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## Agricultural groundwater with high nitrates and dissolved salts given to pregnant mice alters brain development in the offspring

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### ABSTRACT

Groundwater is the main source of drinking water for a significant portion of the human population. In many agricultural areas, diffuse pollution such as high levels of total dissolved salts including nitrate, puts the quality of this resource at risk. However, the effect of exposure to these water contaminants on brain development is currently poorly understood. Here we characterised water from a borewell located in an intensely cultivated area (agricultural) or water from a borewell located in a nearby pristine forest. The agricultural borewell water was rich in nitrates with high total dissolved salts. We then studied the consequence of drinking the agricultural water on mouse brain development. For this, the agricultural borewell water or forest water was given to mice for 6 weeks before and during pregnancy and lactation. The brains of the offspring born to these dams were analysed at postnatal day (P)5 and P21 and compared using immunohistochemistry for changes in glial cells, neurons, myelin, and cell death across many brain regions. Brains from offspring born to dams who had been given agricultural water (versus forest control water) were significantly smaller, and at P21 had a significant degeneration of neurons and increased numbers of microglia in the motor cortex, had fewer white matter astrocytes and an increase in cell death, particularly in the dentate gyrus. This study shows that brain development is sensitive to water composition. It points to the importance of assessing neurodevelopmental delays when considering the effect of water contaminated with agricultural run offs on human health.

**Main finding:** Pregnant and lactating mice were given borewell water from intensely cultivated land. Offspring brains reveal degeneration of neurons and a loss of astrocytes, increase in microglial cells and cell death, pointing to neurodevelopmental problems.

### 1. Introduction

Groundwater is an important source of drinking water for a significant portion of the human population (Morris et al., 2003). However, agricultural activities have had a severe impact on the availability and

the quality of groundwater (Burri et al., 2019). Excessive application of inorganic fertilisers and animal manure on cultivated land has led to widespread contamination of groundwater with nitrate (NO<sub>3</sub>) across the world (Burow et al., 2010; Saha et al., 2014). Also, in the dry tropics, such as parts of India, the high levels of nitrate are often accompanied by

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total dissolved salts (TDS) exceeding 1 g/L due to the combined effects of intense evapotranspiration, rock weathering and recycling processes (Buvaneshwari et al., 2017). Nitrate concentration is included in globally recognised indices of water quality such as the pollution index (Van der Weijden and Pacheco, 2006) and the weighted Groundwater Health Index (wGHI) (Di Lorenzo et al., 2020).

High nitrate concentration in the drinking water is associated with methemoglobinemia (or blue baby syndrome), many types of cancer, impaired thyroid functions, congenital abnormalities, spontaneous abortion, premature birth and intrauterine growth retardation (IUGR) (Blaisdell et al., 2019; Bukowski et al., 2001; Migeot et al., 2013; Vanhatalo and Riikonen, 2001; Vuong et al., 2016; Ward et al., 2018). Despite the potential importance of water contaminant on human health and development, the cellular and molecular effects of contaminants on prenatal brain development is not well understood. For instance, a 1973 study in rats showed decreased pup survival and postnatal growth due to nitrate exposure during pregnancy (Grüener et al., 1973). However, there was no study of neuropathology, there have been no follow-up studies and to the best of our knowledge the impact of exposure to nitrates on brain development has not been examined even though persistent behavioural deficits after prenatal nitrate exposure have been noted suggesting long term effects (Markel et al., 1989). It is critical to understand the impact of nitrate on brain development as the current drinking water limit set by the World Health Organization (WHO) at 50 mg/L has been defined based on the incidence of methemoglobinemia only, and no other considerations.

Nitrate ( $\text{NO}_3$ ) is the most common nitrogen oxide species in the body and its levels are dependent on dietary intake and oxidation of precursors. Humans possess a very low level of nitrate reductase activity (Jansson et al., 2008) but symbiotic oral bacteria that is concentrated in our salivary glands reduce  $\text{NO}_3$  to  $\text{NO}_2$  (Duncan et al., 1995; Hyde et al., 2014).  $\text{NO}_2$  is converted to the highly bioactive NO by enzymatic activity in blood and tissues. NO is widely present as a highly diffusible neurotransmitter in the brain where it has been implicated as important in normal development (Canossa et al., 2002; Gallo et al., 2002; Nott et al., 2008; Takahata et al., 2003) and in several pathophysiological conditions (Dawson and Snyder, 1994; Snyder et al., 1998). Further, pathological levels of NO and associated reactive nitrogen species (RNS) can lead to activation of microglia contributing to neuroinflammation and subsequent brain damage (Yuste et al., 2015). In addition, exposure of the mother to other salts, such as high chloride (often observed with high nitrates) also lead to a dysregulated immune response that can have deleterious effects on brain development (Lawrence and Wynn, 2018).

In this study, we aimed to provide important cellular information about the impact on brain development of groundwater from a region of intense agricultural activity containing high nitrates and also high TDS. This combination is common in semi-arid region and as a control we used water from a nearby groundwater source from a pristine forest region. We found that our agricultural run-off ground water was high in nitrates and TDS and exposure to this water before and during pregnancy and during lactation caused significant disruption to brain development in the progeny. This study has key health policy implications for determining safe levels of fertiliser-induced contaminants in drinking water and is important to ensure that women and their unborn babies are adequately protected.

## 2. Materials and methods

### 2.1. Water collection

Groundwater locations were selected from among the sites monitored in the Kabini Critical Zone Observatory (CZO) in Southern India (SNO M-TROPICS), because of their contrasting chemical composition, particularly nitrates (Buvaneshwari et al., 2017; Riotte et al., 2014). The purpose of the Kabini CZO is comparing the water and nutrient levels of a pristine forested ecosystem, the Mule Hole watershed, with those of an

agrosystem, the Berambadi watershed (Appendix A; (Sekhar et al., 2016)) for increasing our understanding the effect of agriculture on water, soils and biogeochemical cycles. The CZO provides the scientific community with time series of weather, hydrological and geochemical variables in each watershed (<https://mtropics.obs-mip.fr/>, (Riotte et al., 2021)). Groundwater samples were collected in August 2014, i.e., during the monsoon season. Water herein called FOREST water, was collected from pristine conditions in a piezometer near the outlet of the Mule Hole watershed at 3 m depth. The other, further referred as AGRI water, was collected in a borewell located in the downstream part of the Berambadi (Appendix A) at 39 m depth in an area particularly impacted by intensive agriculture. Water in the cultivated area was present at greater depth due to pumping for irrigation which led to the depletion of the resource (Buvaneshwari et al., 2017). A volume of 10 l of each sample was filtered in the laboratory soon after collection (Sartorius cellulose acetate, 0.22  $\mu\text{m}$  membrane) and the fraction destined for mice feeding stored in pre-cleaned 4 l polypropylene cans in a fridge while 100 ml were reserved for chemical analyses. A specific sampling was also undertaken for pesticide analysis, in two-litre glass bottles sheltered from light and stored at 4 °C.

### 2.2. Water analysis

Dissolved major species were analyzed at the analytical platform of the Indo-French Cell for Water Sciences (Indian Institute of Science, Bengaluru) following the protocol described in Riotte et al. (2014, 2021). Conductivity and pH were determined with a WTW metre, anions (F, Cl,  $\text{NO}_3$ ,  $\text{SO}_4$ ) and cations (Na, K, Ca, Mg) with a Metrohm 561 ion chromatograph, alkalinity with a Mettler Toledo DL50Gx titrator, DOC with a Shimadzu TOC 500 and silica by colorimetry (blue method). The quality of the major ion data was checked using reference materials ION-96.4 and PERADE, and by calculating the inorganic charge balance. This latter was better than 3% for both samples. Trace element concentrations, including metals, were determined with an ICP-MS Agilent 7700x at BSIP, Lucknow, with detection limits ranging from 10 ng/L for light trace elements to 1 ng/L for the heavy ones. Pesticide analysis consisted of two independent screenings of the 400 most common molecules by GC-MS/MS by Eurofin (Bengaluru branch) and by LHyGes (Strasbourg University), both after a pre-concentration step on cartridge (Masbou et al., 2018). This yielded an effective detection limit greater than 0.01  $\mu\text{g/L}$  on individual molecules, hence a sensitivity that was within the drinking water analyses standards.

### 2.3. Generation of animals

All animal experiment protocols were approved by the Institutional Animal Ethics Committee of the Indian Institute of Science, Bengaluru. Ten Swiss albino female mice, 6–8 weeks old, were taken from an inbred colony at the Indian Institute of Science. The females were given either FOREST water or AGRI water for 6 weeks and then housed for mating, based on a similar approach employed by us previously (Ranade et al., 2008, 2012). The day on which a vaginal plug was observed was considered embryonic day (E) 0. The females were maintained on their respective water throughout gestation and lactation. Brain and body weight were monitored using standard weighing scales. Animals had access to food and water ad libitum and were housed under a 12 h light–dark cycle. Dams gave birth and pups stayed in their home cage until postnatal day (P)21. Pups analysed born to mothers given AGRI water are referred to as AGRI group and those born to mothers given FOREST water are referred to as FOREST group. The protocol is shown schematically in (Appendix C.a).

### 2.4. Immunohistochemistry (IHC)

Brains were collected at P5 and P21 and processed and prepared for IHC as previously (Ranade et al., 2012; Schang et al., 2014; Van

Steenwinckel et al., 2019). In brief, animals were transcardially perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS under terminal anaesthesia. The brains were rapidly removed and post-fixed in 4% PFA overnight at 4 °C. The following day they were immersed in 30% sucrose in PBS and stored at 4 °C until further processing. Finally, they were embedded in 15% sucrose-7.5% gelatine solution and frozen at -80 °C before cryosections were cut at 14 µm. The primary antibodies used were anti-neuronal nuclear antigen (NeuN) (MAB377 Millipore Sigma; 1:500), anti-Calretinin (CR7697 Swant 1:500), anti-Caspase 3 (CS9961, Cell Signalling; 1:200); anti-ionising adaptor protein 1 (Iba1) (WAKO r019-19741r; 1:1000); anti-myelin basic protein (MBP) (MAB 382, MilliporeSigma;1:500), anti-Glial fibrillary acidic protein (GFAP) (DAKO Z334; 1:500). Appropriate biotinylated secondary antibodies (Vector Laboratories) were used, the antibody was visualised using 3,3'-diamino-benzidine (DAB; Sigma-Aldrich Company) and sections were counterstained with Nissl. Where intensity of immunostaining is reported it was calculated by densitometric analysis using the NIH ImageJ software (Fiji, NIH). Optical density was calculated from grayscale images standardised to the photomicrograph background as previously described (Fleiss et al., 2012b; Rangon et al., 2018). In brief, for each animal, four measurements in each region (4 fields of view) were made across four sections. For cell counts, the number of cells per field under 10x or 20x magnification were performed. Results are expressed as cell counts per field. The different areas of the brain analysed are shown in Appendix C.b.

### 2.5. Statistics

Number of animals per group were as follow: AGRI group at P5 (n = 11); AGRI group at P21 (n = 12); FOREST group at P5 (n = 15); FOREST group at P21 (n = 14). All quantitative data are reported as histograms that are plotted as mean ± SEM. Statistical analysis of all data was performed using GraphPad PRISM version 6.0 (San Diego, CA). The data sets were put through the D'Agostino-Pearson normality test and where data failed the normality test, we used a non-parametric Mann-Whitney to test for significance. Otherwise, a Student's *t*-test was applied to determine statistical significance and  $\alpha = 0.05$  was set as an acceptable significance. For all analyses, the exact *p* value is provided in the figure captions. Further the stars above the graph refer to the following \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.005$ .

## 3. Results

### 3.1. Chemical composition of the water

As expected, the FOREST and AGRI waters were characterised by contrasting chemical compositions, reflected by differences in their overall conductivity, 220 and 1906 µS/cm respectively (Appendix B). While the anionic charge of the FOREST water is largely dominated by alkalinity (89%), the AGRI water also contained high concentrations of Cl<sup>-</sup> and NO<sub>3</sub><sup>-</sup>, 229 and 319 mg/L respectively (30% and 24% of anionic charge). This nitrate concentration in the AGRI water corresponds to 6–7 times the safe drinking limit of 50 mg/L established by the WHO (2011). Cationic charges were dominated by alkali-earth Ca and Mg in FOREST water and Ca, Mg and Na in the AGRI one. The screening of the 400 pesticide molecules with a limit of quantification of 0.01 µg/L did not reveal any trace of pesticide. Most of trace element concentrations were higher in AGRI than in the FOREST water (Appendix B), with the exception of few redox-sensitive metals (Fe, Mn, Ni), showing that anoxic conditions prevail in the FOREST observation well. However, potentially toxic trace elements (Al, As, Cd, U, Ba, Mo, Co, Cu, Cr, Fe, Ni, Mn, Sb, Pb) were all far lower than the guidelines established by WHO, as shown in Appendix A.b. To sum up, the main differences between these AGRI and FOREST waters are a ratio of 500 to 1 in nitrate contents and a ratio of 10 to 1 in TDS.

### 3.2. Body and brain weight of pups

The body weight of the animals was monitored to test for evidence of growth restriction. There was a small but significant reduction in pup body weight in the AGRI group at P1 (Mean±SEM: FOREST, 2.041±0.137 g vs. AGRI 1.471±0.021 g), however by P5 this difference was no longer significant (Mean±SEM: FOREST, 3.041±0.213 g vs. AGRI 2.521±0.103 g) (Appendix C.c). At P5 and P21 brain weights were measured. The average weight of the brains of the AGRI group was smaller than that of the FOREST group at P5 (Mean±SEM: FOREST, 0.302±0.011 g vs. AGRI 0.252±0.003 g) and P21 (Mean±SEM: FOREST, 0.562±0.009 g vs. AGRI 0.540±0.005 g), although the difference reduced with increasing age. (Appendix C.d). There was no difference in the size of the litters born (Appendix C.e). Thus, exposure to AGRI water altered body and brain weights in early life.

### 3.3. Neurons

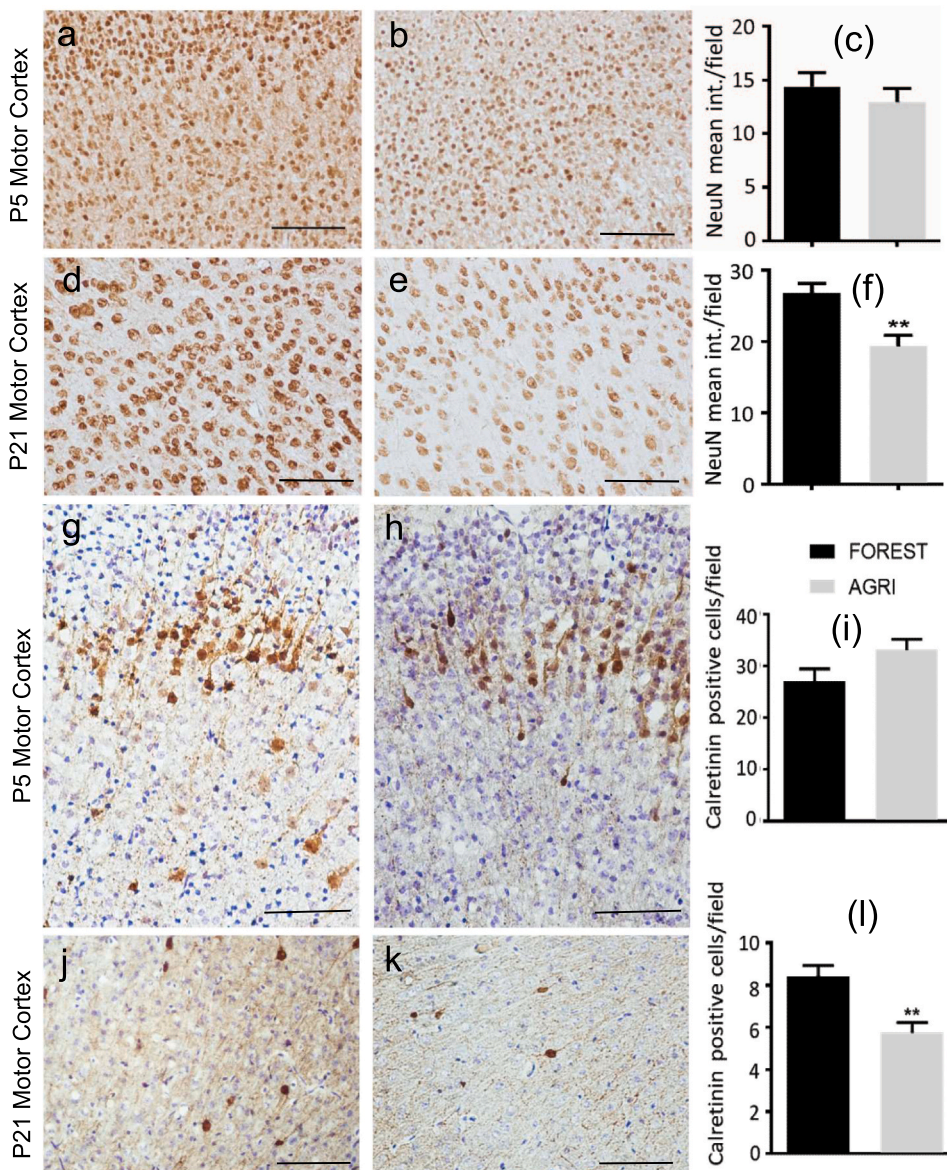
NeuN is a pan-neuronal marker for differentiated neurons and was used as a measure of the total number of neurons present in the motor area. At P5, there was no difference in the intensity of NeuN staining in the motor cortex between the two groups (Fig. 1a, b and c). However, at P21, the intensity of NeuN staining in the motor cortex in the AGRI group was significantly lower than in the FOREST group (Fig. 1d, e, and f) showing that there was degeneration of differentiated neurons in this group.

### 3.4. Number of interneurons

Interneurons are an important part of the cortical excitability circuit (Cauli et al., 2014). Calretinin neurons are a heterogenous population that are present in a subpopulation of GABAergic interneurons that expresses different combinations of neuropeptides. Compared to other interneuron populations, the calretinin population can be visualised in the first postnatal week in the lower layers of the cortex (Stolp et al., 2019). Counting the number of calretinin neurons showed that at P5 there was no difference in the number of calretinin positive cells between the two groups (Fig. 1g, h and i). However, at P21, the number of calretinin positive cells was significantly lower in the AGRI group (Fig. 1j, k and l). This shows that at least part of the reduction in the total number of neurons is due to a decrease in the calretinin subpopulation of interneurons.

### 3.5. Astrocyte area coverage

Astrocytes are fundamental to homeostatic regulation in the central nervous system. Astrocyte density was analysed using the astrocyte marker GFAP and quantifying the intensity of staining. In the white matter tracts, the external capsule (EC), corpus callosum (CC), and anterior commissure (ACC), there was a decrease in GFAP positive staining in the AGRI group. The external capsule connects the cerebral cortex to other cortical structures of the same hemisphere. Here although there was a significantly lower expression of GFAP at P5 (Fig. 2a, b and c) the difference did not persist at P21 (Fig. 2d, e and f). We also analysed the corpus callosum, which crosses the midline and connects the two cerebral hemispheres, and the anterior commissure, another smaller tract that also crosses the midline and interconnects regions of the forebrain. In both these regions there was no difference in GFAP intensity at P5 between the two groups (Fig. 2g, h and i for ACC, and Fig. 3a, b and c for CC). However, at P21 there was a significant decrease in GFAP labelling in these two midline crossing tracts (Fig. 2j, k and l for ACC and Fig. 3d, e and f for CC). No change in astrocyte density was detected in the grey matter of the motor cortex (Fig. 3g, h and i P5 and Fig. 3j, k and l).



**Fig. 1.** (a) to (f) NeuN IHC and quantitation at P5 and P21. Representative IHC images of motor cortex with NeuN (a) and (d) FOREST P5 and P21 motor cortex and (b) and (e) AGRI P5 and P21 motor cortex. Quantitation of mean intensity of NeuN IHC in (c) P5 and (f) P21 motor cortex. (\*\*  $p = 0.0013$  by Student's  $t$ -test). Scale bar = 100  $\mu\text{m}$ . (g) to (l) Calretinin IHC and quantitation at P5 and P21. Representative IHC images of motor cortex with calretinin (g) and (j) FOREST P5 and P21 motor cortex and (h) and (k) AGRI P5 and P21 motor cortex. Quantitation of mean intensity of calretinin IHC in (i) P5 (Mann Whitney) and (l) P21 motor cortex. (\*\*  $p = 0.0013$  by Student's  $t$ -test). Scale bar = 100  $\mu\text{m}$ .

### 3.6. Number of microglia

Microglia are brain resident immune cells of the myeloid lineage that have a critical roles during development (Sharma and Tremblay, 2020) and respond to infection, insult or injury. In these cases, microglia increase in number and express increased amounts of the protein Iba1 (Broad et al., 2016; Fleiss et al., 2012a). The number of Iba1 positive cells was not different between the two groups at P5 in either the cortex (Fig. 4a, b and c) or the underlying white matter (Fig. 4g, h and i). However, at P21 there was a significant increase in the number of Iba1 positive cells in the cortex (Fig. 4d, e and f) but not in the corpus callosum in the AGRI group (Fig. 4j, k and l). Thus, there was an increase in microglial cell number in the cortex.

### 3.7. Cell death

Upon initiation of programmed cell death, a key step is the cleavage of the enzyme caspase 3 which then acts on several substrates driving cell death. The dentate gyrus of the hippocampus, important for its role in learning and memory showed a highly significant increase in cell death (Fig. 5a, b and c) in the AGRI group. In contrast, although we

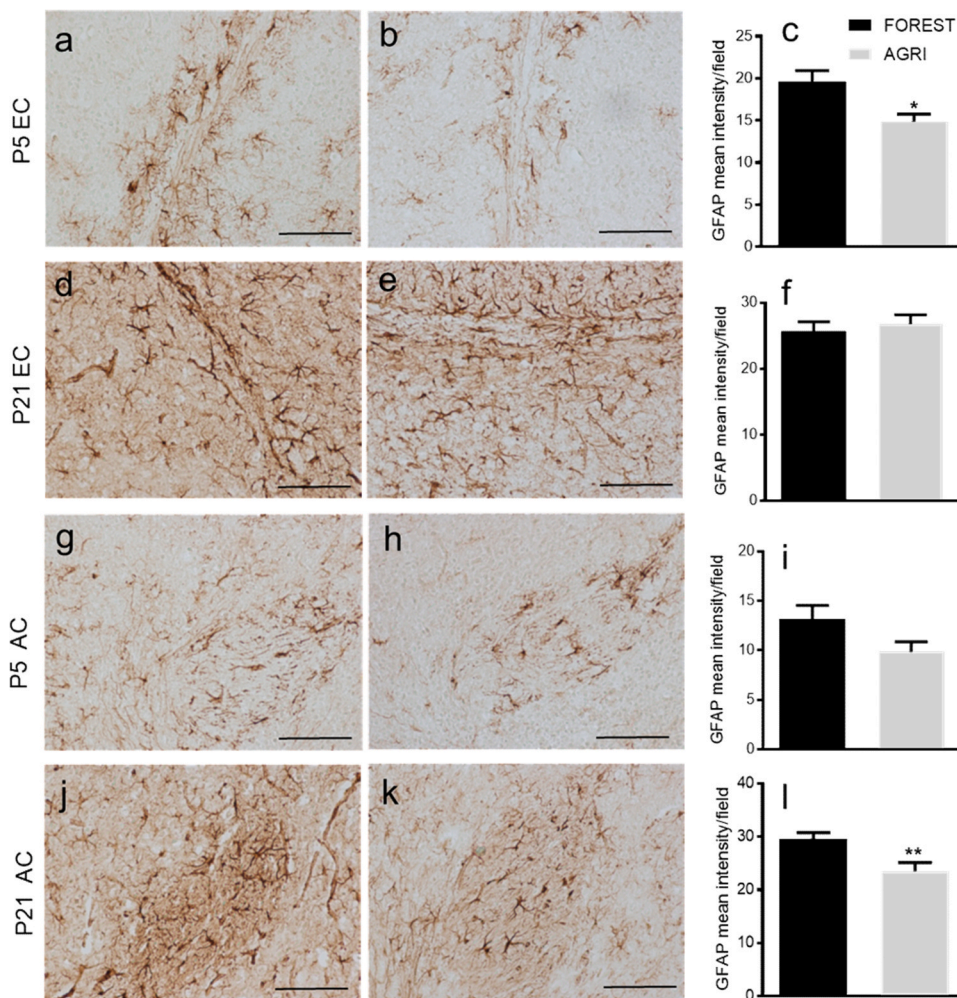
detected a statistically significant increase in cell death in the motor cortex and the white matter corpus callosum and cingulum, the absolute number of cells was still very low (data not shown for white matter and cingulum). Thus, overall a significant increase in cell death in several areas of the brain at P21 was observed in the AGRI group.

### 3.8. Myelination

MBP is an abundant myelin protein as it is an integral part of the myelin sheath. No difference in MBP immunodensity was detected between the two groups in the external capsule (Appendix D.a, D.b and D.c), corpus callosum (Appendix D.d, D.e and D.f) and the cingulate white matter (Appendix D.g, D.h and D.i). However, there was an increase in MBP staining at P21 in the motor cortex of the AGRI group (Appendix D.j, D.k and D.l).

## 4. Discussion

Our study has proven that there are negative effects of exposure to agricultural water on brain development in a mouse model. These results are summarised in Table 1 (including proportions of change and



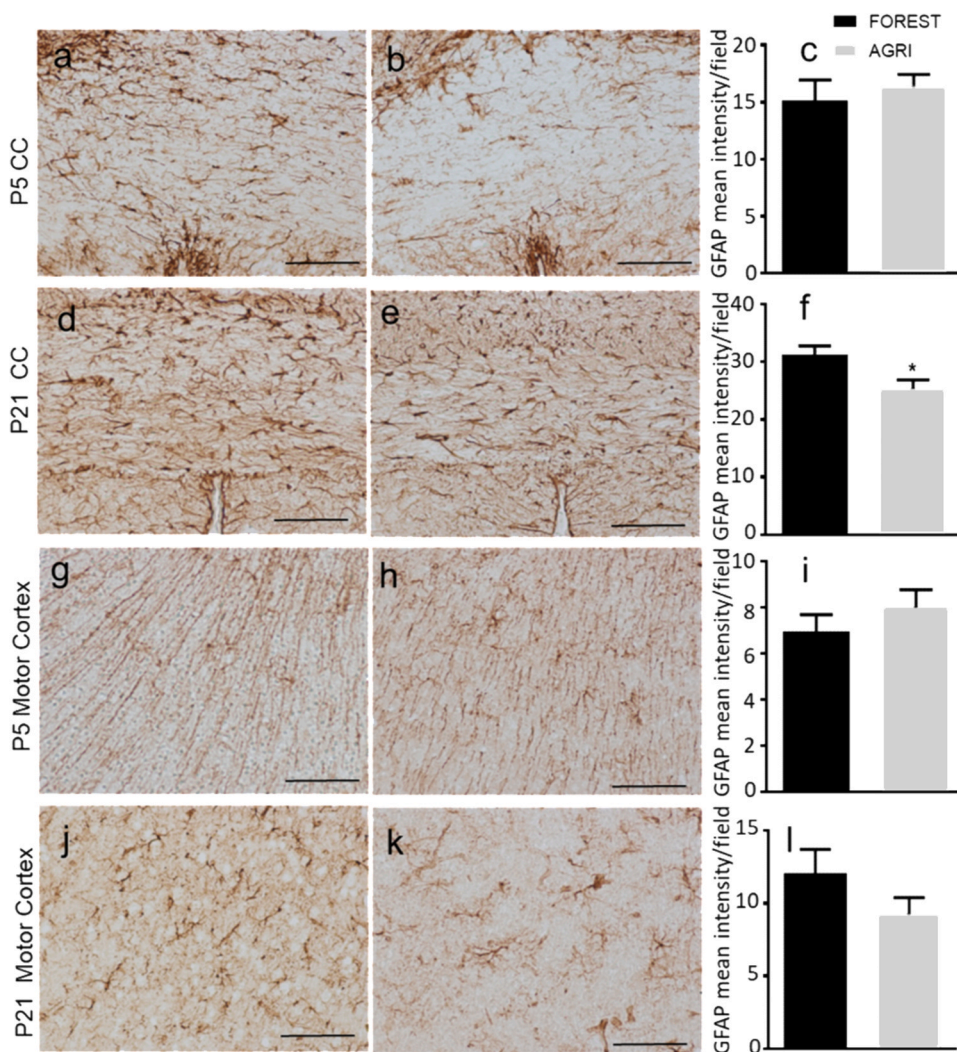
**Fig. 2.** GFAP IHC and quantitation at P5 and P21 (External Capsule and Anterior Commissure). Representative IHC images of External Capsule (EC) with GFAP (a) and (d) FOREST P5 and P21 EC and (b) and (e) AGRI P5 and P21 EC. Quantitation of mean intensity of GFAP IHC in (c) P5 and (f) P21 EC. Histograms are plotted as mean  $\pm$  SEM. (\*  $p = 0.035$  by Student's  $t$ -test). Representative IHC images of Anterior Commissure (AC) with GFAP (g) and (j) FOREST P5 and P21 AC and (h) and (k) AGRI P5 and P21 AC. Quantitation of mean intensity of GFAP IHC in (i) P5 (Mann-Whitney) and (l) P21 AC. (\*\*  $p = 0.006$  by Student's  $t$ -test). Scale bar = 100  $\mu$ m.

significance). The effect of high nitrates with high TDS on brain development has not been studied in detail previously. Water from the borewells located in the cultivated Berambadi catchment contains very high levels of nitrates, up to 6 times the drinkable limit defined by the WHO, along with high TDS. However, such high nitrate concentrations are not new nor exceptional in India (Adimalla and Qian, 2019), in Europe; (Hudak, 2012) in the US (Wick et al., 2012) and in Australia (Adelana et al., 2020). Specifically, pups born to mothers who are fed groundwater procured from the nitrate-rich borewell (versus the pristine forest water) showed several brain abnormalities compared to pups whose mothers were reared on groundwater from the pristine forest area. The defects include degeneration of neurons in the grey matter, a reduction in the density of astrocytes in the white matter, an increase in cell death in both grey and white matter, and an increase in reactive microglia in the white matter. These defects were almost always apparent at P21.

Based on our analysis of the water composition, high nitrate levels and TDS possibly account for the observed negative neurological effects as we did not detect any pesticides or toxic inorganic compounds. Pesticides are massively applied in the Berambadi watershed, but we have previously noted that pesticides such as acetamiprid, emamectin benzoate or imidacloprid are present in the first centimetres of the topsoil of this watershed (unpublished data), observations in agreement with other reports that observed that pesticides are more likely found in surface water rather than deep water (French Ecological Ministry, 2007). In Berambadi, it is likely that the pesticides are not present in deep groundwater because they have decayed during their transit to the groundwater table, or because the topsoil and underlying low

permeability, thick saprolite (weathered rock) layer in these watersheds (5–25 m (Braun et al., 2009)) act as a filter for organic molecules. Indeed, the pesticide molecules found in topsoil are known to be efficiently degraded in soils, within weeks for acetamiprid and emamectin benzoate in pH and temperature conditions similar to Berambadi (Xu et al., 2020) (Chukwudebe et al., 1997). Although the efficiency of pesticide adsorption strongly depends on soil biota, clay minerals composition, organic matter (Clausen and Fabricius, 2001; Davies and Jabeen, 2002, 2003) and pesticide properties particularly whether they are ionic or anionic (Clausen and Fabricius, 2001), it is likely that both the few years of transit time in the vadose zone of Berambadi and the thickness of this layer contributed to both pesticide adsorption and/or complete decay (Buvaneshwari et al., 2020).

The first postnatal week in the mouse corresponds to the third trimester of human pregnancy and P21 in the mouse corresponds to around 1 year of age in a human (Ross et al., 2015; Semple et al., 2013). This study shows several significant changes in the brain at P21 that were not present at P5, indicating a vulnerability of the brain during the stage of early post-natal life in the human. This age dependent effect includes our observation that the AGRI water caused neuronal degeneration in the motor cortex and further that part of this decrease is in the number of calretinin interneurons at P21, but not P5. We have previously shown reductions in calretinin interneuron number due to preterm birth in human infants and due to exposure to early postnatal (P1-P5) inflammation in the mouse (Stolp et al., 2019). These interneurons are present in the brain earlier than other interneuron subpopulations, reviewed in Cauli et al. (2014) and whether their loss is selective and



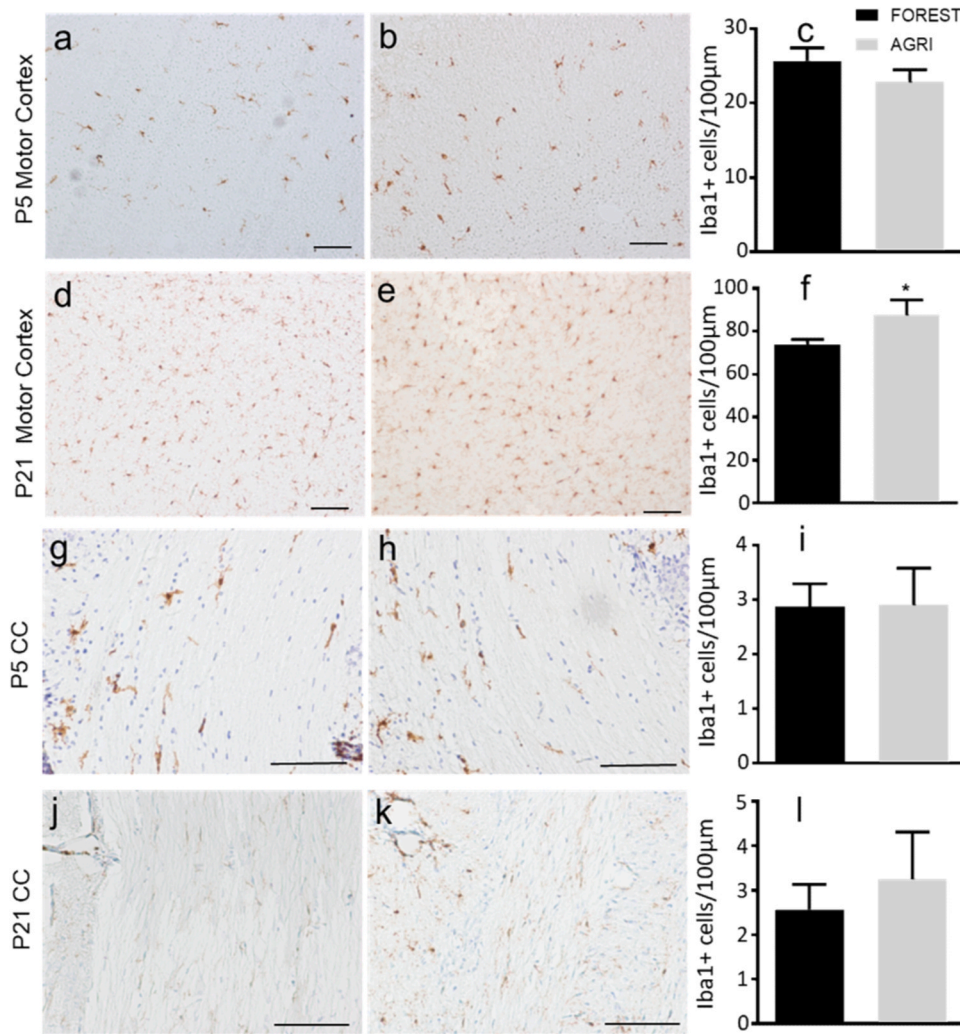
**Fig. 3.** GFAP IHC and quantitation at P5 and P21 (Corpus Callosum and Motor Cortex). Representative IHC images of Corpus Callosum (CC) with GFAP (a) and (d) FOREST P5 and P21 CC and (b) and (e) AGRI P5 and P21 CC. Quantitation of mean intensity of GFAP IHC in (c) P5 and (f) P21 CC. Histograms are plotted as mean  $\pm$  SEM. (\*  $p = 0.02$  by Student's  $t$ -test). Representative IHC images of motor cortex with GFAP (g) and (j) FOREST P5 and P21 motor cortex and (h) and (k) AGRI P5 and P21 motor cortex. Quantitation of mean intensity of GFAP IHC in (i) P5 and (l) P21 motor cortex. Scale bar = 100  $\mu$ m.

any behavioural deficits result from this needs to be further investigated.

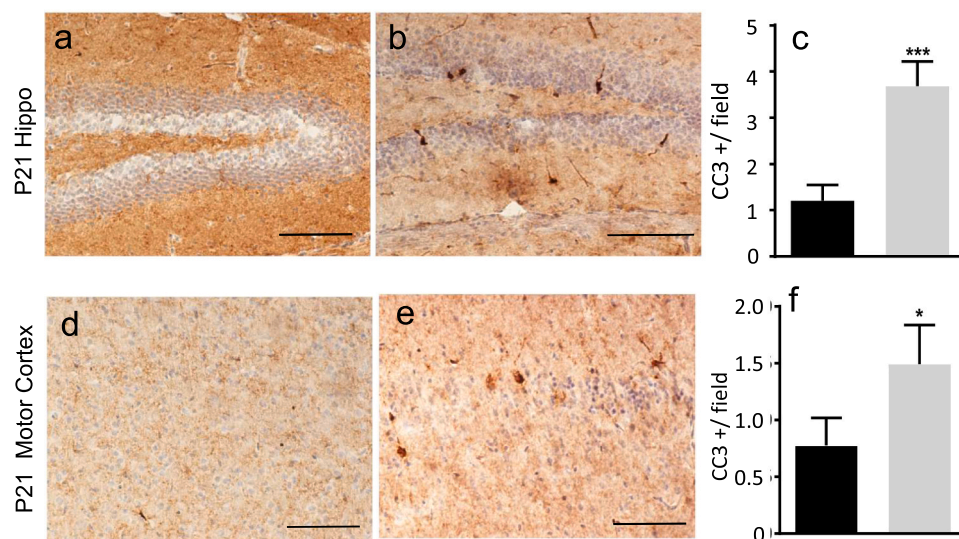
Exposure to agricultural water also caused a decrease in the expression of the reactive astrocyte marker GFAP, in the white matter but not in the grey matter. Key differences in the functions of grey versus white matter astrocytes are reflected in differences in morphology, and a higher expression of glutamate transporters and GFAP itself in white matter astrocytes versus grey matter (Goursaud et al., 2009) and reviewed in Lundgaard et al. (2014). Of note, in GFAP KO mice, there is more damage to the white matter than the grey matter (Liedtke et al., 1996), linked to the specific role of astrocytes as producers of the oligo-trophic factor platelet-derived growth factor- $\alpha$  (PDGF) (Raff et al., 1988). Interestingly, we did not observe a deficit in the production of MBP, a marker of mature oligodendrocytes, which suggests sufficient conserved functions of astrocytes in this paradigm, and on the contrary, we saw increased MBP expression at P21. Hypermyelination is also observed in mice with a KO of transthyretin, a thyroid hormone binding protein that leads to hypothyroidism (Alshehri et al., 2020). As nitrate exposure is similarly reported to cause hypothyroidism, via competing with iodine for uptake by the thyroid (Pesce and Kopp, 2014), further work will be needed to explore the fate of oligodendrocytes and the link to altered thyroid signalling caused by nitrate exposure.

There was an increase in the number of Iba1 positive microglia seen with AGRI water exposure suggestive of neuroinflammation and an increase in cell death. Microglia are the immune cells of the brain and activation of microglia leads to the secretion of pro-inflammatory cytokines and neuroinflammation and can result in neuronal injury

increasing the risk for neurodevelopmental disorders and neurodegeneration (Fleiss et al., 2021; Tay et al., 2017). The primary direct mechanism by which nitrate exposure is proposed to result in neuroinflammation is by leading to excessive production of NO (Yuste et al., 2015) and reactive nitrogen species (RNS) such as pernitrate which is also a potent neurotoxin (Karwowska and Kononiuk, 2020; Kaur et al., 2013; Sunitha, 2013). Further, high sodium chloride intake can lead to immune dysfunction (Binger et al., 2015) and the production of cytokines shown to cause brain injury (Lawrence and Wynn, 2018) can be mediated via a NO-dependent mechanism (Faraco et al., 2018). Neonates have several mechanisms to keep NO<sub>2</sub> levels low to reduce the levels of NO. First, newborns ingest less NO<sub>3</sub> than adults since breast milk (and formula) contains low levels of NO<sub>3</sub> and NO<sub>2</sub> and infants lack the salivary bacteria for reducing NO<sub>3</sub> to NO<sub>2</sub>. Furthermore, the generation of NO from NO<sub>2</sub> is highly pH dependent, making process inefficient in the neonate because the neonate stomach has a higher pH than the adult (Jones et al., 2015). Given how tightly the nitrate, nitrite and NO species are controlled in the newborn, our current study shows that this combination which naturally exists in groundwater of highly cultivated areas can lead to defects in brain development. This calls for additional studies to dissect out this effect and look at the consequences for cognitive function and lifelong brain health. Further, careful epidemiological analysis will clarify its relevance for human health.



**Fig. 4.** Iba1 IHC and quantitation at P5 and P21 (Motor Cortex and Corpus Callosum). Representative IHC images of motor cortex with Iba1 (a) and (d) FOREST P5 and P21 motor cortex and (b) and (e) AGRI P5 and P21 motor cortex. Quantitation of mean intensity of Iba1 IHC in (c) P5 and (f) P21 motor cortex. Histograms are plotted as mean±SEM. (\*  $p = 0.00199$  by Mann-Whitney test). Representative images of corpus callosum (CC) IHC with Iba1 (g) and (j) FOREST P5 and P21 CC and (h) and (k) AGRI P5 and P21 CC. Quantitation of mean intensity of Iba1 IHC in (i) P5 and (l) P21 CC. Scale bar = 100 µm.



**Fig. 5.** Cleaved caspase 3 (CC3) IHC and quantitation at P21. Representative IHC images of (a) FOREST and (b) AGRI P21 hippocampus (Hippo) (c) quantitation (\*\* $p = 0.0006$  by Student's *t*-test). Cleaved caspase 3 (CC3) motor cortex and quantitation at P21. Representative IHC images of (d) FOREST and (e) AGRI P21 hippocampus (Hippo) (f) quantitation (\*\* $p = 0.0446$  by Mann-Whitney test). Scale bar = 100 µm.



**Table 1**

Summary of the neuropathological findings, including percentage change for the significant findings and the significance (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ ), showing greater changes occurring in the later timepoint. +/-, = no significant change. na. = not assessed.

	P5	P21
<b>Body weight</b>	-28% ****	+/-
<b>Brain weight</b>	-17% ****	+/-
<b>Mature neurons (NeuN)</b>		
<i>Motor cortex</i>	+/-	-26% **
<b>Interneurons (Calreticulin)</b>		
<i>Motor Cortex</i>	+/-	- 31% **
<b>Astrocytes (GFAP)</b>		
<i>External capsule</i>	- 24% *	+/-
<i>Corpus callosum</i>	+/-	- 19%*
<i>Anterior commissure</i>	+/-	- 20% **
<i>Motor cortex</i>	+/-	+/-
<b>Myelin (MBP)</b>		
<i>External capsule</i>	na.	+/-
<i>Corpus callosum</i>	na.	+/-
<i>Motor cortex</i>	na.	+53% **
<b>Microglia (IBA1)</b>		
<i>Motor cortex</i>	+/-	+ 16% *
<i>Corpus callosum</i>	+/-	-
<b>Cell death (CC3)</b>		
<i>Dentate gyrus</i>	na.	+ 245% ***
<i>Motor cortex</i>	na.	+ 100% *
<i>Corpus callosum</i>	na.	+
<i>Cingulum</i>	na.	+

## 5. Conclusion

This study shows that in a mouse model, when dams were given water from an area of high agricultural activity with resulting high nitrate content and TDS, the brains of the pups born to these dams had degeneration of neurons and reduced astrocyte density and an increase in the number of Iba1 positive microglial cells. These findings highlight that the neuropathological effects were greater at P21 suggesting a vulnerability to nitrates and TDS of processes occurring in the period equivalent to early neonatal life in humans. However, it may also be that exposure altered brain development earlier and these effects were simply exacerbated over time. Further studies are envisaged to clarify this effect of timing, understand the pathways that lead to the abnormalities seen herein and whether they can be reversed and the behavioural consequences of these changes. Another important question is whether such a high nitrate and high salt exposure during development would alter the immune response in the brain such that a subsequent infection or brain injury can have a worse consequence. In addition, the relevance of these results to human health needs to be investigated by conducting epidemiological studies that analyse behavioural consequences including neurodevelopmental disease related to water quality/contaminant type. However, this study clearly raises a red flag, suggesting that current water safety limits might not be appropriate to protect the developing brain from toxicity related to high nitrates and TDS.

## CRediT authorship contribution statement

Initial conception and design – S. Mani, LR, PG. Data generation and analysis - LS, IS, JR, GPG, SB, S. Mishra. Interpretation and Manuscript writing - LR, PG, S. Mani, JR, BF, MS.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Contaminants".

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112635.

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