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Associations among exposure to methylmercury, reduced Reelin expression, and gender in the cerebellum of developing mice

Filippo Biamonte [a,1,3], Laura Latini [b,1], Filippo Sean Giorgi [c], Maria Zingariello [d], Ramona Marino [a], Roberto De Luca [a], Sonia D'Ilio [e], Costanza Majorani [f], Francesco Petrucci [f], Nicola Violante [f], Oreste Senofonte [f], Marco Molinari [b,2,**], Flavio Keller [a,2,*]

[a] Laboratory of Developmental Neuroscience and Neural Plasticity, University Campus Biomedico, Via A. del Portillo 21, 00198 Rome, Italy
[b] Santa Lucia Foundation, I.R.C.C.S., Via del Fosso di Fiorano 64, 00143 Rome, Italy
[c] Section of Neurology, Department of Clinical and Experimental Medicine, University of Pisa, Via Roma 67, 56126 Pisa, Italy
[d] Laboratory of Histology, University Campus Biomedico, Rome, Italy
[e] Istituto Superiore di Sanità, Centro Nazionale Sostanze Chimiche, Viale Regina Elena 299, Rome, Italy
[f] Istituto Superiore di Sanità, Dipartimento di Ambiente e Prevenzione Primaria, Viale Regina Elena 299, Rome, Italy

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ABSTRACT

Genetic risk factors acting during pregnancy or early after birth have been proposed to account for the exponential increase of autism diagnoses in the past 20 years. In particular, a potential link with exposure to environmental mercury has been suggested. Male sex constitutes a second risk factor for autism. A third potential genetic risk factor is decreased Reelin expression. Male heterozygous *reeler* ($rl^{+/-}$) mice show an autism-like phenotype, including Purkinje cells (PCs) loss and behavioral rigidity. We evaluated the complex interactions between 3 risk factors, i.e. genetic status, sex, and exposure to methylmercury (MeHg), in $rl^{+/-}$ mice. Mice were exposed to MeHg during the prenatal and early postnatal period, either at a subtoxic dose (2 ppm in Dams' drinking water), or at a toxic dose (6 ppm Dams' drinking water), based on observations in other rodent species and mice strains.

We show that: (a) 2 ppm MeHg does not cause PCs loss in the different animal groups, and does not enhance PCs loss in $rl^{+/-}$ males; consistent with a lack of overt neurotoxicity, 2 ppm MeHg per se does not cause behavioral alterations (separation-induced ultrasonic calls in newborns, or sociability and social preference in adults); (b) in stark contrast, 6 ppm MeHg causes a dramatic reduction of PCs number in all groups, irrespective of genotype and sex. Cytochrome C release from mitochondria of PCs is enhanced in 6 ppm MeHg-exposed groups, with a concomitant increase of μ-calpain active subunit. At the behavioral level, 6 ppm MeHg exposure strongly increases ultrasonic vocalizations in all animal groups. Notably, 6 ppm MeHg significantly decreases sociability in $rl^{+/-}$ male mice, while the 2 ppm group does not show such as decrease.

At a subtoxic dose, MeHg does not enhance the autism-like phenotype of male $rl^{+/-}$ mice. At the higher MeHg dose, the *scenario* is more complex, with some "autism-like" features (loss of sociability, preference for sameness) being evidently affected only in $rl^{+/-}$ males, while other neuropathological and behavioral parameters being altered in all groups, independently from genotype and sex. Mitochondrial abnormalities appear to play a crucial role in the observed effects.

* Corresponding author. Tel.: +39 06225419195; fax: +39 0622541456.
** Corresponding author at: Experimental Neurorehabilitation Laboratory, Direttore UO A e Sezione Mielolesi, Fondazione S. Lucia, Via Ardeatina 306, 00179 Roma, Italy. Tel.: +39 0651501600; fax: +39 0651501679.
E-mail addresses: m.molinari@hsantalucia.it (M. Molinari), f.keller@unicampus.it (F. Keller).
[1] These authors contributed equally to this work.
[2] Equally senior authors.
[3] Present address: Institute of Histology and Embryology, School of Medicine, Catholic University of the Sacred Heart "A. Gemelli", Rome, Italy.

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1. Introduction

In the past decade, concepts regarding the role of genes in organism development have changed profoundly, by incorporating dynamical considerations. One example of this new paradigm is the "triple helix model" of organism development proposed by evolutionary biologist Richard Lewontin (Lewontin, 2000). The main concept of this model is that we will never fully understand living organisms (in health and disease) if we continue to think of genes, organisms, and environments as separate entities, each with its distinct and independent role in the history and operation of organic processes. According to Lewontin, an organism is a unique consequence of both genes and environment, of both its internal and external contingencies. The present paper represents an attempt to apply this thinking to a mouse model of autism, to understand the potential interactions between an environmental toxicant, mercury, and an autism candidate gene.

There are several lines of evidence for a neurotoxic effect of environmental mercury (Hg), first described after mass intoxications occurring in the 1950s in Japan (Minamata) and in the 1970s in Iraq (see the review by Clarkson, 2002). Even though these events represented extraordinary situations, in the modern era a constant chronic exposure to Hg has been shown to occur worldwide (Environmental Protection Agency, 1997). Since Hg is metabolized mainly to methylmercury (MeHg) in living organisms, the main form of Hg intake via food is MeHg, e.g. through consumption of large fishes (Mahaffey, 1999; Clarkson, 2002). It has been demonstrated in rats and mice that the neurotoxic effects of MeHg significantly involve the cerebellum and several mechanisms have been proposed for such neurotoxicity (Castoldi et al., 2001).

A variety of studies showed a significant lower threshold of the immature brain to the toxic effects of MeHg as compared with the adult brain, after reports of severe brain malformations in offspring of apparently healthy mothers from the Minamata area (Castoldi et al., 2003). In addition, several epidemiological studies have described more subtle cognitive and behavioral effects in the offspring of pregnant/lactating mothers after Hg intoxication (see e.g. Trasande et al., 2005, 2006), suggesting that developmental toxicity of MeHg has been underestimated (Grandjean and Herz, 2011). However, all the above-mentioned studies refer to frankly toxic exposures of Hg, while none of them tested the effects of exposure to lower levels of MeHg in sub-populations with potentially increased susceptibility to neurotoxic insult.

Developmental exposure to Hg is considered to play a potentially significantly role in the pathogenesis of autism at least in some sub-populations of patients (Bello, 2007), in light of epidemiological data (Palmer et al., 2009), as well as re-evaluation of the results of large cohort studies (Desoto and Hitlan, 2007; Ip et al., 2004). However, as expected, studies in humans are far from being fully useful in clarifying the existence of such a link, since human studies would need prolonged prospective observation in which exposure of dams to MeHg and other environmental factors can be precisely ascertained.

As regards genetic vulnerability among different factors, different lines of evidence suggest the involvement of the *reelin* gene in autism. Four main lines of evidence support Reelin involvement in autism: (a) decreased function of Reelin appears to be a risk factor for autism (Fatemi et al., 2002, 2005); (b) the *reelin* gene is affected in several autistic pedigrees (Persico et al., 2001; Zhang et al., 2002; Skaar et al., 2005; Serajee et al., 2006) but see (Dutta et al., 2007); (c) Reelin expression has been shown to be altered in the *post-mortem* brains of autistic subjects (Fatemi et al., 2001; Chow et al., 2012); (d) data from Reelin-deficient mice highlight the importance of cerebellar damage in developing autism-like behaviors. Heterozygous male $rl^{+/-}$ mice, displaying 50%-reduced Reelin expression in the brain, show a decreased number of Purkinje cells (PCs) compared to wild-type ($rl^{+/+}$) littermates and $rl^{+/-}$ female mice (Biamonte et al., 2009). In addition, infant $rl^{+/-}$ mice show reduced motivation for social stimuli, and adult $rl^{+/-}$ male mice exhibit reduced cognitive flexibility (Macri et al., 2010).

There is a large consensus that male sex constitutes a third important factor for autism. Particularly, researchers have focused on the influence of sex hormones during development as a key element for developing autism (for recent reviews on this topic, see Keller and Ruta, 2010; Fanelli et al., 2013). In the above cited model of Reelin deficiency, it is relevant that both neuroanatomical and behavioral abnormalities observed in $rl^{+/-}$ males are reversed by neonatal estradiol administration (Biamonte et al., 2009; Macri et al., 2010).

Thus, it is possible that reduced Reelin expression may confer a genetic vulnerability that, by interacting with epigenetic factors, such as environmental toxic agents and prenatal or perinatal levels of sex hormones, would yield the full-blown autism phenotype.

We therefore decided to assess the potential interactions between genetic vulnerability and developmental exposure to Hg in the heterozygous *reeler* mouse model.

In this study we assessed: (1) neuroanatomical and behavioral changes induced by chronic exposure of the mothers to MeHg, at two different doses (2 ppm and 6 ppm in drinking water); (2) whether Reelin haploinsufficiency influences MeHg effects; and (3) whether MeHg effects are sex-dependent.

We observed that exposure to 2 ppm MeHg does not cause neuropathological changes or behavioral alterations over and above those already observed in male $rl^{+/-}$ mice. In stark contrast, exposure to 6 ppm MeHg led to overall neurotoxicity in all animal groups; however, behavioral alterations were more consistently observed in $rl^{+/-}$ male mice.

2. Material and methods

2.1. Animals

Parental animals were purchased from Jackson Laboratories (Bar Harbor, Maine, USA) and were bred in an approved vivarium. The genetic background of this line carries a spontaneous mutation of the *reelin* gene (D'Arcangelo et al., 1995). Affected mice were obtained by crossing two heterozygous animals (B6C3Fe *a*/*a*-*Reln*^{rl}/J × B6C3Fe *a*/*a*-*Reln*^{rl}/J – for detail on gene and strain nomenclature by Jackson see http://jaxmice.jax.org/strain/000235.html).

The expected rate of animals was 50% of heterozygotes and 25% of wild type. Homozygous (25%) affected animals ($rl^{-/-}$) were not used in the present study because these mice present a profound hypoplasia of the cerebellum, in which the normal cerebellar *folia* are missing. Moreover the $rl^{-/-}$ mice showing ataxia, eating complications, and survival difficulties for the majority of animals, in the absence of appropriate handling.

Two females and one male at 8 weeks of age were mated in a 33 cm × 13 cm × 14 cm Plexiglas box. Pregnancy was confirmed by the presence of a vaginal plug the following morning. After ca. 2 weeks, the male was removed and the females were housed individually and daily checked for delivery. Mice were housed in a temperature-controlled room at 21 ± 1 °C (relative humidity 60 ± 10%) under a reverse 12:12 h light–dark cycle, with lights off at 7:00 a.m., and fed by Enriched Standard Diet (Mucedola, Settimo Milanese, Italy). Mice were genotyped at P8 for the rl^{ix} mutation and sex (Sry gene) as previously described (Biamonte et al., 2009). All animal handling and experimental procedures were performed according to European Communities guidelines (EC Council Directive 86/609), Italian legislation on animal experimentation (D.L. 116/92) and the NIH guide for the care and use of laboratory animals. The experimental protocol was approved by the Ethical Committee of Tor Vergata University.