Serotonin abnormalities in *Engrailed-2* knockout mice: New insight relevant for a model of Autism Spectrum Disorder

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ABSTRACT: Autism spectrum disorder (ASD) is a congenital neurodevelopmental behavioral disorder that appears in early childhood. Recent human genetic studies identified the homeobox transcription factor, *Engrailed 2* (*EN2*), as a possible ASD susceptibility gene. *En2* knockout mice (*En2−/−*) display subtle cerebellar neu-ropathological changes and reduced levels of tyrosine hydroxylase, noradrenaline and serotonin in the hippocampus and cerebral cortex similar to those ones which have been observed in the ASD brain. Fur-thermore other similarities link *En2* knockout mice to ASD patients. Several lines of evidence suggest that serotonin may play an important role in the pathophysiology of the disease. In the present study we measured, by using an HPLC, the 5-HT levels in different brain areas and at different ages in *En2−/−* mice. In the frontal and occipital cortex, the content of 5HT was reduced in *En2−/−* 1 and 3 months old mice; in 6 month old mice, the difference was still present, but it was not statistically significant. The 5-HT content of cerebellar cortex was significantly reduced at 1 month old but significantly high when the KO mice reached 3 months of age. The increase was present even at 6 months of age. A similar trend was highlighted by SERT immunolabeling in *En2−/−* mice compared to control in the same areas and age analyzed.
Our findings, in agreement with the current knowledge on the 5-HT system alterations in ASD, confirm the early neurotransmitter deficit with a late compensatory recovery in *En2* KO-mice further suggesting that this experimental animal may be considered a good predictive model for the human disease.

INTRODUCTION: Autism Spectrum Disorder (ASD) is a major mental health dis-order of childhood; it is devastating for morbidity, outcome, impact on the family and costs on the society. In the past it was considered an emotional disturbance resulting from early attachment experi-ences (Bettelheim, 1967), but now ASD is recognized as a brain development disorder in the first or second year of life (DiCicco-Bloom et al., 2006). Once considered rare, but, according to recent epide-miological data, ASD is now reported to affect approximately 1% of children (Elsabbagh et al., 2012; Kim et al., 2011). The rapid advance in genomic technologies, during the years, has given a new impetus to genetics of ASD. Although the genetic risk factors remain diffi-cult to identify, it is possible to state that a few chromosomal disorders and some single gene disorders are clearly associated with an increased risk for autism. Among them, there are the *serotonin trans-porter gene* (*5HTT*), *reelin* (*RELN*), and *Engrailed-2* (*En2*) (Persico and Napolioni, 2013).

A number of neural structures, as cerebellum, limbic system, ce-rebral cortex and brainstem, are identified as compromised in ASD (Bauman and Kemper, 2005). MRI and post-mortem studies show an altered intracortical connectivity affecting cerebellum. Interest-ingly, the cerebellum of ASD is hypoplastic and shows a reduced number of Purkinje cells (Amaral et al., 2008). Furthermore a recent review indicates that brain lesions in ASD are due to alterations of serotonin signaling during early CNS development (Yang et al., 2014).

The *En2* homeobox transcription factor regulates the expression of genes during embryonic and postnatal CNS development and con-tinues to be expressed in a subset of differentiated neurons in the adult (Joyner, 1996). *En2* gene is necessary for the correct develop-ment of the cerebellum and of the monoaminergic neurotransmitter pathways as serotoninergic (5-HT), noradrenergic (NA) and dopa-minergic (DA) neurons (Benayed et al., 2009). A growing number of studies have highlighted the possibility that Engrailed gene dysfunc-tion may involve the metabolism of serotonin in ASD

(Chugani, 2004) and a study on *En2*−/− mice describes the behavioral and the neu-rochemical DA and 5-HT alterations in some brain areas at a specific time of adult mice (Cheh et al., 2006).

We have therefore investigated the neurochemical alterations of 5-HT and its metabolite 5HIAA in some brain areas during the adult life of *En2*−/− mouse. In this study we have described the results obtained for 5-HT content and its immunoreactivity and its trans-porter (SERT) in different brain areas.

DISCUSSION: Several studies demonstrate a number of social and learning al-terations in *En2* knockout mice that are particularly relevant for the first diagnostic symptoms of ASD. In particular they describe reduced social behavior as playing and sniffing, but not social communica-tion in *En2*−/− mice compared to controls (Brielmaier et al., 2012; Cheh et al., 2006). This decrement in social and playing behavior is observed during the period in which normal mice behavior is evident in peer interaction (Terranova et al., 1993). Likewise, in ASD, social deficits become prevalent when children, at about 3–4 years of age, begin to interact with each other (Wetherby et al., 2004). Therefore, the *Engrailed* KO-mice model seems to represent many aspects of the ASD brain and potentially, it may open new knowl-edge (Kuemerle et al., 2007).

Many genetic studies concur with reports that describe the wide similarities between the neuropathology of the *En2*−/− mouse and some of the features usually found in ASD (Benayed et al., 2005; Brielmaier et al., 2012; Gharani et al., 2004; Provenzano et al., 2012).
In particular, it has been described a significant decrease in the number of Purkinje, granule, deep nuclear and inferior olive cells in the *En2* KO-mouse (Kuemerle et al., 1997) that was previously reported as neuroanatomical abnormalities observed in autistic pa-tients (Bauman, 1991). Also Saitoh and Courchesne (1998) described that the folial abnormalities in the *En2*−/− mouse are similar to the alterations seen in autistic subjects.

Regarding neurochemical alterations, several studies reported that a number of transmitter systems are affected in autism, such as serotoninergic, dopaminergic, noradrenergic and others (Lam et al., 2006). In particular several studies report gene dysfunction of se-rotonin metabolism in autism. Whitaker-Azmitia (2001) underlined the role of serotonin in early neural development as a possible primary pathochemical alteration in the growth of ASD. The authors suggested that high levels of serotonin during early brain devel-opment may produce a fall of 5-HT terminals and subsequent impairment of neural development. It is interesting to note in ASD that the changes in monoamine neurotransmitter levels are one of the most consistent reported neurochemical phenotype, sup-ported by physiological, pharmacological and genetic studies (Anderson et al., 1990; Chugani et al., 1999; Cook et al., 1997; Lam et al., 2006; Yirmiya et al., 2001). At this regard, neurochemical studies describe modification in monoamine transmitter pathway. Cheh et al. (2006) have described that 5-HT levels, and its metabo-lite 5-hydroxyindoleacetic acid (5-HIAA), are significantly high in cerebellum, but not in the other brain areas studied. Other authors confirmed partially these data and they describe reduced levels of noradrenaline and serotonin in the hippocampus and cerebral cortex (Benayed et al., 2005; Genestine et al., 2011; Gharani et al., 2004).

In the present study, our neurochemical analysis have de-scribed in detail for the first time how the levels of 5-HT changed in some brain areas and at different ages in *En2*−/− mice. The 5-HT levels, measured with HPLC analysis and immunostaining quanti-fication, were significantly high in cerebellar cortex at the age of 3 and 6 months in KO-mice comparable with the previous reported data by Cheh et al. (2006). However, when 1 month old, the KO-mice showed a 50% reduction of 5-HT level in the same area. This reduction, which was present in all the four brain assessed areas at the age of 1 month, could reflect a defect in 5HT neuron matu-ration and migration, although *En2* expression in these neurons is extinguished by embryonic day 17.5, as pointed out by Fox and Deneris (2012). These authors claim that *En 1/2* are required for the cytoarchitecture and survival of the dorsal nucleus, where *En1*, however, is the predominant functional *En* paralog in the 5HT neuron maturation

(Fox and Deneris, 2012). Our data further revealed the complexity of the role of *En2* in the development and/or matura-tion of the 5HT system.

In agreement with our data, in different areas and at different mouse ages, we showed a partial recovery 5HT levels with a co-herent opposite temporal trend of the turnover rate.

In all the observed areas, except for cerebellum and hippocam-pus, the 5-HT turnover rate increased at P30 with a following recovery. This phenomenon was further strengthened by the SERT immunostaining and quantification in all the four areas, suggest-ing that a remarkable sprouting of 5-HT fibers takes place as a compensatory mechanism in adult KO-mouse life in comparison with wild type animals. The observed fluctuations of 5-HT and SERT levels in these brain areas of KO-mice clearly correlated with the early behavioral al-terations observed by Cheh et al. (2006) although other neurotransmitter deficits may be operative in these animals. Con-sistently previous findings in our laboratory indicated that NA was also affected in *En2*$^{-/-}$ mice and could account for the behavioral deficit observed at early stages of life.

In this study we have described the remarkable alterations of serotoninergic neurons in crucial brain areas observed during most part of the *En2* KO-mouse life. Nevertheless, as already reported earlier, altered 5-HT levels are relevant to ASD for several reasons. For example, 20–25% of individuals with autism and their first-degree relatives have platelet serotonin levels elevated (Anderson, 2002; Cook et al., 1993; Schain and Freedman, 1961) and a recent review points out a new insight on the role of platelet 5HT in ASD (Janušonis, 2014). The maladaptive behaviors associated with ASD are treated with selective serotonin reuptake inhibitors (SSRIs) and the response is effective (Posey and McDougle, 2000). Chugani et al. (1999) described an abnormal 5-HT synthesis capacity in the ASD brain. Furthermore, an increased risk to develop ASD is related to serotonin transporter gene (encoded by SLC6A4) as emerging from several human genetic studies (Cook et al., 1997; Sutcliffe et al., 2005; Yirmiya et al., 2001).

In conclusion, our findings, in agreement with the current knowl-edge on the 5-HT system alterations in ASD, confirm that the early neurotransmitter deficit followed by a late compensatory recov-ery in *En2* KO-mice is indicating that this animal model may be relevant for the human disease. Further correlations with other neu-rotransmitter systems in this KO-animal may help to understand all the complexity of the behavioral alterations observed in human pathology.