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Short communication

Long-term effects of prenatal allopurinol treatment on brain plasticity markers in low and normal birth weight piglets

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ABSTRACT

In this study, we investigated the effect of antenatal allopurinol (ALLO) treatment on levels and expression of plasticity markers in the dorsal hippocampus of low (LBW) and normal (NBW) birth weight piglets. ALLO treatment given daily in the last trimester to pregnant sows had a protective effect on neuronal plasticity markers in their piglets. ALLO increases protein levels of BDNF and the postsynaptic marker PSD95 in LBW and NBW piglets. ALLO treatment increases the pCREB/CREB ratio in LBW piglets to a similar level as that found in untreated NBW piglets. In conclusion, antioxidant treatment administered in the last trimester might be a promising treatment for LBW neonates.

1. Introduction

Low Birth Weight (LBW) children are at risk for several diseases, and have a higher incidence of neonatal mortality and morbidity (Gagnon, 2003). LBW children born at full term are at risk for developing cognitive deficiencies such as learning problems (Chaudhari et al., 2004) and reduced brain volume (Toft et al., 1995). More specifically, the intelligence and academic performance of LBW children (<2000 g) at the age of 12 years is shown to be significantly lower than that of normal birth weight (NBW) controls (Chaudhari et al., 2004).

Brain-derived neurotrophic factor (BDNF) is a pivotal regulator of neuronal plasticity and cognitive functioning (Benraiss et al., 2001). Prenatal malnutrition, related to LBW, has been shown to have an adverse influence on spatial navigation and cerebral BDNF levels in rats, pointing to an impaired neuronal plasticity (Wang and Xu, 2007). A fetus suffering from hypoxemia or from reduced nutritional supply will slow down its growth rate (Gagnon, 2003). Pharmacological treatment with neuroprotective substances, preventing the formation of free-radicals, or scavenging the free-radicals produced, could improve neurological outcome in these cases.

Allopurinol (ALLO) is a xanthine oxidase inhibitor which, together with its active metabolite oxypurinol (OXY), acts as a scavenger for toxic hydroxyl free radicals, and chelates non-protein-bound (pro-radical) iron. ALLO has been found to reduce free-radical formation in both pig and human fetuses (Boda et al., 1999). In clinical settings, ALLO is currently only experimentally administered to asphyxiated neonates during, or shortly after, birth. ALLO is not yet available for use in preventative therapies. One recent study monitored responsiveness to induced hypoxic insults in neonatal mice, serving as a model for the hypoxic insults observed in the very low birth weight premature infant population. Results showed that brain plasticity markers, in particular those involved in the BDNF signaling pathways, were significantly reduced, whereas hypoxic markers were increased (Li et al., 2011).

In the present study, we investigated whether prolonged antenatal ALLO treatment during the last trimester of pregnancy would improve neuronal plasticity in LBW pigs as compared to NBW pigs, thereby potentially preventing, or reducing, long-term effects on cognitive abilities. Recently, we showed that LBW piglets display transiently retarded learning (reversal) in the hole-board discrimination task (Gieling et al., 2012). Although generally less severe

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Table 1
Characteristics of ALLO treated LBW and NBW piglets and sows.

Animals	Group	Parity	Litter size	Av. Lit.	♂/♀	LBW and NBW piglets selected for testing											
						LBW (birth weight (g)/gender)						NBW (birth weight (g)/gender)					
Batch 1																	
Sow 1	ALLO	7	14 (+2†)	1303.21	10/4	755	♂ 1040	♂				1300	♂ 1375	♂			
Sow 2	ALLO	4	13 (+4†)	1276.54	7/6	890	♀ 980	♂				1400	♀ 1480	♀			
Sow 3	ALLO	2	12 (+2†)	1275.83	5/7	875	♂					1410	♂				
Sow 4	Control	6	18 (+1†)	1362.67	11/7	715	♂ 720	♂	1080	♀		1660	♂ 1710	♀	1750	♂	
Sow 5	Control	4	17	1338.82	13/4	930	♂ 1070	♂	1085	♂		1370	♂ 1440	♂	1340	♂	
Sow 6*	Control	3	10	1855.50	7/3	–	–		–			–	–		–		
Batch 2																	
Sow 7	ALLO	9	9 (+4†)	1607.79	5/4	1155	♂					1740	♂				
Sow 8	ALLO	2	18	1439.44	11/7	860	♀					1700	♂				
Sow 9	ALLO	2	12	1546.67	6/6	825	♀					1665	♀ 1590	♀			
Sow 10	Control	8	8	1304.38	7/1	870	♂					1460	♂				
Sow 11*	Control	2	5	2028.00	2/3	–	–		–			–	–		–		
Sow 12	Control	10	19	0858.89	12/7	470	♂					950	♂				
Summary	ALLO	4.9	13.0	1408.2	7.3/5.6												
Summary	Control	5.5	12.9	1458.0	8.6/4.1												

All animals are a (Terra × Finnish landrace) × Duroc mix.
* No piglets from these litters were selected as no proper LBW animals were present in those litters. Gender was balanced over treatment (Fisher exact test; $p = 0.12$) and birth weights (Fisher exact test; $p = 0.77$).

than experimentally induced asphyxia, the birth weight comparison paradigm is an interesting model to study prenatal ALLO treatment of term born LBW neonates. Hippocampal brain tissue was investigated for expression of relevant markers in the BDNF-mediated neuronal plasticity pathway of 5 months old piglets, i.e. the long-term consequences of a low birth weight and the eventual protective effects of antenatal allopurinol treatment were assessed.

2. Materials and methods

2.1. Animals and housing

The experiments were approved by the local ethics committee at Utrecht University, and conducted in accordance with the recommendations of the EU directive 86/609/EEC.

Within sow comparison between full-term LBW piglets and their NBW littermates is considered to be an appropriate model to study effects of birth weight on later development (Gieling et al., 2011).

Sows: Twelve pregnant sows (divided over two batches of six sows, with two months between batches, due to practical feasibility) were housed as previously described (Gieling et al., 2012). Of the twelve pregnant sows, six were treated once a day p.o. with ALLO (15 mg/kg, Ratiopharm, The Netherlands; based on a previous pharmacokinetic study (van Dijk et al., 2008), supplemented with in house orienting pharmacokinetic studies (unpublished results)) for 30 days, and six were used as control subjects (Table 1) until the day of farrowing.

Piglets: The average birth weights were determined per litter. Piglets weighing at least the mean litter weight minus 1× the SD were classified as LBW. After excluding all LBW piglets from the litter, a new litter mean was derived. Animals with a weight that was closest to this new mean, and with the same sex as the LBW animal(s) from the litter, were selected as normal birth weight (NBW) animals. One to three LBW and one to three NBW animals were selected per litter, depending on availability (Table 1). Piglets were weaned at 3.5–4 weeks.

2.2. Tissue processing

At the age of 5 (batch 1), or 5.5 months (batch 2), the pigs were transported to a local slaughterhouse, where they arrived approximately 24 h before slaughtering. They stayed in a pen as a group (i.e. there was no mixing with other animals), and entered the lairage, only a few meters from the pen, approximately 30 min before slaughtering started. The order of slaughter was randomized. A pig was stunned with an electrical stunner, bled, immediately decapitated and the dorsal hippocampi were dissected and stored at −80 °C until further processing.

Approximately 100–130 mg dorsal hippocampus tissue was homogenized in 1 ml ice-cold homogenizing buffer (100 mM Tris, 200 mM NaCl, 1 mM EDTA, 2 mM DTT, 0.05% Triton (vol/vol) containing phosphatase and protease inhibitors (Roche #04906837001 and #11836153001, Vilvoorde, Belgium). The following primary antibodies were applied to perform western blot: 1:500 rabbit anti-BDNF (H-117), 1:1000 β-Actin (Santa Cruz Biotechnology, Santa Cruz, USA), 1:250 rabbit anti-TrkB, 1:3000 mouse anti-CREB, 1:100 rabbit anti-pCREB, 1:1000 rabbit anti-GSK-3β, 1:1000 rabbit anti-pGSK-3β (Cell Signaling Technology, Beverly, USA), 1:2000 mouse anti-PSD95 (QED Bioscience Inc., #0711, San Diego, USA), mouse anti-Synaptophysin (Millipore MSxsynaptophysin, MS, #MAB5258, Billerica, USA), and 1:2,000,000 mouse anti-GAPDH (#10R-G109A, Fitzgerald, Huissen, The Netherlands) for normalization. Secondary antibodies were respectively 1:5000 goat-anti-rabbit IRDye 800 and 1:10,000 donkey-anti-mouse IRDye 680 (Li-Cor). Intensities of specific bands were quantified using ImageJ (http://rsbweb.nih.gov/ij/), corrected for β-actin or GAPDH signal.

2.3. Statistical analysis

The data were analyzed with SAS 9.2. NBW and LBW piglet data were averaged per sow (allopurinol or control). Each sow was considered to be an experimental unit with or without ALLO treatment (the between subjects factor). The averaged values of the NBW and LBW piglets were considered to be a within sow or litter (within subjects) factor.

Data were first tested for normality using a Shapiro–Wilk test. Subsequently, a repeated measures analysis of variance (SAS GLM procedure) was performed to verify the effect of ALLO treatment, effects of birth weight, and the interaction between treatment and birth weight. If the data, after exclusion of outliers (http://www.graphpad.com/quickcalcs/Grubbs1.cfm), still did not meet the prerequisite of normality, the data were log10 transformed. No batch specific effects were detected.

3. Results

Analysis of the mature BDNF concentrations of the dorsal hippocampus revealed a significant ALLO treatment effect on LBW and NBW pigs ($F_{1,7} = 16.93$, $p = 0.0045$; Fig. 1B). BDNF concentration was increased in the treated animals as compared to the untreated animals. We observed a significant effect of ALLO treatment for PSD95 ($F_{1,7} = 7.79$, $p = 0.0269$; Fig. 1E). The PSD95 protein level was higher in ALLO treated animals as compared to untreated animals. No birth weight effect was detected. Only a marginal birth weight effect by ALLO treatment interaction for the ratio of pCREB/CREB was found ($F_{1,8} = 4.1$, $p = 0.0801$); Fig. 1C). ALLO treatment increased the pCREB/CREB ratio in LBW piglets, to levels similar to those found in untreated NBW piglets. No effects of ALLO treatment or birth weight were found for the mBDNF high affinity receptor TrkB ($p = 0.94$), the pGSK-3β/GSK-3β ratio ($p = 0.70$) and Synaptophysin ($p = 0.97$) (Fig. 1A, D and F).

4. Discussion

In this study, we observed significantly increased BDNF and PSD95 protein levels in ALLO treated piglets, independent of birth