Lithium Administered to Pregnant, Lactating and Neonatal Rats: Entry into Developing Brain.

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Keywords: pregnancy, bipolar-disorder prophylaxis, placental transfer, postnatal, brain barriers

Posted Date: September 10th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-74064/v1

Abstract

Background

Little is known about the extent of drugs administered to pregnant and lactating women entering the developing brain. Lithium is one of the most commonly prescribed drugs for bipolar disorder. Here we studied transfer of lithium given to dams, into blood, brain and cerebrospinal fluid (CSF) in embryonic, postnatal and adult rats.

Methods

Lithium chloride in a clinically relevant dose (3.2mg/Kg body weight) was injected intraperitoneally into pregnant (E15-18) and lactating dams (birth-P16) or directly into postnatal pups (P0-P16). Acute treatment involved a single injection; chronic treatment involved twice daily injections for the duration of the experiment. Following terminal anaesthesia blood plasma, CSF and brains were collected. Lithium levels and brain distribution were measured using Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS).

Results

Lithium was detected in blood, CSF and brain of all fetal and postnatal pups following lithium treatment of dams. Its concentration in pups' blood was consistently below that in maternal blood (30-35%) indicating significant protection by the placenta and breast tissue. Levels of lithium in plasma fluctuated in different treatment groups but its concentration in CSF was stable at all ages, in agreement with known stable levels of endogenous ions in CSF. Only a small non-significant increase of lithium transfer into CSF occurred following application of Na^+/K^+ ATPase inhibitor (Digoxin) *in vivo*, indicating that Na^+/K^+ ATPase is at most only a minor mechanism for lithium transfer across choroid plexus cells into CSF early in development. Presumably lithium transfer across choroid plexus epithelial cell is occurring predominantly via other ion channels.

Conclusions

Information obtained on the distribution of lithium in developing brain provides a basis for studying possible deleterious effects on brain development and behaviour in offspring of mothers undergoing lithium therapy.

Background

Lithium salts have been used since the 18th century to treat a wide variety of conditions and ailments (1. Brown, 2019). It was introduced by Cade (2. 1949) for the treatment of bipolar disorder, although he did not use it for many of his patients because of concerns about toxicity (1. Brown, 2019). It is now recognised that lithium is the "gold standard" treatment for bipolar (3. Cipriani et al., 2013; 4. Oruch et al., 2014) and depressive disorders (5. Bschor, 2014). For reasons that seem unclear, lithium is prescribed for oral use in the form of lithium carbonate capsules with strict instructions that they should not be bitten (6. Australian Medicines Handbook, 2019), presumably because it is highly alkaline and would burn the mouth. Most published studies on lithium in rats have used the more neutral LiCl (e.g. 7. Ebadi et al., 1974; 8. Pearce et al. 2004; 9. Hillert et al., 2012) which we have also used in our studies (see Methods).

Lithiumasatreatmentforbipolardisorder

Lithium's mechanism of action in treating bipolar disorder is not understood. A range of intracellular mechanisms has been proposed, including modulation of neurotransmission via disruption of some Na^+ bound receptor/transporter

activity and other G-protein-mediated intracellular mechanisms, as well as inhibition of glycogen synthase kinase (10. Malhi et al., 2013; 11. Dudev et al., 2019).

There appear to be no studies that provide a definitive answer to the question of the mechanism of action of lithium in bipolar disorder; as this is not relevant to understanding mechanisms involved in permeability of lithium across brain barrier interfaces, this will not be considered further. Of relevance is the long-established observation that lithium can substitute for sodium in cellular permeability mechanisms involving specific channels and ion exchange systems (see below).

Lithiumduringpregnancyandlactation

Medications taken by pregnant and lactating women carry a risk to the fetus and neonate by potentially having untoward effects if transferred across the placenta or breast tissue. Accordingly, many pregnant women, and those planning to become pregnant, are advised to limit taking of medications, both prescription and over the counter, to mitigate potential risks, some of which may be unknown in the current state of knowledge (12. Australian Government Department of Health, 2020; 13. Bakker et al., 2006).

At present, there is a dearth of evidence-based guidelines for clinical practitioners and patients regarding the safety of maternally administered drugs in terms of risks of adverse effects on the fetus and neonate (14. The Royal Women's Hospital Australia, Pregnancy and Breastfeeding Medicines Guide 2020). For women with bipolar and other psychiatric disorders that require long-term treatment, avoiding medications could result in more harm than good for both the mother and for the baby. Studies have shown that relapse of bipolar episodes are common when lithium treatment is discontinued (15. Viguera et al., 2007; 16. Volkmann et al 2020)

Effectsoflithiumonfetalandneonataldevelopment

There is only limited information on possible effects of lithium administered during pregnancy and breast feeding. Much of the available information is summarised in 17. Briggs et al. (2017). As with other drugs the focus has tended to be on whether lithium can induce teratogenic malformations in the fetus. The evidence is mixed because it is based mainly on retrospective uncontrolled studies of small numbers of patients. One prospective comparative study found no differences in rates of major congenital malformations between treated and control groups (18. Diav-Citrin et al., 2014).

Entry of lithium into the adult and developing brain

There have been several studies of lithium entry into the adult brain, mainly in rats (7. Ebadi et al., 1974; 19. Mukherjee et al., 1976; 8. Pearce et al., 2004; 9. Hillert et al 2012) but also a few in human patients (20. Sachs et al. 1995; 21. Soares et al., 2001). Two studies used imaging methods to study the distribution of lithium administered to adult rats (22. Sandner et al., 1994; 23. Stout et al., 2017). These and the quantitative studies of lithium in different regions agree in showing that there is considerable regional variation in the distribution of lithium. These studies and their quantitative aspects will be considered in the Discussion in relation to the results of the present study.

In contrast to the adult, there seems to be a lack of studies of lithium entry into the developing brain. For maternally administered lithium to enter the fetal brain it needs to: (i) cross the placental interface between maternal and fetal circulations; (ii) from the fetal circulation, lithium has to cross fetal brain barriers: the blood-brain barrier proper (between the blood and brain parenchyma) and the blood-cerebrospinal fluid (CSF) barrier (24. Saunders et al., 2018). In the case

of breast feeding the lithium would need to enter the milk, cross the gut into the blood of the neonates and then cross the same barriers into the brain.

Lithium ions interact with sodium and to a lesser extent potassium, magnesium and calcium ions in channels and transport systems in a variety of tissues (e.g. 25. Prokop & Marcus, 1972; 26. French & Shoukimas, 1985; 27. Kemp et al., 2008; 28. Boda et al., 2009). However, generalisations are difficult to make due to tissue differences (29. Jakobsson et al., 2017). There have been a few studies of lithium transfer across the mammalian choroid plexus involving experimental evidence for the transfer of lithium from blood to CSF and in the reverse direction, but disagreement exists about the mechanisms involved (30. Hesketh, 1977; 31. Reed & Yen, 1980; 32. Yen & Reed, 1981). However, recent studies investigating the localisation and function of ion channels and transporters in choroid plexus epithelial cells, in both adult and during development (33. Damkier et al., 2013; 34. Liddelow et al., 2013, 35. 2016) are beginning to provide a better understanding.

Materials And Methods

Chemicals

Anaesthetics: Isoflurane (Pharmachem Queensland); Urethane > 99% (Sigma-Aldrich, Australia, CAS number 51-79-6)

Heparin: Heparin Sodium (Porcine Mucous) Injection BP (Hameln pharmaceuticals GmbH, Germany)

Lithium Chloride Anhydrous LR (Chem-Supply, CAS number: 7447-41-8)

Optimal Cutting Temperature media: TissueTek OCT from Sakura Finetek

Isotonic sodium chloride: 0.9 g/100 mL

Sodium Chloride (Baxter Healthcare, New South Wales)

Digoxin 98.9% purity (Sigma-Aldrich, CAS number: 20830-75-5)

Animals and surgical procedures

Sprague Dawley rats were sourced from The Biological Research Facility at The University of Melbourne. All rats were subjected to a 12 h light/ dark cycle with ad libitum access to food and water. All experimental procedures were conducted in accordance with National Health and Medical Research Committee (NHMRC) guidelines and approved by The University of Melbourne Ethics Committee, Ethics ID 1914793 (Entry of Anti-Epileptic and Psychotic Drugs into the Developing Brain).

Table 1
Ages and numbers of rats used

Age (pups)	E18	P0	P2	P4	P7	P12	P16/17
n	26	7	9	33	13	9	10
Weight (g) SD	N/A	5.0 0.4	6.8 1	8.3 1.6	17 3	28 6	47 4

Adults/ Dams	Control E18 dam	Treated E18 dam	Control breast feeding dam	Treated breast feeding dam
n	1	1	2	1
Weight (g)	431	389	312, 354	352

E = embryonic, P = postnatal, n, number of animals at each age, SD = standard deviation. For sex and weight of individual pups see Additional file 1.

As our experiments required administration by intraperitoneal (i.p.) injection, we used the more neutral chloride salt rather than the strongly alkaline clinical preparation of lithium carbonate (see Introduction).

Animals were randomly allocated to two experimental protocols:

i. acute experiments where animals received a single injection of lithium chloride (LiCl),
ii. chronic exposure experiments where animals received multiple injections of LiCl over several days.

Therapeutic concentrations of lithium in the blood of patients on lithium therapy are in the range of 0.4–1.2 mmol/L (6. Australian Medicines Handbook 2019) indicated in this paper as mM. Doses and injection volumes were standardised to the animal's body weight (3.2 mg/ Kg Li, see Fig. 1A) to achieve the lowest therapeutic concentration of lithium in plasma, injection volumes were limited to < 1% of body weight to avoid any significant increase in the circulating blood volume. LiCl was dissolved in 0.9% sterile sodium chloride and administered by intraperitoneal (i.p.) injection.

Injection protocols

(i) Fetal animals

Fetal animals were exposed to lithium via placental transfer only. This was achieved by i.p. injections into pregnant dams. For acute exposure experiments, time mated E18 pregnant rats were administered a single i.p. injection of 3.2 mg/Kg LiCl. For chronic exposure experiments, twice daily 3.2 mg/Kg LiCl i.p. injections (early morning and late afternoon) were administered to time-mated dams from E15-E18.

(ii) Postnatal animals

For acute experiments, pups and dams were injected i.p. with 3.2 mg/Kg LiCl and left for 90–120 min (duration where plasma and CSF levels are stable, see Fig. 1B-D). For long-term experiments, pups were exposed to lithium via breast milk from dams injected twice daily (early morning and late afternoon) with a standard dose of LiCl (3.2 mg/Kg body weight) and collected after 2, 4, 7, 12 or 16 days of exposure to breast milk. Dams were collected together with P16 pups (17 days of treatment). Pups in the long-term experiments were separated from the dam 2–3 h after dam's last injection.

Untreated control animals were age-matched to all experimental animals.

Treatment protocols and surgery

Fetal animals

Pregnant rats were deeply anaesthetised (urethane 2.5 g/ Kg i.p.), a tracheal cannula inserted to maintain the airway and left femoral artery catheterised for collection of maternal blood samples. Animals were placed on heated plate (33ºC) and a uterine horn exteriorised via a mid-line abdominal incision. Pups, starting at the ovary end of the uterine horn were removed from their amniotic sac and samples collected as described below. Any pups with visible signs of haemorrhages were not collected, but their position in the uterine horn was recorded.

Postnatal animals

Pups were terminally anaesthetised with an overdose of inhaled anaesthetic (> 5% isoflurane), before samples were collected (see below).

Inhibition of sodium/ potassium ATPase in P4 pups

One litter of pups at P4 was used for the experiment investigating if partial blocking of $Na^{+/k+}$ ATPase could influence lithium transfer into the CSF. Three pups were injected with digoxin (300 mg/ Kg body weight in 75 µL, dissolved in ethanol followed by isotonic sodium chloride solution) and 3 were injected with equal volume of isotonic sodium chloride solution, all injections were given i.p. Pups were carefully monitored for 30 min, before they were given the same lithium injection protocol as other postnatal animals (see injection protocol) with samples collected. Samples were all collected within 90–105 min after lithium injection and coded for blinded determination of lithium analysis (see below).

Collection and processing of blood and CSF samples

Pup blood samples were taken directly from the right ventricle of the heart into heparinised glass capillaries. For pregnant rats, a small sample of maternal arterial blood (0.2 mL) was collected from the femoral arterial catheter at the same time as each pup was removed and the catheter then flushed with an equal volume of isotonic sodium chloride solution to maintain circulating volume (~ 2 mL of blood was collected from each dam in total).

All blood samples were stored on ice until processed.

Blood samples were centrifuged for 5 min (5,000 rpm), plasma separated, diluted in 0.9% NaCl (1:10 v/v) and 0.5 µL spotted in onto a glass slide in triplicate (Superfrost glass slides, Thermo Scientific), air dried for 24 h before analysis using laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) see below.

Samples of CSF were collected from the cisterna magna using a glass micropipette, with care taken to avoid rupturing blood vessels. Any sample that was visibly contaminated with blood was discarded (contamination as small as 0.2% can be visibly detected, (36. Habgood et al., 1993). Samples of undiluted CSF (0.5 µL) were spotted in triplicate onto glass slides and dried for 24 h before analysis with LA-ICP-MS (see below).

Collection and processing of brain samples

Whole brains, including olfactory lobes, were dissected out of the skull and bisected along the sagittal plane into two equal halves. Each half was randomly assigned to either (1) extraction and quantification of lithium or (2) sectioning for LAICPMS.

1. *Lithium extraction*: half of the brains were frozen on dry ice and stored at -80 °C until use. Brain samples were defrosted for a minimum of 30 min at room temperature, homogenised in isotonic sodium chloride solution (1:2 w/v) using a glass pestle, mixed vigorously and incubated at 37°C for 20 min. Samples were mixed briefly, centrifuged for 10 min (5000 rpm) and supernatant collected (total extracted volumes were recorded). The supernatants were diluted with isotonic sodium chloride (1:5 and 1:10 v/v) and replicates spotted onto glass slides, air dried and analysed using LA-ICP-MS (see below).

2. *Sectioning of brain samples*: the half brain samples were immediately placed in a mould containing Optimal Cutting Temperature media (OCT), frozen on dry ice and stored at -80 °C until sectioning. The frozen brain blocks were temperature-equilibrated for at least an hour before sectioning (30 µm serial sagittal sections) in a cryostat. Sections were mounted on glass slides and air dried overnight before LA-ICP-MS analysis.

Laser ablation-inductively coupled-mass spectrometry (LA-ICP-MS)

Samples (0.5 µL "droplets" or 30 µm brain sections) were air dried on standard microscope slides for 24 hours before being placed into a 10 × 10 cm ablation cell where the laser was tuned to the topography and dimensions of the sample slides together with matrix-matched elemental standards for quantitative analysis (37. Hare et al., 2012). Brain sections were ablated with a 213 nm laser (NWR213 ablation system, Kennelec Scientific) by a series of rasters using a 60 × 60 µm square spot size and a scanning speed of 240 μms^{-1} for low resolution scans, and 30 × 30 µm square spot size and a scanning speed of 120 μms^{-1} for higher resolution scans. For droplet (CSF, plasma and brain homogenate) analysis 100 μm^2 spot size and speed of 200 μms^{-1} was used. Ablated material was swept into Agilent 8800 QQQ-ICP-MS (Mulgrave, Victoria, Australia) by argon gas flow at 1.2 L/min and directed through the plasma torch for ionisation. Ionised material was analysed for lithium (^{7}Li), carbon (^{13}C), sodium (^{23}Na), phosphorus (^{31}P), potassium (^{39}K) and iron (^{56}Fe) for a dwell time of 0.045 s per element. Carbon and phosphorus were recorded for tissue structure identification and iron for detection of residual blood in the tissue and CSF. Approximate analysis time for each brain section was 5–6 h and for each microscope slide of droplets (100–150 droplets on average) 20–24 h. Two dimensional elemental maps were constructed using the Iolite analysis software (School of Earth Sciences, University of Melbourne) operating under the Igor Pro suite (38. Paul et al., 2015).

As there are no polyatomic interferences for lithium (39. May and Wiedmeye et al.,1998), good limits of detection (LoD; 4.014 µg/L calculated as 3 × SD of 8 replicates blank) and quantitation (LoQ; 13.381 µg/L calculated as 10 × SD of 8 replicates blank) were obtained.

Certified ICP-MS standards and LiCl-based standards were used for droplet lithium quantitation. Certified ICP-MS standards (Agilent) of 500 parts per billion (ppb or µg/L) and LiCl of 240 mg/mL were serially diluted in a matrix-matched artificial CSF solution (148 mM NaCl, 3 mM KCl, 1.2 mM $MgCl_2$, 10 mM glucose, 1 mg/mL bovine serum albumin) (Fig. 2) (40. Kysenius et al., 2019).

From the scans, regions of interest (each spot of plasma, CSF or brain homogenate sample) were selected and average counts per second for each residue were measured and calibrated to standard curve constructed from standards on the same slide. Information on the standards and quantitation used for brain sections and droplets can be found in 40. Kysenius et al., 2019 and 41. Hare et al., 2013, respectively. All droplets were analysed in technical triplicates.

Experimental design

Randomisation

In acute experiments, littermates were randomly assigned to the control and experimental groups. In long-term experiments, because all pups within the one litter were exposed to lithium via the mother, separate litters from untreated dams were used as controls.

Blinding of the study

Analysis of plasma, CSF and brain sections by LA-ICP-MS: samples were identified only by code which was decoded at the end of the experiment.

Statistical analysis

Statistical differences between levels of lithium in plasma, CSF and CSF/ Plasma ratios in acute and long-term treatment group at each age (see Additional file 2) were determined using two-tailed Student's t-tests with F tests for equal variance (Graphpad Prism 6).

Results

Therapeutic concentrations of lithium in the blood of patients on lithium therapy are in the range of 0.4 -1.2 mM/L (6. Australian Medicines Handbook, 2019). In order to achieve similar concentrations in the animal model, an injection protocol was established. The first step was to find an appropriate dose, followed by establishing an experimental timeframe that would be consistent across all ages.

Determining lithium concentrations in rat blood plasma

Three doses of LiCl (3.2, 6.4 and 16 mg lithium/ Kg body weight) were injected i.p. into individual P4 pups from the same litter, left for 1.5 h, terminally anaesthetized and blood and CSF samples collected as described in Methods. Lithium concentrations in each individual pup's plasma, CSF and CSF/ Plasma ratios are illustrated in Figure 3.

Concentrations of lithium in CSF were lower than in plasma in all animals, but reflected the dose administered (Figure 3B). CSF/ Plasma ratios were similar (around 25-30%) in all pups regardless of the injected dose (Figure 2C). As can be seen in Figure 3A the dose of 3.2 mg Li/Kg body weight was sufficient to achieve the lowest concentration (around 0.4 mM) that was within the clinical range of 0.4-1.2 mM (6. Australian Medicines Handbook, 2019). This dose was adopted for the rest of the study.

Establishing experimental time frame for postnatal animals

Levels of injected markers in an animal's circulation are inherently variable therefore an established method for determining the entry of markers into CSF is to express the concentration in CSF as a ratio of that in plasma when at steady-state (42. Saunders, 1992). The steady-state CSF/ Plasma ratio is defined as the period when both CSF and plasma concentrations are stable. In order to establish this period in rats at different developmental ages a litter-based model approach was used (36. Habgood et al., 1993). Standardised amounts of lithium (3.2 mg/Kg body weight) were injected i.p. to a litter of P4 pups and samples of blood and CSF collected at different time points from 30-170 min (see Additional file 4).

The period when the CSF/ Plasma ratio appeared stable was between 90 and 150 min (Figure 1).

In order to establish if this time frame also applies to animals at different postnatal ages, a similar experiment was conducted in a limited number of pups at P2, P7 and P16. Results are illustrated in Figure 1.

As shown in Figure 1 the CSF/ Plasma ratios at the postnatal ages studied were relatively stable over the time up to 150 min after an i.p. injection of lithium. Therefore, a dose of 3.2 mg/Kg body weight and an experimental timeframe of 90-120 min was used for all subsequent experiments.

Entry of lithium from blood plasma into the CSF in acute experiments

Rats at different developmental ages (E18, P0, 2, 4, 7, 12, 16 and adult) were injected i.p. with 3.2 mg/Kg lithium and blood, CSF and brain samples collected within 90-120 min (Figure 4.

The dam at E18 and pups at P0-16 received an i.p. injection of 3.2 mg lithium/Kg body weight. The E18 pups received lithium via placental transfer only. This accounts for the much lower plasma level of lithium in the E18 fetuses and in turn their much higher CSF/Plasma ratios. Note the similarity of CSF lithium concentrations in fetuses and postnatal animals. Each dot in (A) and (B) represent samples from individual animals. CSF/ Plasma ratios (%) are calculated for the same animal. Error bars are ±SD when n>2. For control values, see Additional file 1.

As shown in Figure 4, plasma (Figure 4A) concentrations in pups (P0-P16/17) following a single i.p. injection were stable and within the therapeutic window in plasma (~0.4 mM). In the E18 fetuses the plasma concentration was less than half that in the postnatal animals; this is because the lithium was administered i.p. to the dam and would have distributed to all of the fetuses resulting in a larger distribution volume and therefore lower concentration. CSF concentrations across ages were also stable at ~0.15 mM. CSF/ Plasma ratios were similar at all postnatal ages (~35% Figure 4C) with the exception of E18 where ratios were much higher (~100%) because of the lower plasma value. The adult CSF lithium concentration appeared to be less than in the fetuses and postnatal animals. This may reflect a greater degree of control between blood and CSF in the adult, but is based on a single value.

Entry of lithium from blood plasma into the CSF in long-term experiments

To compare results from pups following acute exposure to those exposed to lithium over a prolonged period of time, lithium was administered by i.p. injections to the dam over the course of pre- and post-natal rat development (E15-E18 and P0-P16 respectively). This was in order to mimic clinical situations where pregnant and lactating mothers need to take daily lithium. Fetuses were exposed to lithium via the maternal circulation and placental transfer (levels estimated at E18); postnatal animals were exposed via breast milk (P0-P16). The results are illustrated in Figure 5.

The plasma and CSF concentration of lithium in postnatal pups that received lithium via breast milk of treated dams was much lower compared to those acutely treated (cf Figure 4) at around 0.1 mM up to P4 after which it declined (acute vs long-term plasma and CSF: $P<0.01$ and $P<0.05$ respectively across all ages). Lithium concentration in the CSF appeared to decline with increasing age of the pups falling from 0.2 mM at E18 to 0.07 mM at P2 and 0.009 mM at P16 (Figure 5B). This decline in CSF lithium concentration was reflected in similar age-dependent decline in CSF/ Plasma ratios, which ranged from 54-92% at P2-4 down to 27-55% at P12-16 (Figure 5C). Due to the high values for lithium in plasma at E18 the CSF/ Plasma ratios at this age was much lower (7%, Figure 5C).

Entry of lithium into the developing brain

Entry of lithium into the developing brain was investigated using two independent methods: (i) extraction of brain samples to obtain overall quantitative estimates of its concentration compared to plasma and (ii) LA-ICP-MS to map its distribution and obtain regional estimates of concentrations of lithium.

i. Quantitative estimates of lithium in brain homogenates

To quantify lithium in whole brain, homogenates were prepared and lithium measured (see Methods). Two separate brains were measured at each age. The results are in Table 2.

Table 2

ConcentrationofLithiuminBrainHomogenates

	E18	P0	P2	P4	P7	P12	P16	adult
Acute experiment (mM)	0.22	0.10	0.99	0.39	0.23	N/A	0.37	0.45
Long term experiment (mM)	0.21	N/A	0.17	0.18	0.07	0.12	0.06	0.40

Average concentrations of lithium in brain homogenates from acute and long-term treatment groups. P0 not included in long-term experiments as the dam's treatment started from day of birth. Individual values are in Additional file 3. Control values, are in Additional file 1.

The concentration of lithium in brains from control un-injected animals was considerably less than in all the treated animals and was similar at all ages examined in including adult (0.037 0.01 mM, n=8). Values for lithium in brain from acute experiments are from the same animals as in Figure 4. For long-term experiments the brain values are from the animals in Figure 5. In animals exposed to lithium via the breast milk the concentrations of lithium in acutely treated animals were consistently higher than in animals exposed to lithium via breast milk. In the fetuses in contrast both acute and long-term treated groups had similar concentrations (0.22 and 0.21 mM respectively). To assess how entry of lithium into the brain was related to its concentration in plasma, brain to plasma concentration ratios (%) were calculated and results are shown in Figure 6.

In the acutely treated animals the brain/plasma ratios were similar in the fetuses and postnatal animals except at P2 when the ratios were higher. This is in contrast to the CSF/plasma ratios which were similar at the fetal and postnatal ages (Figure 4). The higher ratio reflects a higher brain content of lithium, as the plasma concentration was similar to that at other postnatal ages.

ii. LA-ICP-MS analysis of brain sections

A summary of the distribution patterns of lithium in brain is shown in Figure 7; other scans obtained, including control scans are in Additional file 3.

Lithium in both acute and long-term treated pups showed a regional pattern of localisation, with a distinct accumulation in the olfactory bulb, which was present at E18 but more prominent in postnatal ages particularly in the brains of acutely treated animals. In the rest of the brain there was a more generalised distribution. The concentration of lithium in the brains of pups that received lithium via breast milk following i.p. injection to the mother was much less at all ages than in acutely exposed pups which received an i.p. injection. In addition, the concentration of lithium in the brain decreased in long-term exposed pups as they got older and it was barely above background by P16. These differences between the acute and longer-term experiments are probably a consequence of the lower concentrations of lithium in plasma in breast-fed pups (c.f. Figures 5 & 6).

ProtectiveFunctionof the Placenta

To measure the degree of protection provided by the placenta, concentrations of lithium in the fetal plasma were compared to maternal plasma from dams that received either a single i.p. injection of 3.2 mg lithium/ Kg body weight at E18 (acute experiment) or twice daily i.p. injections of 3.2 mg lithium/ Kg body weight from E15 to E18 (long-term

experiment). Maternal blood samples were collected from the cannula inserted into the femoral artery (see Methods) and were time-matched to each pup as it was removed from its amniotic sac. Results are shown in Figure 8.

As can be seen in Figure 8 the ratio of fetal to maternal plasma lithium concentration in acute and long-term experiment was about 15-40% and did not appear change much during the experimental period (4 h). This means that following administration to the dam about 70% of maternal lithium did not reach the fetal circulation. The placenta thus seems to be providing a substantial level of protection for the fetus. Nevertheless around 30% of the injected dose was reaching the fetus and much of this was reaching the developing brain (Table 2 and Figures 7- 9) but less into the CSF (Figures 5 and 6).

Lithium entry into the developing brain following inhibition of sodium/ potassium ATPase pump

To examine whether sodium/ potassium ATPase pump is involved in lithium entry into the developing brain, digoxin, an inhibitor of the enzyme was injected i.p. to P4 pups. A group of six littermates were randomised and three received digoxin 30 minutes prior to an i.p. injection of lithium, while three that served as control pups received an equal volume of isotonic sodium chloride for the same duration of time (Figure 9). There was a small non-significant difference in CSF/plasma ratios ($p>0.05$). The dose of digoxin was deliberately chosen to be large. The minimal effect suggests that sodium/potassium ATPase may not play a functionally significant role in entry of lithium into the CSF early in brain development (see Discussion).

Discussion

This study aimed to estimate the extent and distribution of entry of lithium into the brain during development from E18 to P17 compared to the adult, following administration of clinically revevant doses of lithium either to the pregant and lactating mothers or directly into the postnatal animals. This approach allows determination of the degree of protection provided by the placenta and breast tissue against maternally administered lithium as well as the protection provided by blood brain barriers during rat development. Acute (single dose i.p.) experiments were used to study directly the entry of lithium across the blood-brain and blood-CSF barriers in adult and developing animals. For technical reasons only i.p. injections were use in the E19 experiments. In longer-term treatment experiments injections (i.p.) were only made into the pregnant and lactating mothers. This mimicked the clinical situation of continuous treatment and allowed estimation of the role of placental and breast tissue protection. Samples of blood plasma, CSF and brain were collected from fetuses, postnatal animals and dams for analysis of lithium distribution between the dam, fetuses and pups and between blood and tissues at different stages of development. The period of treatment (E15-E19) is about 25% of the gestational period of the rat (21 days). E19 is approximately equivalent to 22–24 weeks gestation in humans (43. Clancy et al., 2001; 44. Workman et al 2013), corresponding to the earliest age of viability (45. Fischer et al., 2009; 46. Stoll et al., 2010).

Transfer of maternally administered lithium across the placenta

The extent of protection provided by placenta against lithium entry into the fetus was assessed from lithium concentration ratios between fetal and maternal plasma. In E18 pups from an acutely injected dam, lithium concentrations in the fetal plasma at 100-250 min were lower than the dam's plasma, with fetal/maternal ratios between 15-30%. In E18 pups from a pregnant dam receiving daily injections of lithium from E15-E18, the fetal/maternal concentration ratios were between 20-35% within same timeframe (Figure 8). Thus rat placenta appears to significantly restrict lithium entry into fetal blood late in gestation by about 70%.

There have been few studies of placental transfer of lithium in humans. In ten patients with bipolar disorder treated with lithium throughout pregnancy, on delivery in late gestation fetal/maternal concentration ratios were around 100% (47. Newport et al. 2005). In 10 patients in a region where drinking water contained high levels of lithium (NW Argentina) in infants born at 39-weeks' gestation the cord/maternal plasma ratios averaged about 150% (48. Harari et al 2015). In another study (49. Krachler et al. 1999) a large number of naturally occurring elements, the cord/maternal ratios for lithium showed wide variation with a mean of about 80% (n=29). These findings perhaps suggest that in the case of lithium therapy or ingestion of water with high naturally occurring levels of lithium there may be unrestricted transfer between maternal and fetal blood in humans. This contrasts with the much lower ratios observed in the present study. Factors that might contribute to this difference could include differences in where blood was sampled (cord blood in humans and heart right ventricle in fetal rats), different lengths of exposure (4 days in the rats compared to continuing lithium treatment during pregnancy in humans) and species-specific differences in placental structure. The rat and human placentas are both classed as hemochorial (50. Blood et al., 2007; 51. Dawe et al., 2007) but there are differences in morphology, in particular that the rat placenta has more morphological layers between the fetal and maternal circulations. Also, there may be age-dependent differences in placental function. This is suggested by a single case example of a fetus delivered from a mother with lithium toxicity by caesarean section at 28-weeks' gestation where the cord/maternal blood ratio was 86% (52. Zamani et al 2017).

Transfer of maternally administered lithium across breast tissue and milk

Transfer of drugs through breast milk can be affected by a number of factors such as characteristics of the drug itself: molecular size, degree of ionization, lipid solubility and for many drugs but not lithium, extent of plasma protein binding (53. Newton & Hale, 2015) and maternal factors such as maternal plasma concentrations and pharmacogenomics (54. Hotham and Hotham, 2015). Lithium concentrations in human colostrum and breast milk have been found to be around 50% of the maternal blood concentration (49. Krachler et al. 1999; 15, Viguera et al. 2007; 53. Newton & Hale, 2015) although large variations were observed (30-70%, 15. Viguera et al. 2007; 49. Krachler et al. 1999). These studies also reported a wide range of lithium concentrations in infant serum. These variations perhaps relate to the time of breast feeding compared to the time of ingestion of lithium by the mother, whether as medication or in drinking water.

A key finding in the present study was detection of lithium in the plasma of pups from lactating dams administered long-term lithium therapy. Compared to pups directly injected with lithium (3.2 mg lithium/Kg i.p.) and sampled at steady-state (90-120 min), plasma and CSF lithium concentrations in the breastfed pups were much lower (~0.4 mM vs ~0.1 mM in plasma and ~0.15 mM vs ~0.05 mM in CSF respectively, Figures 5 and 6). Concentrations of lithium in breast milk were not measured in this study, nor how much milk was consumed by each pup, so it was not possible to quantitate the extent of protection provided by breast tissue.

Mechanism of lithium entry into the CSF

Due to its very small size (hydrodynamic radius 0.079 nm, 55. Mähler and Persson, 2011) lithium would be predicted to enter the CSF from blood either by passive diffusion or by transfer mechanisms via ion channels or exchange transporters where Li^+ substitutes for Na^+, see Introduction.

Previous studies of blood to CSF transfer have suggested that entry of molecules is dependent on molecular size, lipid solubility and stage of development (age) with smaller molecular radius and younger age correlating with higher apparent rates of entry. This relationship has been demonstrated in a number of different species (42. Saunders, 1992) including the rat (36. Habgood et al., 1993). However, the level of lipid insoluble molecules is heavily dependent on the

turnover of CSF, which is much less in the developing brain. Thus, the much higher levels of these molecules in brain and CSF early in development, should better be referred to as an index of “apparent” permeability (24. Saunders et al. 2018). The smallest molecular marker previously investigated in rat was L-glucose (molecular size 180Da, molecular radius 0.43nm) which is much larger than the 0.079nm hydrodynamic radius of lithium (55. Mähler and Persson, 2011). Nevertheless, if lithium predominately enters CSF by passive diffusion, it would be expected that its CSF/plasma concentration ratio would fall on the correlation lines predicted by other passively transferred markers (36. Habgood et al., 1993).

In the acute exposure experiments, lithium CSF/ Plasma ratios fell below the levels predicted for passive markers across all ages studied (Figure 10A). In contrast, CSF/ Plasma ratios from the steady-state (long-term) lithium exposure experiments did fall on the lines predicted for passive permeability (Figure 10B) at each stage of development investigated (R^2 values of 0.97, 0.99 for P2 and P16-20 respectively).

The much lower apparent entry of lithium into CSF in acute experiments, compared to steady-state and compared to other markers, is inconsistent with transfer by simple passive diffusion. The CSF/ Plasma ratios for the passive markers used to compare with the results of present study were all measured at steady-state (i.e. approaching the equilibrium between the rates of entry into and out of CSF). Times to reach steady-state for L-glucose, sucrose and inulin were between 4-5 h. Based on the very small molecular size of lithium it would be expected to approach steady-state within 1.5- 2 h, but the ratios reached were well below those predicted (Figure 10A).

There are numerous ion channels exchangers in choroid plexus epithelial cells (33. Damkier et al., 2013). Many of these are expressed in immature rat choroid plexus (34, 35. Liddelow et al., 2013; 2016). However, their permeability to lithium appears not o have been investigated.

The much lower CSF/ Plasma ratios for lithium in the acute experiments could perhaps be explained by a restriction on passive entry at the blood/CSF barrier interface. It seems unlikely that Na^+/K^+ ATPase activity (56. Naylor et al., 2016) was involved as inhibition with a large dose of digoxin had only a marginal effect on the entry of lithium into CSF (Figure 9). Consistent with this is the finding that Na^+/K^+ ATPase activity is low in fetal and newborn rats (57. Johansson et al 2008a)

In the long-term treated animals, lithium reached the predicted steady state ratios for the age groups studied (Figure 10B) presumably reflecting the much longer period of exposure.

Steady-state CSF/ Plasma ratios in developing rats for sodium were reported to be just below 100% in the age range of our study (58. Amtorp and Sørensen, 1974).

In the long-term treated animals there was an age-dependent decrease in entry of lithium into CSF; steady-state CSF lithium concentrations decreased from 0.2 mM at E18 to 0.07 mM at P2 and 0.009 mM at P16 (Figure 5B). Consistent with this were age-dependent decreases in CSF/plasma ratios falling from 54-92% at P2-4 down to 27-56% at P12-16. These age-related decreases are consistent with developmental increases in the rate of CSF turnover (CSF sink effect) with increasing age (59. Johansson et al., 2008b).

Entry and distribution of lithium in developing brain

The concentration of lithium in brains of treated dams, both acute and long-term, were similar (0.45 and 0.40 mM respectively, Table 2). These are consistent with previous studies (60. Smith, 1976; 61. Wraae, 1978), and also indicate there is no accumulation of lithium in brain with long-term exposure. Pups in the acute treatment group had more variable concentrations in brain homogenates, but were on average similar to the dams, mirroring concentrations in plasma. For

pups receiving lithium via breast milk, concentrations of lithium in brain homogenates were much lower than in the dam, but these animals also had lower plasma concentrations (Table 2, Figure 4). This is consistent with the suggestion that the concentration of lithium in brain is a function of the concentration in plasma (Figure 5).

The Brain Homogenate/ Plasma ratios (Figure 6) in pups from acute experiments and long-term treatments had ratios of around 100% and 150% respectively which is much higher than CSF/ Plasma ratios in the same animals (around 35%, Figure 4C and <90%, Figure 5C respectively). This suggests a faster rate of lithium excretion from CSF. Previous reports have described greater rates of lithium loss from CSF compared to brain tissue (61. Wraae 1978).

The distribution of lithium within the brain has been investigated following lithium exposure using various imaging techniques. Sandner et al (22. 1994) used ^{6}Li(n.□)^{3}H nuclear reaction in the presence of a dielectric a particle track detector to image lithium in adult rat brain, but the resolution was poor. Lithium-7 nuclear magnetic resonance (^{7}Li-NMR, 23. Stout et al., 2017) and 3D ^{7}Li magnetic resonance (62. Smith et al 2018) are non-invasive *in vivo* methods that allow low resolution brain mapping of lithium and regional quantitation of lithium levels in patients.

In the present study, LA-ICP-MS was used as it provides a higher resolution of anatomical features and patterns of distribution compared to MRI. Brain sections from this study revealed even distribution of lithium within the brain, but with notable accumulation in the olfactory lobes in all treated animals (Table 2). Preferential accumulation of other metal ions in the olfactory lobe has been reported in human and rats for manganese, aluminium, nickel, zinc and cobalt (63. Bonilla et al., 1982; 64. Henriksson et al. 1999; 65. Fechter et al., 2002; 66. Chalansonnet et al. 2018; 67. Calderón-Garcidueñas et al. 2013; 68. 69. Perrson et al. 2003a, 2003b). High resolution ion imaging in the rat have provided images showing accumulation of lithium in the frontal lobe of the brain (70. Zanni, 2017). Accumulation of lithium in the olfactory lobe of rats detected in the present study could be of clinical relevance as loss of smell (hyposmia) and altered taste sensation (dysgeusia) in patients on lithium treatment (71. de Coo and Haan, 2016; 72. Terao et al., 2011) have been reported.

Additional observations that are outside the scope of this study of lithium entry into the developing brain were that there was a prominent increase in the number of neutrophils in the blood of animals exposed to lithium (see Additional file 4) which is consistent with leucocytosis following lithium therapy described in adults and children (73. Schou et al., 1970; 74. Chan et al., 1981; 75. Ishii et al., 1983). Another known side effect in patients on lithium therapy is weight gain (76. Kerry et al., 1970). This was evident in pups chronically exposed to lithium via breast milk (Additional file 4).

Limitations Of Study

1. Number of different litters and littermates.

Due to time constraints, data from long-term treated animals were obtained in only one litter of pups that were all exposed to the milk from the same dam, potentially influencing the results if they turn out to be litter specific. However, this is unlikely as results from acute treatment group appear to have no litter specific differences (six litters).

2. Potential effects of kidney excretion

An explanation of the variability of lithium in the blood would likely be the rate of secretion through the kidneys, this is age dependent as in a study that looked at tracers in rats, plasma concentration of tracer was much steadier in postnatal rats who received nephrectomy (36. Habgood et al., 1993).

3. Limitations of using only LA-ICP-MS

LA-ICP-MS is highly sensitive for lithium detection in samples, however, heavily reliant on the accuracy and consistency of standards (see Methods). To decrease the possibility of error in this study all CSF/ Plasma ratios were calculated within same run with same set of the standards. However, LA-ICP-MS was the sole method of lithium quantitation in all samples of present study, therefore an alternative method to measure lithium could be used in future studies such as the colorimetric method using Quinzarin (77. Gracia et al. 1997) or optical and impedence spectroscopy (78. Qassem et al. 2018) .

Conclusions

Lithium entry into brain and CSF was demonstrated in fetuses (E18) and postnatal animals (P0-P16) when lithium was administered via the mother or directly to the postnatal pups. The concentration of lithium in CSF at all ages and in all treatment groups was similar in spite of variations in plasma levels. This indicates that the mechanisms controlling lithium entry into developing CSF are well developed early on as is known to be the case for endogenous ions such as Na^+, K^+ and Ca^{++}. Inhibition of Na^+/K^+ ATPase with a large dose of digoxin had only a marginal effect on reducing lithium entry into CSF. This indicates that other channels and ion exchange mechanisms are involved in lithium entry into the developing brain. In the pregnant animals the fetal plasma lithium concentration was only 30–35% of that in maternal plasma indicating a substantial degree of placental protection at this stage of gestation. Similar restriction on lithium entry into postnatal pups via the breast milk of the lactating dam was observed. Never the less in both cases lithium did enter the brain of the pups. This information on entry of lithium and its distribution in the developing brain provides background for studies of possible deleterious effects of lithium on brain development and behaviour in offspring of mothers on lithium therapy.

Abbreviations

CSF: cerebrospinal fluid. E: embryonic. i.p.: intraperitoneal.

LA-ICP-MS: Laser Ablation Inductively Coupled Plasma Mass Spectrometry

LiCl: lithium chloride. ^{7}Li-NMR: Lithium-7 nuclear magnetic resonance

Na^+/K^+ ATPase: sodium/ potassium adenosine triphosphatase

P: postnatal. SD: standard deviation

Declarations

Authors' contributions

The study was conceived following a discussion between PJC, KMD, KK and NRS.

The study was devised and designed by KMD, MDH, NRS.

SY-SC & KMD wrote the first draft of the manuscript. KMD and NRS wrote the second draft. All authors contributed to the preparation of the manuscript.

SY-SC Carried out animal experiments and preparation of samples with YH under supervision of KMD, MDH. Preparation of Figures, Tables and Additional file material (1,2,4)

KK measured lithium in samples using Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS) analysed data and prepared images.

MDH Supervised animal experiments

YH Carried out animal experiments and preparation of samples with SY-SC under supervision of KMD, MDH

LMK assisted with the animal experiments.

PJC supervised Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS) lithium measurements, data analysis and image preparation.

KMD supervised experiments, analysis and preparation of data.

NRS overall supervision.

Funding

PC and KK supported by grants from The University of Melbourne

[Research costs paid for by NRS and KMD with a small contribution from the Department]

Availability of data and materials. All data generated or analysed during this study are included in this published article and its supplementary information files.

Ethics approval

All experimental procedures were conducted in accordance with National Health and Medical Research Committee (NHMRC) guidelines and approved by The University of Melbourne Ethics Committee, Ethics ID 1914793 (Entry of Anti-Epileptic and Psychotic Drugs into the Developing Brain).

Competing interests

The authors declare that they have no competing interests.

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Figures

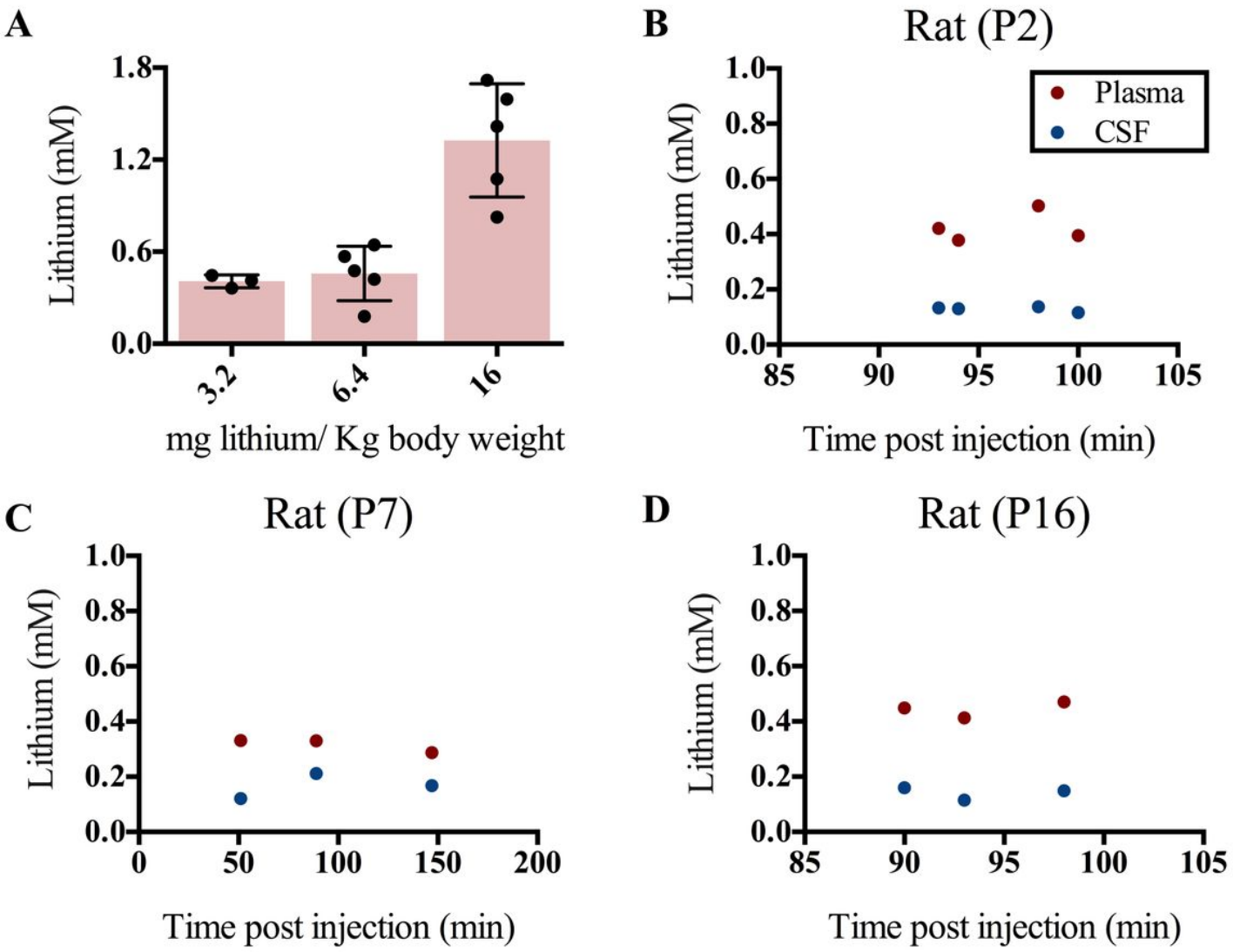

Figure 1

Determination of Injection Protocol. A. Concentration of lithium in plasma of a litter of P4 rats administered 3.2, 6.4 or 16 mg lithium/ Kg body weight via i.p. injection, samples were collected 90 min after injection. Each dot represents an individual animal. Error bars are ± standard deviation (SD) when n>2. B-D. Standardised amounts of lithium 3.2 mg lithium/ Kg body weight were injected to litters i.p.at the ages indicated; plasma and CSF were collected from pups at different times. Each dot in (B), (C) and (D) represent samples from individual animals, paired plasma and CSF for each pup are aligned vertically on graphs. Concentrations of lithium in un-injected control rats (P4) were below the limit of quantitation (LoQ).

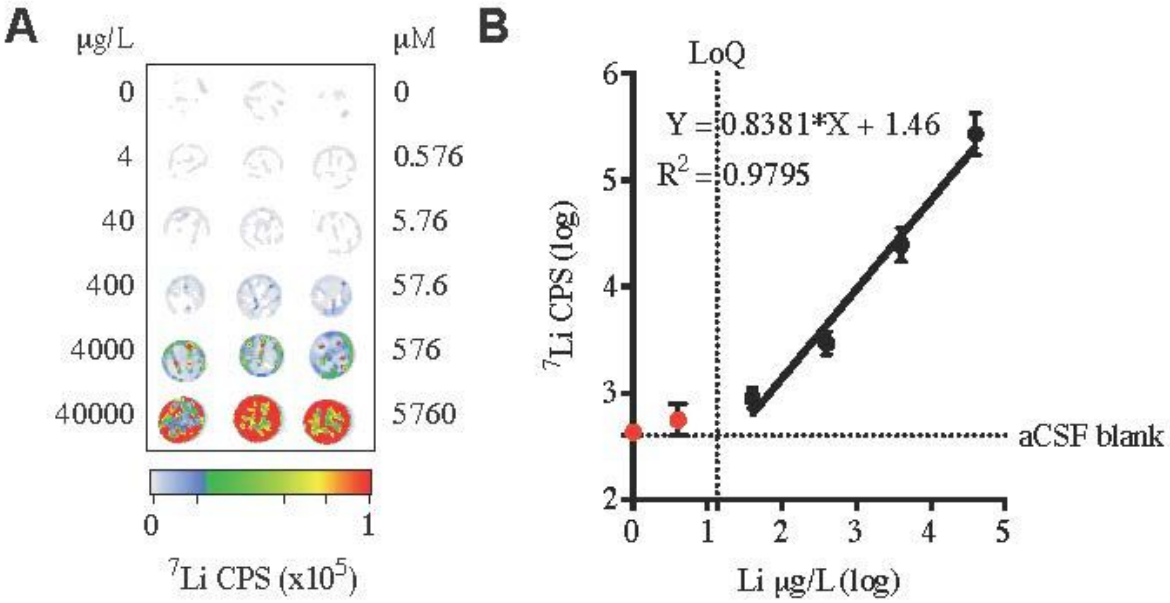

Figure 2

Figure 2

(A) Representative elemental map of lithium standards in CSF matrix residues (µg/L and µM) (B) resulting standard curve for these samples. Lithium standard concentration curves were prepared independently for each experiment.

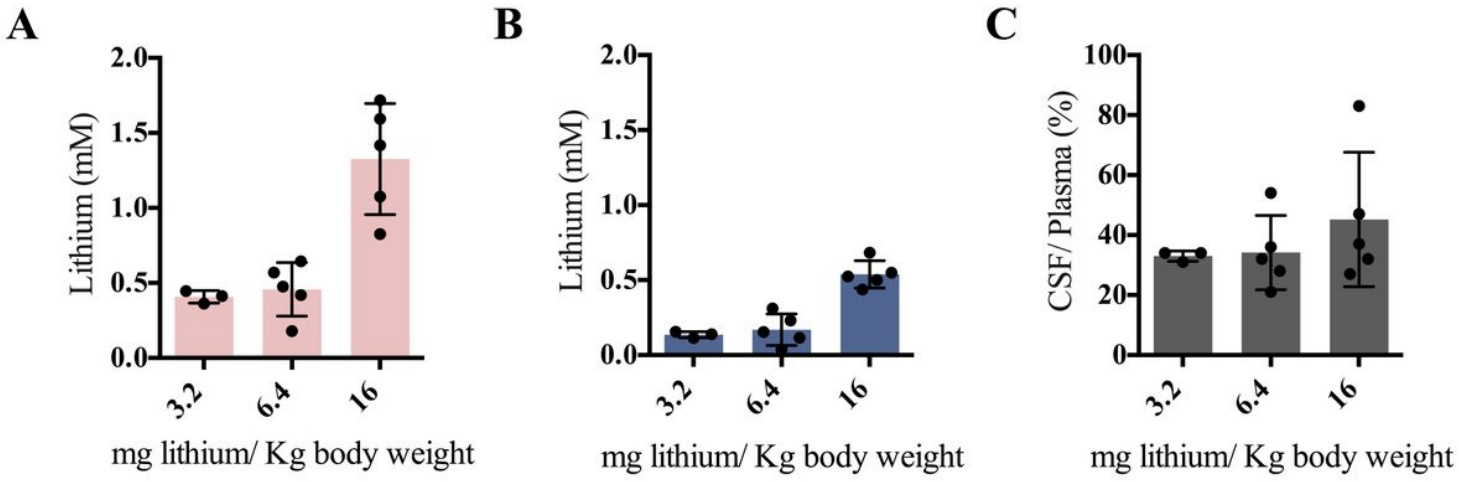

Figure 3

Concentration of lithium in plasma and CSF. (A) plasma (same data as Fig 1A), (B) CSF and (C) CSF/ Plasma ratios (%) of P4 rats administered 3.2, 6.4 or 16 mg lithium/ Kg body weight via i.p. injection. Each dot in (A) and (B) represents an individual animal. CSF/ Plasma ratios (%) are calculated for each animal. Error bars are ±SD when n>2. Differences in mean CSF/plasma ratios for different doses of lithium were not statistically significant, p=0.05. Concentration of lithium in un-injected rats (P4): 4-13 µM.

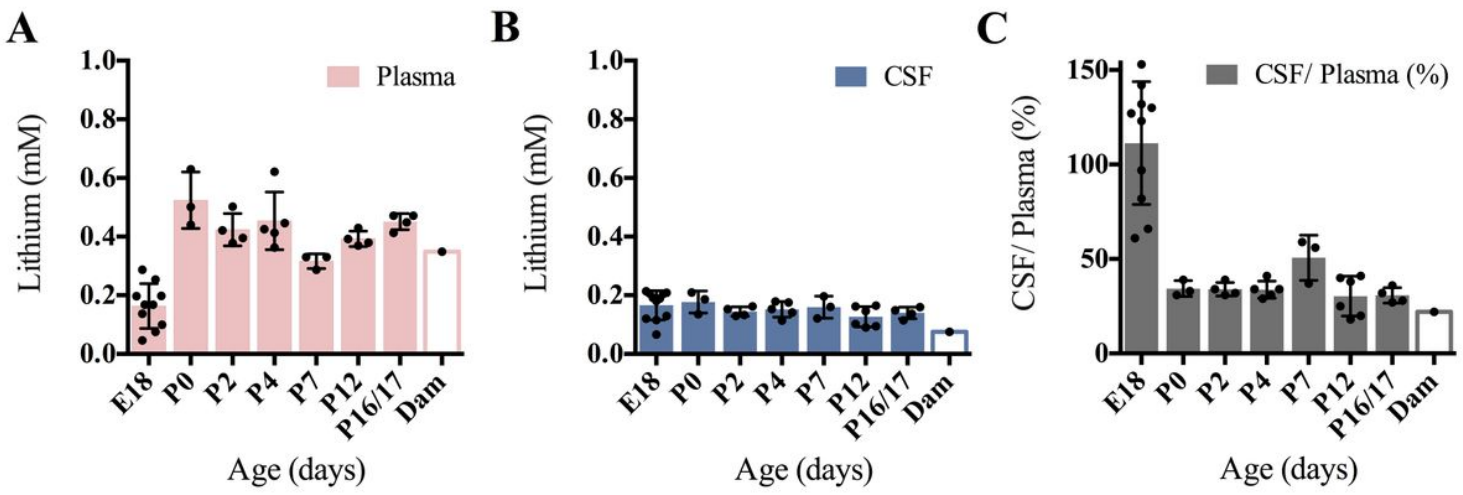

Figure 4

Concentration of lithium. (A) plasma, (B) CSF (B) and (C) CSF/ Plasma ratios (%) of animals following a single dose of lithium.

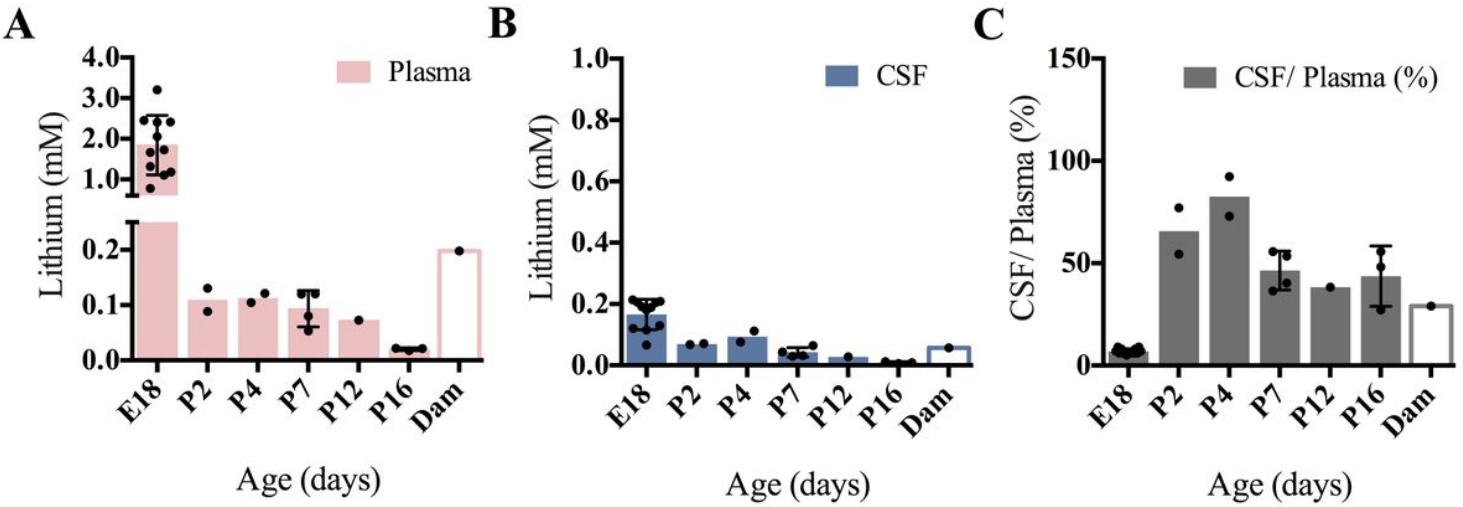

Figure 5

Concentration of lithium (A) plasma, (B) CSF and (C) CSF/ Plasma ratios (%). Rats exposed to chronic lithium treatment via placental transfer (estimated at E18) or breast milk (estimated at P2-P16) from treated dams. P0 is not included as in long-term treated pups the dam's treatment started from the day of birth (P0). Note the difference in y axis scale in (A) and (B) and that the plasma levels of lithium in the fetuses were much higher than in the single injection experiments but this was not reflected in a higher concentration in fetal CSF; also the lithium concentrations in these postnatal animals were less than in the acute experiments and declined with age (cf Figure 4). Each dot in (A) and (B) represents a sample from individual animals. CSF/ Plasma ratios (%) are calculated for the samples from the same animal. Error bars are ±SD when n>2. For control values, see Additional file 1.

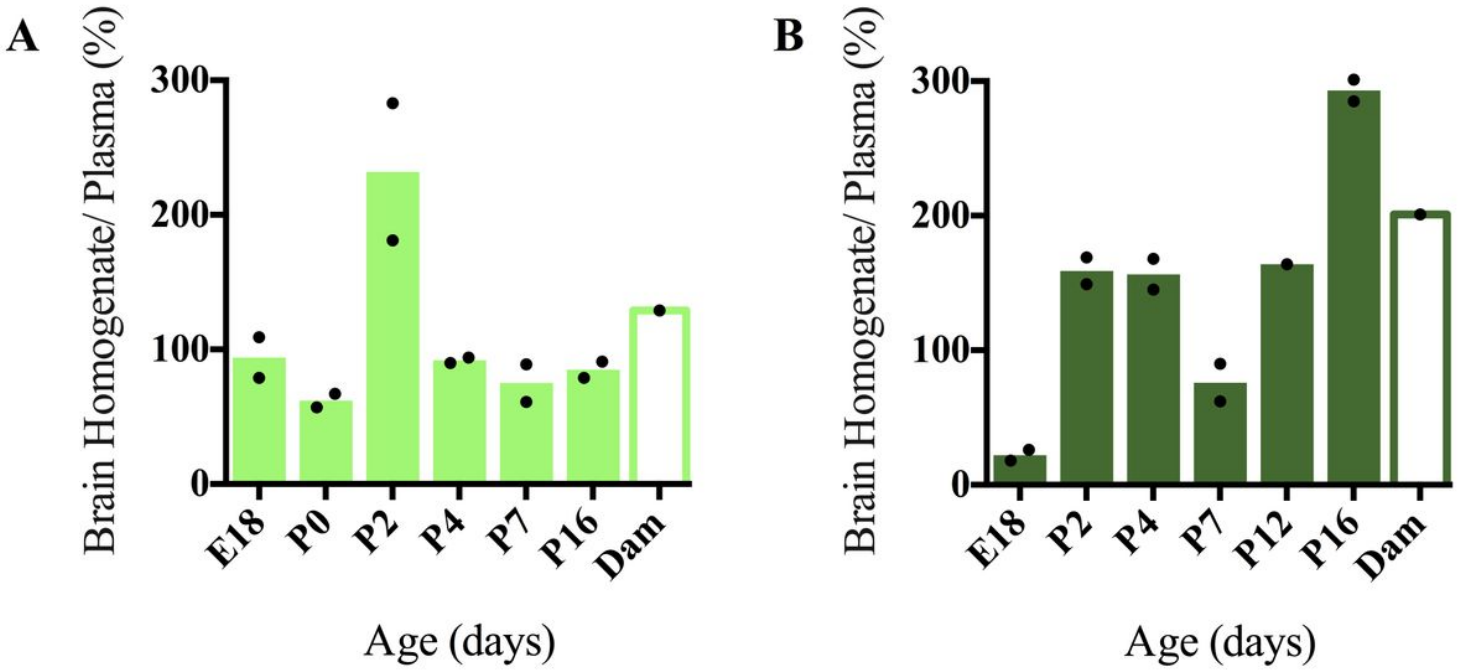

Figure 6

Brain / Plasma lithium concentration ratios (%). (A) acute and (B) long-term experiments. Brain / Plasma ratios (%) estimated from values of same animal. P0 is not included in (B) as in long-term treated pups the dam's treatment started from the day of birth.

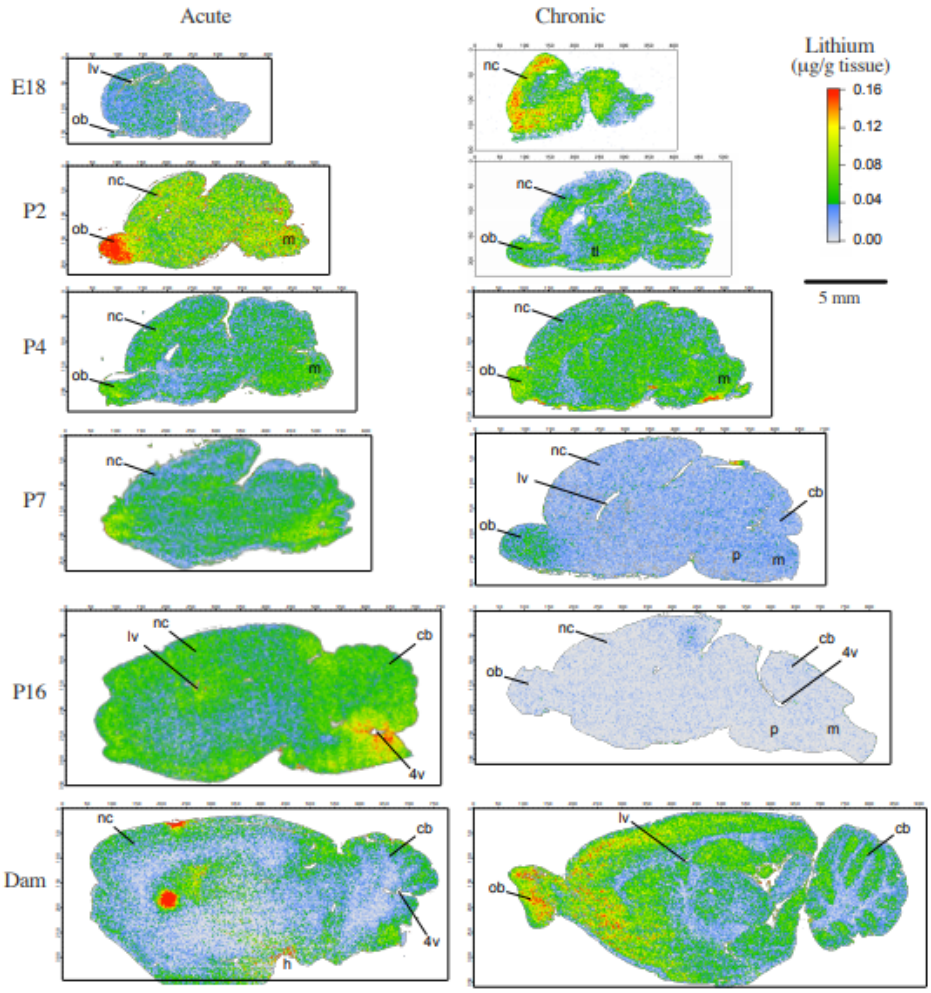

Figure 7

Distribution of Lithium in brain following acute and long-term exposure to lithium at different ages. Representative sagittal sections of brains at different ages from animals exposed to lithium acutely (left hand panels) and long-term (right hand panels). Olfactory bulbs are to the left in all sections. The dimensions of the brain sections are approximately to scale but due to dehydration in preparation of tissue they can vary between ages. At most postnatal ages there is a particular concentration of lithium in the olfactory bulbs and more generalised lower level distribution in the rest of the brain. There was a similar distribution in the postnatal brains of animals treated long-term, but at lower intensity. More sections of treated and control (which were always blank) rat brains are in Additional file 3. P0 is not included in long-term treated group (right hand panels) as in long-term treated pups the dam's treatment started from the day of birth. The "hotspot" in the acute dam section is due to lithium contamination. See Additional file 3 for additional images. Abbreviations: ob, olfactory bulb; fc, frontal cortex; lv, lateral ventricle; nc, neocortex; tl, temporal lobe; p, pons; m, medulla; cb, cerebellum; 4v, 4th ventricle; h, hypothalamus.

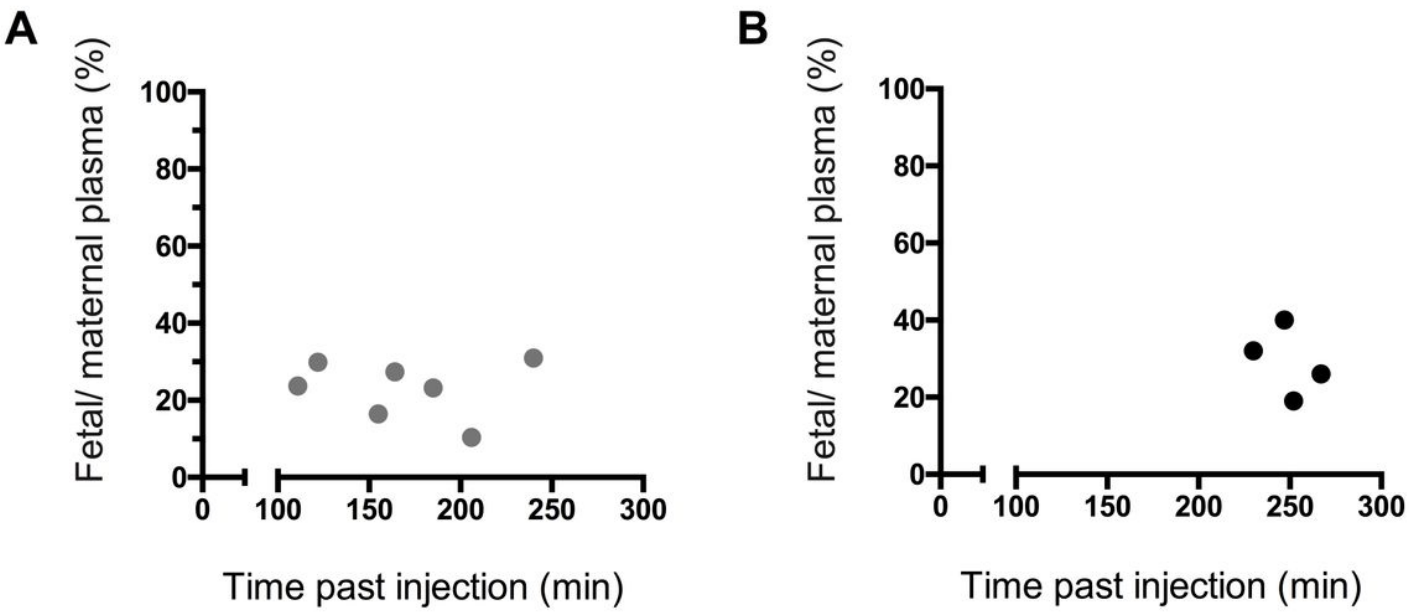

Figure 8

Fetal/maternal plasma lithium concentration ratios at E18. (A) acute treatment and (B) long-term treatment. Each point on the graphs represents a ratio calculated using fetal plasma levels in an individual fetus and the corresponding plasma of the dam.

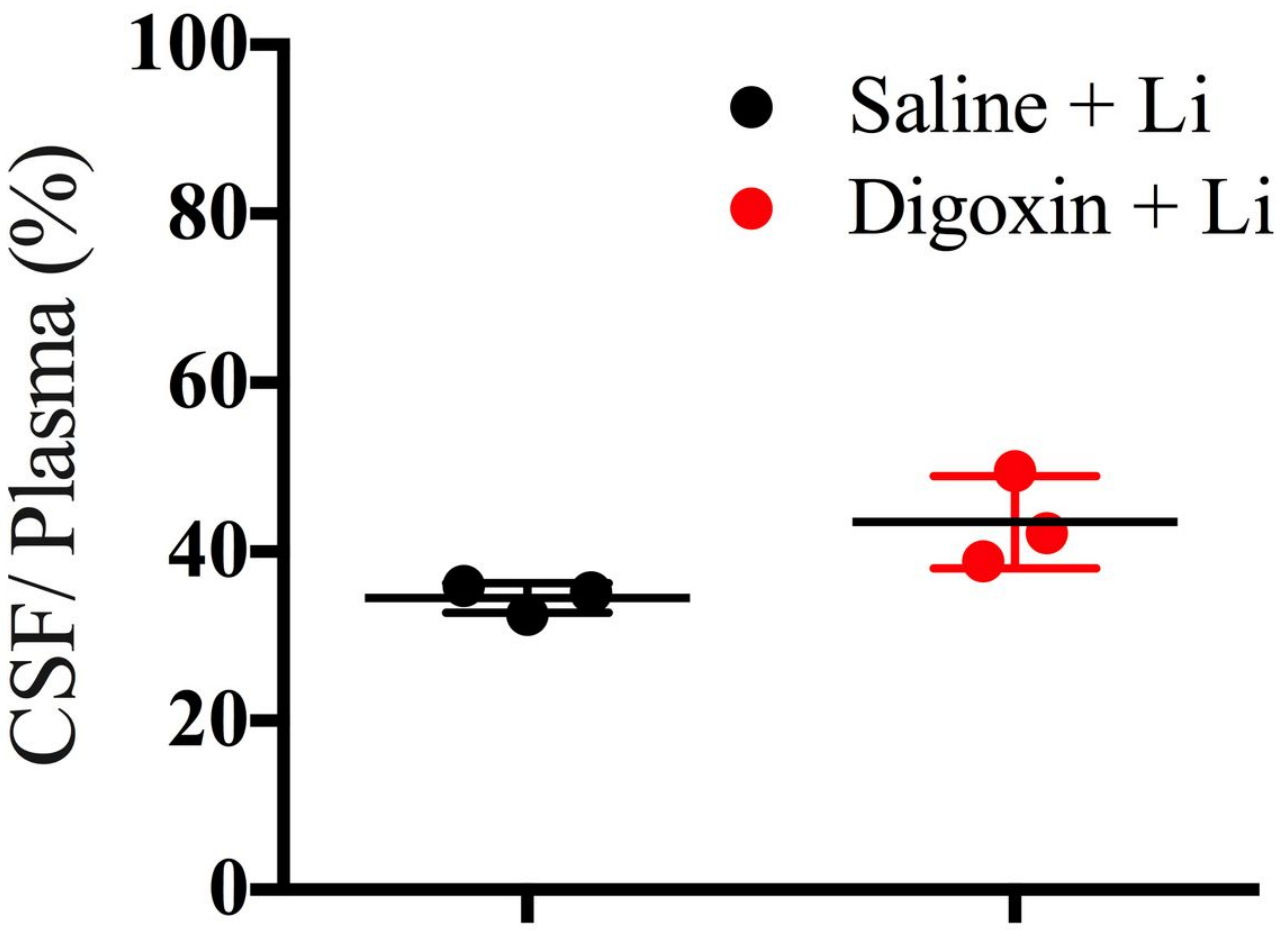

Figure 9

Effect of digoxin on entry of lithium into CSF of P4 rats. CSF/ Plasma ratios (%) of P4 littermates acutely exposed to lithium following treatment with a large dose of digoxin (300 mg/Kg body weight, red dots) compared to isotonic sodium chloride (black dots). Each dot represents a sample from individual animals. Error bars are SDs) $p>0.05$.

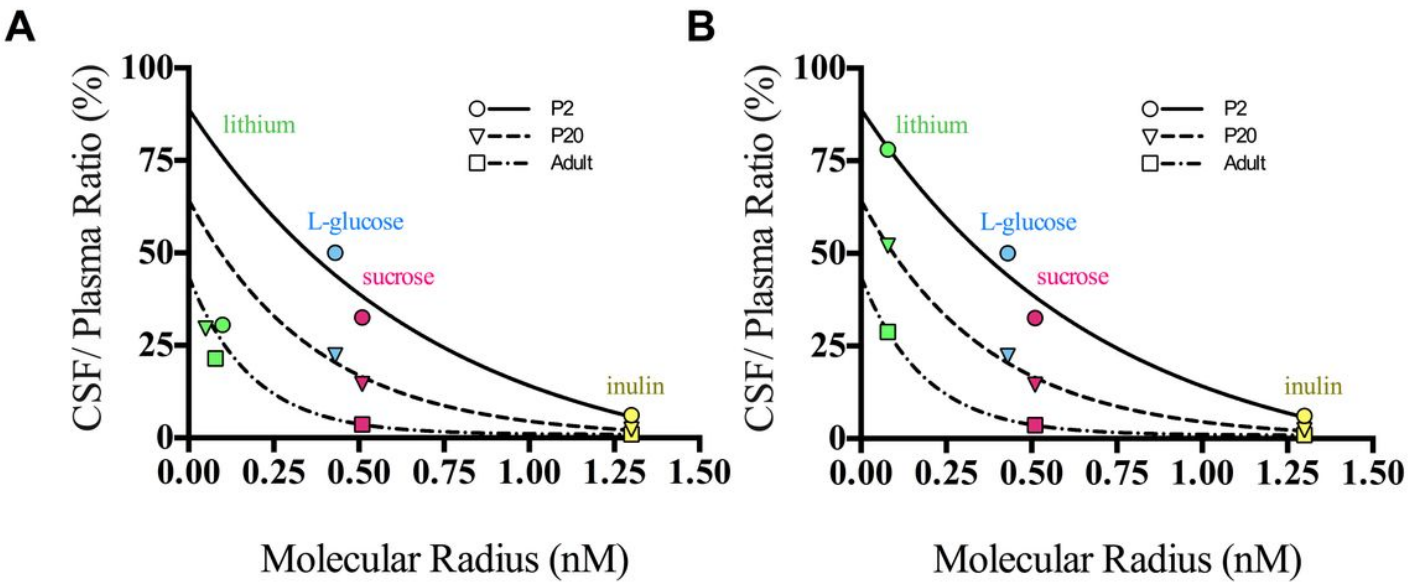

Figure 10

Comparison of CSF/ Plasma concentration ratios (%) for lithium and passive permeability markers. A. following acute exposure (n=3-10 for lithium, postnatal animals, n= 1 adult; data from Figure 4) and B. long-term exposure in rats at different stages of development (n= 1-3 postnatal animals, n=1 adult; data from Figure 5). CSF/plasma ratios are plotted against the molecular radius of the markers and the curves were fitted by non-linear regression analysis (Graphpad Prism). Each point represents the average CSF/ Plasma ratio (%) for each age within each treatment group. Permeability data for markers other than lithium from 36. Habgood et al. (1993).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile4.docx
- Additionalfile3Figure1Table1.docx
- Additionalfile2.docx
- AdditionalFile1.xlsx