed. For iron export, the amount ratio of T_1 to T_3 in the brain relative to that in the diet should be lower than that of T_2 to T_3, since T_1 is administered first, followed by T_2 and lastly T_3. Differences in amount ratios between tracers, i.e. $n(T_1)/n(T_3)$ and $n(T_2)/n(T_3)$ can be used to model kinetics of iron release from brain.

Animals

Eight male Wistar rats (6 to 8-month old, body weight 625 ± 48 g) were obtained from a local supplier (Laboratory Animals Centre, LAC) and housed individually at a temperature of 21 ± 1 °C, with a humidity of 55 ± 5 % and a 12 hour light/dark cycle. Rats were randomly assigned to the two different feeding groups, *DI* (dual-isotope feeding scheme) group (n=3) and *TI* (triple-isotope feeding scheme) group (n=5). The body weight of the rats and the amount of water consumed by the rats were monitored weekly. All experimental protocols were reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of National University of Singapore (NUS).

All rats had *ad libitum* access to a standard rat chow diet (Teklad 2018S Rodent Diet, Harlan, Indianapolis, USA; iron concentration 200 mg/kg) and isotopically enriched drinking water during the course of the study. In the *DI* group, the feeding period lasted for 4 months. The first tracer T_1 (^{57}Fe) was fed during month 1-3 while the second tracer T_2 (^{54}Fe) was fed during month 2-4 via the drinking water (see Fig. 1). The average concentrations of ^{54}Fe and ^{57}Fe in the drinking water for the *DI* group was 11.215 ± 0.021 and 5.207 ± 0.045 µg/g respectively. In the *TI* group, the feeding period was extended to 5 months to allow for feeding of an additional enriched

stable iron isotope. As for the *TI* group, all tracers were administered via drinking water. The first tracer T_1 (^{57}Fe) was fed during months 1 to 3, the second tracer T_2 (^{54}Fe) during month 2-4 and the third tracer T_3 (^{58}Fe) from month 3 to 5, respectively (see Fig. 2). The average concentrations of ^{54}Fe, ^{57}Fe and ^{58}Fe in the drinking water for *TI* group were, 11.201 ± 0.025, 5.216 ± 0.030 and 3.004 ± 0.016 μg/g, respectively.

In order to maintain dietary iron supply in both groups constant over the course of the study, differences in iron intake induced by differences in tracer concentration in the drinking water were balanced by adding iron of natural isotopic composition to the water. As in our previous study, the pH of the drinking water was maintained at 2.5 in order to ensure no precipitation of iron. Water acidity should not affect water consumption as rats do not have any taste receptors for water [19]. Isotopic enriched water was changed every 3-4 days. The masses of the drinking bottles before and after each change of water supply were recorded in order to accurately assess dietary iron tracer intake over the course of the study.

Stable isotope labels

All isotopic labels were prepared from iron metal (Chemgas, France), isotopically enriched in ^{54}Fe (99.873 ± 0.010 % ^{54}Fe), ^{57}Fe (95.910 ± 0.011 % ^{57}Fe) and ^{58}Fe (99.9109 ± 0.0013 % ^{58}Fe). Isotopically enriched iron spikes were dissolved in 37% HCl and diluted with water to yield 5 M HCl solutions. Iron concentrations were determined against a commercially available standard (Titrisol®; FeCl$_3$ in 15% HCl, 1000 mg Fe; Merck). Negative thermal ionization mass spectrometry (NTI-MS) was used to determine the isotopic composition of the spike solutions using the method developed by [20] with modifications. The concentrations of iron in the spike solutions were determined by reversed isotope dilution mass spectrometry against the prepared iron standard.

All sample preparation work was performed in a class 10,000 clean room equipped with class 10 fume hoods. Only teflon labware or disposable plastic labware was used. Plastic lab ware was pre-cleaned by soaking in 10% (v/v) HNO$_3$ (Merck, USA) for 12 hrs and teflon labware by boiling in half-concentrated HNO$_3$. Acids and reagents used were of analytical-reagent grade. Acids were purified further by sub-boiling distillation before use. Water used throughout the study was ultrapure 18.2 Ω Milli-Q® water (Millipore, Billerica, Massachusetts, USA).

Blood and tissue sampling

Blood samples were collected monthly for the determination of hemoglobin concentration and to assess changes in iron isotopic compositions in the rat blood throughout the feeding period. During the study, blood was collected from the tail artery of the rats using 23G needles under light isoflurane anesthesia (Webster Veterinary Patterson

Company, USA). At the end of the study, blood samples were obtained via cardiac puncture. Blood samples were collected in EDTA coated vaccutainers and stored at -20 °C before elemental and isotopic analysis.

Tissue sampling followed the procedure as described for our earlier study [15]. In brief, animals were sacrificed by hyper-perfusion, i.e. effective removal of blood from tissues by washing of vessels and organs *in situ* with excessive volumes of Ringer's solution (NaCl 0.85%, KCl 0.025%, CaCl$_2$ 0.03%, NaHCO$_3$ 0.02%). Heparin (5 I.U. heparin/mL) was added to the solution to prevent blood clotting during the perfusion process. Organs such as heart, kidneys, liver, muscle and brain were harvested and stored at -20°C prior to isotopic analysis.

Iron elemental and isotopic analysis

The collected samples were freeze dried and grinded into fine powders. For *DI* group samples, a weighed aliquot of the homogenized tissue was transferred into a teflon vessel together with a known amount of enriched ^{58}Fe label to determine the amount of natural iron in the sample following isotope dilution principles (see Data analysis). The mixture was mineralized in a microwave digestion system (Ethos 1, Milestone, Sorisole, Italy). Tissues collected from the *TI* group were digested directly without addition of an additional tracer for iron quantification. After mineralization, iron in the solution was separated by ion-exchange chromatography using a strongly basic ion-exchange resin (Dowex AG 1-X8; 200 - 400 mesh, Sigma, St. Louis, USA). The eluate obtained was alkalized by addition of 25% ammonium hydroxide (Merck, Darmstadt, Germany) for precipitating sample iron as ferrihydrite. The iron precipitate was stored dry until isotope ratio analysis. The iron isotopic composition of the isotopic labels and the prepared samples were determined by negative thermal ionization mass spectrometry (NTI-MS) using FeF$_4^-$ molecular ions and a rhenium double-filament ion source with details described earlier [20]. Further technical details are described in an earlier publication [15].

Concentration of haemoglobin in the rat blood sample was indirectly determined in triplicate through iron concentration in the blood [15]. Iron content of blood samples was analyzed by graphite furnace atomic absorption spectrometry (GF-AAS) (Varian AA240 Zeeman/GTA120, Varian Inc., Palo Alto, CA, USA) by external calibration with an iron standard solution.

Data analysis

The amount ratio of tracers in the brain described were determined following established procedures for isotope dilution analysis using Eqn. (1) [20, 21] and the abundance of the different isotopes in each enriched isotopic label *a* and the measured isotope ratios in the sample *R*.

$$
\begin{bmatrix}
^{54}a_{nat} & ^{54}a_{iso,54} & ^{54}a_{iso,57} & ^{54}a_{iso,58} \\
^{57}a_{nat} & ^{57}a_{iso,54} & ^{57}a_{iso,57} & ^{57}a_{iso,57} \\
^{58}a_{nat} & ^{58}a_{iso,54} & ^{58}a_{iso,57} & ^{58}a_{iso,58} \\
^{56}a_{nat} & ^{56}a_{iso,54} & ^{56}a_{iso,56} & ^{56}a_{iso,56}
\end{bmatrix}
\begin{bmatrix}
\dfrac{n\left(^{nat}Fe\right)}{n\left(^{tot}Fe\right)} \\[8pt]
\dfrac{n\left(^{54}Fe\right)}{n\left(^{tot}Fe\right)} \\[8pt]
\dfrac{n\left(^{57}Fe\right)}{n\left(^{tot}Fe\right)} \\[8pt]
\dfrac{n\left(^{58}Fe\right)}{n\left(^{tot}Fe\right)}
\end{bmatrix}
=
\begin{bmatrix}
\dfrac{^{54/56}R_{sample}}{1+\sum^{i}R_{sample}} \\[8pt]
\dfrac{^{57/56}R_{sample}}{1+\sum^{i}R_{sample}} \\[8pt]
\dfrac{^{58/56}R_{sample}}{1+\sum^{i}R_{sample}} \\[8pt]
\dfrac{^{56/56}R_{sample}}{1+\sum^{i}R_{sample}}
\end{bmatrix}
\qquad (1)
$$

In a sample spiked with three iron isotopic labels (enriched ^{54}Fe, ^{57}Fe and ^{58}Fe), the amount of each of iron isotopes ^{54}n, ^{57}n, ^{58}n and ^{56}n is determined by the isotopic abundances a and the amounts (in moles) of each of the tracers in the blend ($n(^{nat}Fe)$, $n(^{54}Fe)$, $n(^{57}Fe)$ and $n(^{58}Fe)$). The iron isotopic abundance ^{W}a (W=54, 56, 57 and 58) for both isotopically enriched tracers (*iso,54; iso,57; iso,58*) and natural iron (*nat*) can be obtained from the sum of all measured isotope abundance ratios $\sum{}^{i}R$ with ^{56}a as the common denominator. The molar amount of the tracers is related to the measured isotope ratios R_{sample} via the total amount of iron in the sample $n(^{tot}Fe)$ and the abundance ^{W}a of the isotope in natural iron and the tracers. In sum, the abovementioned relationship can be expressed in a matrix form which can be solved to deliver the molar ratio of a given spike relative to natural iron in the sample.

Efflux Rate Index Based on Eqn. (1), the amount ratio of two tracers in a tissue sample with the amount of the last administered tracer as the common denominator, e.g. $n(^{57}Fe)/n(^{54}Fe)$, can be calculated from the molar ratio of the tracers [$n(^{nat}Fe)$, $n(^{54}Fe)$, $n(^{57}Fe)$ and $n(^{58}Fe)$] relative to the total amount of iron in the sample [$n(^{tot}Fe)$]. The calculated amount ratio of tracers in the brain relative to that in the diet was designated as the Efflux Rate Index. The higher the Efflux Rate Index, the less of the tracer taken up by brain was transported out of the brain after termination of tracer administration and the lower is the efflux rate.

Tissue iron uptake (*DI* group) Based on Eqn. (1), the amount of total iron $n(^{tot}Fe)$ in the sample aliquot in the *DI* group was calculated from the $n(^{58}Fe)/n(^{tot}Fe)$ ratio and the known amount of added ^{58}Fe spike (in moles) before digestion. The total amount of ^{57}Fe tracer in the sample could then be calculated from the $n(^{57}Fe)/n(^{tot}Fe)$ ratio and $n(^{tot}Fe)$ in the analyzed sample. Total amount of ^{54}Fe tracer in the sample could be calculated in the same way as that for the ^{57}Fe tracer. Total amount of tracer in the organ was calculated by taking the mass of tissue analyzed and the total mass of the organ/sample into account. Absolute tissue iron uptake (% of iron fed) was calculated as total moles of tracer recovered in the tissue relative to total moles of tracer consumed.

The amount of iron transferred into each tissue is expressed relative to the amount of total iron in the analyzed tissue as relative tissue iron uptake (% of tissue iron coming from feed). For the *TI* group, all available enriched stable iron isotope tracers had already been used during the feeding and were not available any more to quantify tissue iron following isotope dilution principles. Iron concentrations were therefore determined by graphite furnace absorption spectrometry and using standard addition techniques.

Evaluation of kinetics of iron efflux (*TI* group) A single compartment model was used to estimate tracer efflux rates for each individual organ in the *TI* group. The amount of the last administered tracer (T_3; ^{58}Fe) in the organ at sacrifice was set to 100% (Efflux Rate Index of 1.0). Efflux rate indices for the first administered tracer (T_1; ^{57}Fe) and the second administered tracer (T_2; ^{54}Fe) were expressed relative to the efflux rate index of tracer T_3 and plotted against the time period between termination of tracer administration and sacrifice. The obtained data points were fitted by the Windows version of the SAAM/Consaam software, WinSAAM (Simulation, Analysis and Modelling) to determine tracer efflux rates of iron from the respective organ.

Statistical analysis

Comparisons of absolute/relative tissue iron uptake and iron efflux between different tissues were conducted by performing an independent sample *t*-test for equality of means using SPSS version 20 software (SPSS Inc., Chicago, USA). In all tests, a value of $P\leq0.05$ was considered statistically significant.

Results and discussion

Results

No premature deaths were observed among the treated rats. The total amount of consumed feed, water and iron isotopes of each feeding group is summarized in Table 1**.** The rats showed a moderate increase in body weight in both groups over the course of the study from 643 ± 43 g at the beginning to 692 ± 49 at the end in for the *DI* group and from 613 ± 49 g to 713 ± 61 g for the *TI* group which is consistent with adult aging. The blood hemoglobin concentrations of each rat were within the reported normal range of $130 - 170$ g Hb/L [22] at the beginning (161 ± 13 g Hb/L) and end of the studies (159 ± 12 g Hb/L) for the *DI* group and 148 ± 8 g Hb/L and 149 ± 8 g Hb/L for the *TI* group, with minor variations within each study.

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Table 1 Total amount of consumed feed, water and iron tracers for the *DI* group (4 month of feeding) and the *TI* group (5 month of feeding)

Group	Consumed feed [kg]	Consumed water [L]	Consumed natural iron [mmol]	Consumed iron tracers from water		
				Tracer ^{57}Fe [mmol]	Tracer ^{54}Fe [mmol]	Tracer ^{58}Fe [mmol]
DI	2.93 ±0.15	2.57 ±0.20	10.49 ±0.54	0.350 ±0.030	0.800 ±0.080	-
TI	3.80 ±0.20	3.56 ±0.54	13.61 ±0.70	0.400 ±0.060	0.89 ±0.13	0.220 ±0.033

Tracer analysis of blood samples The measured amount ratio of tracer to total iron in blood carries information about the bioavailability of tracer iron. For comparison of data obtained for different tracers, amount ratios of tracer to total iron were adjusted for dose to that of the ^{57}Fe tracer for each individual rat using the tracer concentration ratios in the drinking water for normalization. After dose adjustment, the amount ratios of tracer to total blood iron were comparable for the different iron tracers in blood ($p=0.91$ for ^{54}Fe tracer in *DI* group; $p=0.33$ for ^{54}Fe tracer and $p=0.08$ for ^{58}Fe tracer in *TI* group; see Fig. 3 and 4). As can be expected, amount ratios of tracer to total iron increased steadily over time until termination of tracer feeding on from which the ratios declined due to blood iron turnover. A trend towards a slower increase in amount ratios of tracer to total iron over time was observed.

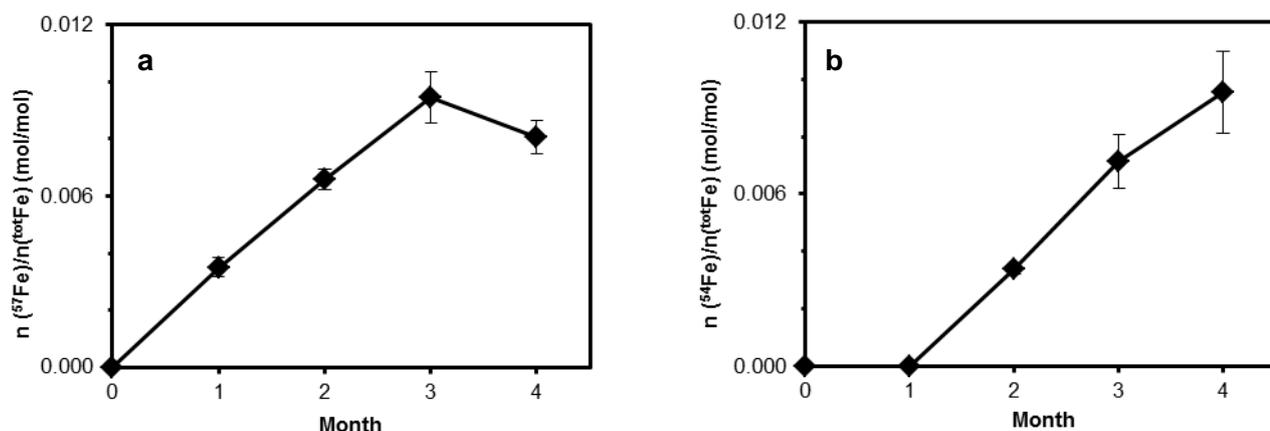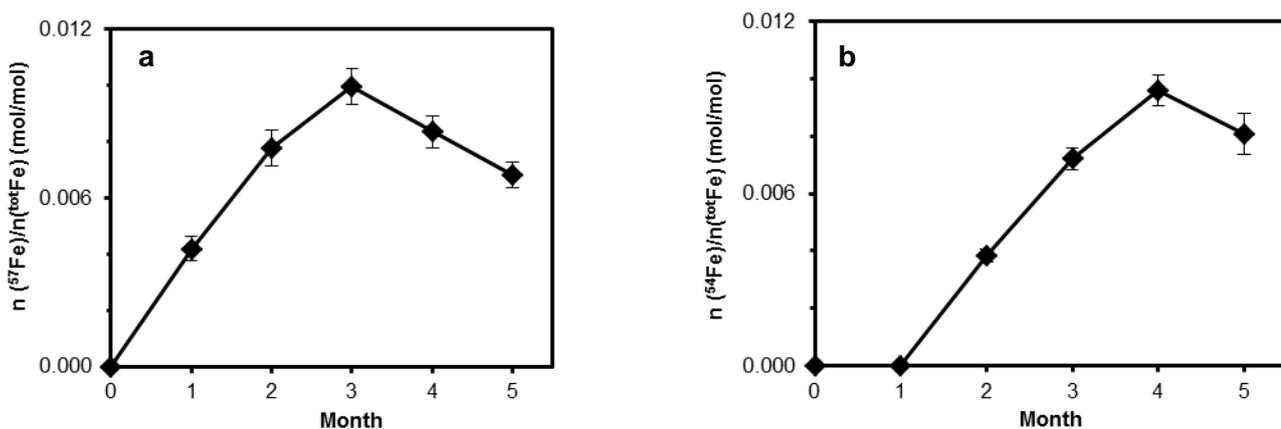**Fig. 3** Amount ratios of tracer (Fig. 3a: ^{57}Fe tracer; Fig. 3b: ^{54}Fe tracer) to total iron (mol/mol) in blood against time for the *DI* group (n = 3 rats). Feeding of the ^{57}Fe tracer was terminated at the end of month 3. Amount ratios were adjusted to differences in tracer concentration in the diet
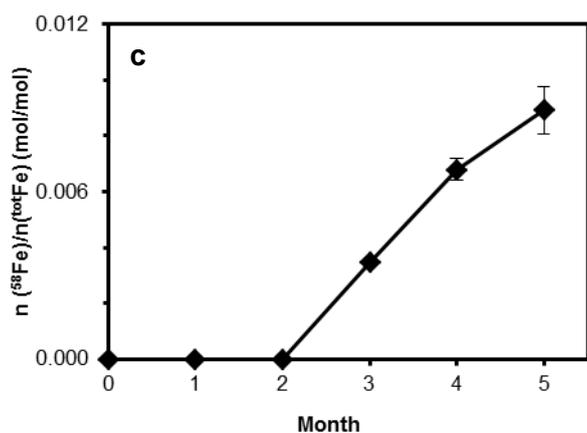
Fig. 4 Amount ratios of tracer (Fig. 4a: ^{57}Fe tracer; Fig. 4b: ^{54}Fe tracer; Fig. 4c: ^{58}Fe tracer) to total iron (mol/mol) in blood against time for the *TI* group (n=5 rats). Feeding of the ^{57}Fe tracer was terminated at the end of month 3 and that of the ^{54}Fe tracer at the end of month 4. Amount ratios were adjusted to differences in tracer concentration in the feed.

Tissue iron uptake for the *DI* group Fig. 5 shows the absolute and relative iron uptake by different tissues in the *DI* group. Absolute iron uptake by different tissues was significantly different from each other and ranged from 0.00062% in brain to 0.102 % in liver in the present study (see Fig. 5a). Fig. 5b shows the percentage of tissue iron in rat organs of the *DI* group coming from feed (relative tissue iron uptake)**.** Relative iron uptake of the analyzed tissues was of the same order of magnitude (9.2% - 32.8%) despite the marked differences in absolute iron uptake (0.000621% - 0.102% of the tracer dose). Relative iron uptake of brain was comparable to that of heart (p=0.10) and that of liver was comparable to that of the kidneys (p=0.46) while relative iron uptake of both brain and heart was significantly different from liver and kidneys (p=0.001).
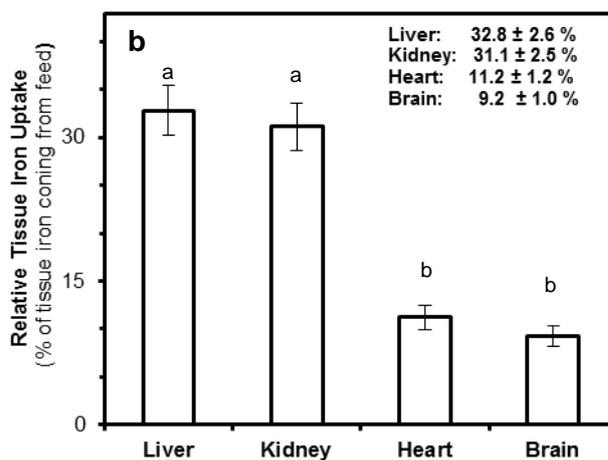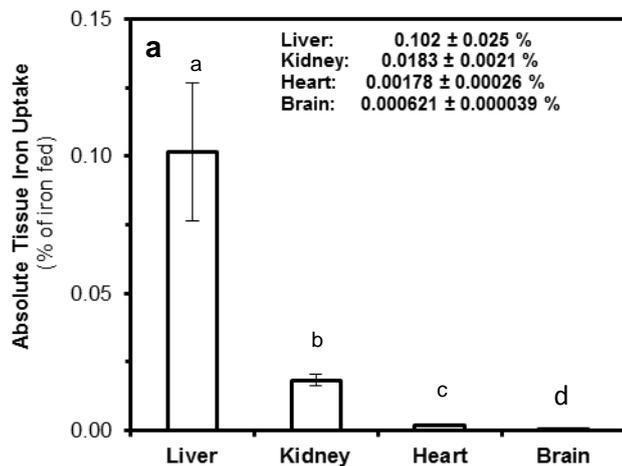

Fig. 5 Tissue iron uptake. Fig. 5a shows the percentage of dietary iron recovered in rat brains of the *DI* group (n=3 rats). Recoveries were calculated as the amount of ^{54}Fe tracer recovered in the organ relative to total amount of ^{54}Fe tracer given (in %). Fig. 5b shows the percentage of tissue iron in rat organs of the *DI* group coming from feed**.** Relative tissue iron uptake was calculated as the amount of ^{54}Fe tracer recovered in the organ coming from feed relative to the total amount of iron in the organ (in %). Different lower case letters indicate statistical significant differences between tissues (p≤.0.05). Values are given as arithmetic means ± SD (n=3)

Iron turnover in tissue - Qualitative assessment (*DI* group)
Muscle tissues exhibited tracer amount ratios that were the closest to that of the diet, demonstrating the lowest iron turnover rate among all analyzed tissues. After normalization of Efflux Rate Indices to muscle tissue, blood exhibited the highest turnover rate among major body iron pools (see Fig. 6). Efflux Rate Indices were all lower than 1.0 which indicates that more iron was replaced in the studied organs over the observational period than in muscle tissue as the iron pool showing the slowest iron turnover. The Efflux Rate Index of brain of 0.93, was higher than that of the other tissues, showing that iron was exported from brain at a slower rate as compared to other organs. The Efflux Rate Index of brain exhibited no difference with that of heart (p=0.18) whereas the Efflux Rate Index of liver showed no significant difference to those of kidney (p=0.46) and blood (p=0.23) but was significantly lower than that of heart (p=0.01) and brain (p=0.001), respectively.

Iron turnover in tissue - Quantitative assessment (*TI* group)
For the triple-isotope feeding scheme, iron turnover of different tissues was quantitatively evaluated by plotting the normalized Efflux Rate Index against time difference between terminations of tracer feeding and sacrifice (see Fig. 7). Dietary iron was found to be released from liver, kidney, heart, brain and blood at different efflux rate. Efflux rates expressed as % of tracer in brain released per month and tracer half-lives in the studied tissues was calculated using a single compartment model (see Table 2). Consistent with findings for the *DI* group, brain iron exhibited the lowest efflux rate (8.2 ± 3.1 % of tracer per month) among the measured tissues in the *TI* group. The half-life of dietary iron taken up by the brain was estimated to be 9.4 ± 3.2 months, the longest half-life among all analyzed tissues.

Table 2 Efflux rates and half-lives of dietary iron in the rat tissues in *TI* group*

	Liver	Kidney	Heart	Brain	Blood
Efflux rate (% tracer / month)	20.5 ± 6.6^a	19.8 ± 6.8^a	11.5 ± 2.1^b	8.2 ± 3.1	37.6 ± 9.6^a
Half-life of dietary iron (month)	3.7 ± 1.3^a	4.2 ± 1.9^a	6.2 ± 1.2^b	9.4 ± 3.2	2.0 ± 0.6^a

* The values of efflux rate and half-life of dietary iron in different tissues were compared with those in brain. Lower case indicates statistical difference ($p \leq 0.05$) whereas lower case b means no statistical difference

Discussion

In the present study we confirmed the major finding of our previous study [15]. The marginal amount of brain iron taken up from diet (0.00062%) after 4 months of feeding constituted ca. 9% of brain iron at sacrifice. While this does not challenge the finding from earlier radioisotope experiments that the absolute amount of iron uptake by brain is very low, it points to brain iron homeostasis and dietary iron intake as key factors in brain iron accumulation in ageing and possibly in neurodegeneration.

Our studies differ in many ways from earlier radiotracer studies due to the different scope of our experiments. Research interests in the past lied mostly in the developmental stage with limited focus on the effect of dietary iron on brain iron balance [17, 23-27]. Because brain iron uptake is age-dependent [28, 29] and dietary iron taken up by brain and its later release is a long-term process, it was imperative for us to study brain iron dynamics by feeding tracer continuously to adult and not to weaning/young rats. Our studies were conducted in 6-8 months old rats, corresponding to twenty-odd human years. The postmortem study by Hallgren and Sourander (1958) [4] demonstrated that the rate of brain iron accumulation increases steeply during the first two decades, slows down in the third decade, and reaches a "plateau" or may increase very slowly thereafter. The change in brain iron accumulation rate suggests a change in brain iron dynamics including iron import/export over the course of life. As relatively large amounts of iron are required early in life for brain development, it is possible that the amount of iron accumulating during adulthood when brain development can be considered complete, contributes to the development of iron-related neurodegenerative diseases. Similar to humans, iron was found to increase steeply in rat brain early in life but slows down at about the fourth month of life [23, 30-33].
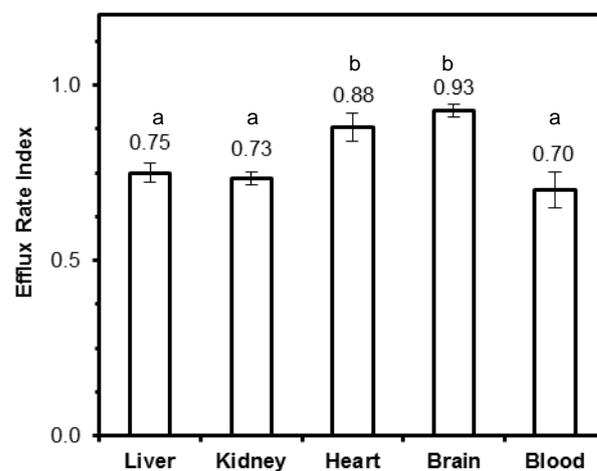
Fig. 6 Efflux Rate Index of different tissues for the *DI* group (n=3 rats). The lower the index, the higher the iron efflux rate (see text). Different lower case letters indicate statistical significant differences between tissues ($p \leq 0.05$). Same letter means no statistical difference.

Fig. 7 Changes in Efflux Rate Indices over two months in the *TI* group (n=5). Efflux Rate Indices at month 1 and 2 were normalized to its value at month 0 (Experimental section)

Our understanding of brain iron dynamics is largely based on short-term radiotracer studies. However, due to their instability and radiation safety aspects they are less useful for tracing l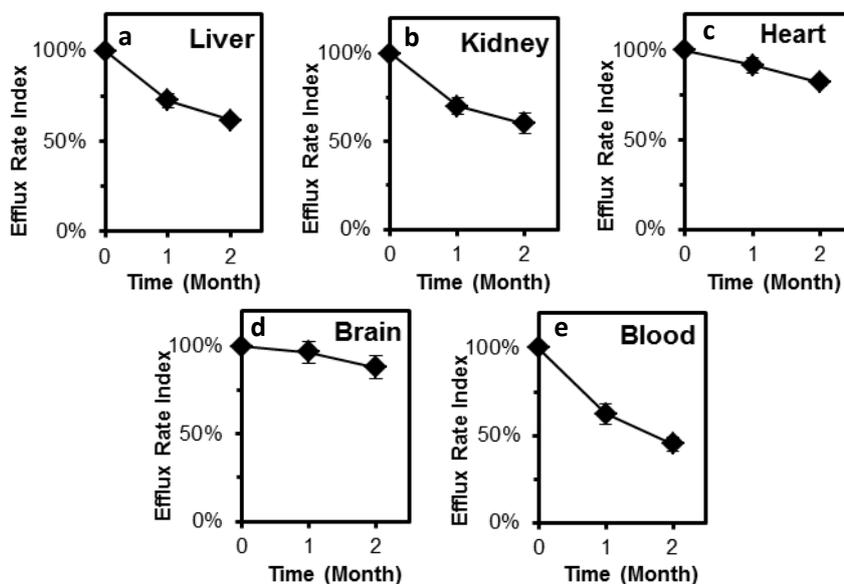ong-term dietary iron uptake by brain. Long-term feeding of non-decaying stable isotope tracers along with diet, as in our study, allows studying brain iron dynamics over time. Furthermore, the single dose of radiotracers, usually by injection, neglects the homeostatic response of the body and possibly brain to changes in dietary iron supply at the systemic level. This is of significance when studying the role of dietary iron intake in brain iron accumulation.

By feeding iron stable isotopes continuously with diet we were able to assess brain iron balance relative to total brain iron and to consider a possible homeostatic response which was not possible in earlier radiotracer experiments. Because iron has three stable isotopes that can be used as tracers (^{54}Fe, ^{57}Fe and ^{58}Fe), it is possible to use each rat as its own control when studying brain iron dynamics by staggering tracer administration over time (see Fig. 1 and 2). This allows reducing sample size significantly as compared to earlier radiotracer experiments [23, 24, 30] in which a single tracer was used. Groups of animals in those studies had to be sacrificed at different time points and compared to each other which require naturally larger number of animals to control for inter-individual variations.

Iron balance of an organism/organ depends both on iron influx and efflux rate. Iron accumulates inevitably at the moment that influx rate exceeds efflux with the rate of iron accumulation being determined by their difference. Our understanding of mechanisms of brain iron uptake is still limited despite considerable efforts have been made. Dietary iron was proposed to be taken up by brain either across the blood-brain barrier (BBB, main pathway) or the blood-cerebrospinal fluid barrier (BCB, secondary pathway) [34, 35]. Many studies suggested that iron uptake across BBB is mainly via transferrin receptor-mediated endocytosis [36], while the detection of H-ferritin receptors on BBB suggested an additional pathway [36, 37]. Expression of iron-related proteins e.g. divalent metal transporter 1, ferroportin, ceruloplasmin and hephaestin were higher in choroid plexus in BCB than virtually all other brain regions [38], pointing to alternative mechanisms of brain iron uptake. Our knowledge on iron export is even more limited as it has been studied to a much lower extent. BCB was proposed to be responsible for not only brain iron uptake [39] but also iron efflux owing to its structure and functions [26, 40]. Structurally, tight junctions of BCB are leakier than those of BBB. This is supported by rat studies in which radio-labeled iron was injected into the lateral cerebral ventricle to study iron efflux from cerebrospinal fluid to blood [27, 40, 41].

In agreement with earlier radiotracer experiments [24-27, 40], our findings support the hypothesis that brain iron uptake is a bi-directional process. Two other studies did not report loss of tracer iron from brain which could be either due to sample size or age of animals [23, 42]. Observations by Holmes-Hampton et al. (2012) [29] point to iron incorporation into brain being biphasic and age-dependent. During early life, iron influx must exceed efflux significantly to cover the high demands for brain development. In the first two years, human brain iron is ca. 50% of the adult brain and approximately doubles within the following decade while it slows down later [43].

In good agreement with findings from our earlier study [15] and Bradbury (1997) [26], the rank order of the amount of tissue iron uptake was blood> liver> kidney> heart> brain (see Fig. 5). Brain iron turnover was found to be the lowest among the studied organs. The Efflux Rate Index of brain was 0.93 (see Fig. 6), showing that iron turnover rate for brain is slightly higher than for muscle but the lowest of all other studied tissues. This observation is in good agreement with findings by Dallman and Spirito (1977) [24] who injected radio-iron into young rats (2-60 days) and monitored brain radioactivity up to 135 days. Their results showed that brain iron radioactivity remained at its peak until the end of the observational period, indicating little/no loss of brain iron during that period. They therefore concluded that brain iron turnover is at an extremely slow rate based on the assumption that mechanisms for brain iron export exist.

In the present study we could estimate that iron that has been transferred from diet to brain is exported at an efflux rate of $8.2 \pm 3.1\%$. However, this figure must be considered the first gross approximation of iron efflux rate of brain in a mammalian organism as only three data points were available per rat for calculation (see Fig. 7). The corresponding half-life of iron tracer in brain, and thus that of dietary iron taken up by brain during adulthood, was 9.4 ± 3.2 months. This can be considered relatively long with regard to the short life time of rats [44]. As iron turnover rate in rat organs cannot be simply extrapolated to humans based on the ratio of their life spans, the data on iron turnover rate in blood in these two species can be of use to extrapolate findings to human brain. The half-life of iron tracer in blood was found to be approximately 83 months in adult men [45] as compared to ca. 2 months for adult rats (Table 2). At an average human life time of 75 as compared to 3 years in rats, the half-life of iron in human brain can be reasonably estimated to be of the order of decade(s).

Conclusion

In agreement with our earlier study, dietary iron taken up by rat brain amounted to ca. 9% of brain iron after 4 months of tracer feeding. Half of this iron will leave brain within one half-life, i.e. 9 months. Assuming that brain iron uptake and its half-life are constant during adulthood, brain iron of the rats can be estimated to increase by ca. 30% from early adulthood (6-8 months) to end of their life (ca. 36 months).

The present study, for the first time ever, revealed that dietary iron enters adult brain at a significantly higher influx rate than efflux which automatically leads to brain iron accumulation during adulthood. This permits us now to study the effect of dietary iron intake on brain iron accumulation in healthy organisms and in conditions where body iron homeostasis is impaired. Furthermore, the developed methodologies can be used to evaluate treatments to reduce iron burdens in adult brain and thus iron-implicated neurodegeneration.

Acknowledgements

Notes and references

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