European Neuropsychopharmacology (▮▮▮▮) ▮, ▮▮▮–▮▮▮

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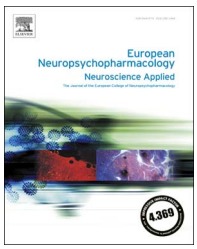

Increased methylation at an unexplored glucocorticoid responsive element within exon 1_D of *NR3C1* gene is related to anxious-depressive disorders and decreased hippocampal connectivity

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Received 31 October 2017; received in revised form 16 February 2018; accepted 22 March 2018

KEYWORDS
NR3C1;
Anxious-depressive disorders;
Epigenetics;
Hippocampal connectivity;
Monozygotic twins

Abstract
Among the major psychiatric disorders, anxious-depressive disorders stand out as one of the more prevalent and more frequently associated with hypothalamic-pituitary-adrenal (HPA) axis abnormalities. Methylation at the exon 1_F of the glucocorticoid receptor gene *NR3C1* has been associated with both early stress exposure and risk for developing a psychiatric disorder; however, other *NR3C1* promoter regions have been underexplored. Exon 1_D emerges as a suggestive new target in stress-related disorders epigenetically sensitive to early adversity.

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http://dx.doi.org/10.1016/j.euroneuro.2018.03.015
0924-977X/

After assessment of 48 monozygotic twin pairs (n=96 subjects) informative for lifetime history of anxious-depressive disorders, they were classified as concordant, discordant or healthy in function of whether both, one or neither twin in each pair had a lifetime diagnosis of anxious-depressive disorders. DNA for epigenetic analysis was extracted from peripheral blood. Exon 1_F and exon 1_D CpG-specific methylation was analysed by means of pyrosequencing technology. Functional magnetic resonance imaging was available for 54 subjects (n=27 twin pairs). Exon 1_D CpG-specific methylation within a glucocorticoid responsive element (GRE) was correlated with familial burden of anxious-depressive disorders ($r=0.35$, $z=2.26$, $p=0.02$). Right hippocampal connectivity was significantly associated with CpG-specific GRE methylation ($\beta=-2.33$, $t=-2.85$, $p=0.01$). Exon 1_F was uniformly hypomethylated across all subgroups of the present sample. GRE hypermethylation at exon 1_D of the *NR3C1* gene in monozygotic twins concordant for anxious-depressive disorders suggests this region plays a role in increasing vulnerability to psychosocial stress, partly mediated by altered hippocampal connectivity.

1. Introduction

Hypothalamic-pituitary-adrenal (HPA) axis abnormalities have been reported in a number of psychiatric disorders, with a preponderant involvement in stress-related disorders such as depression, anxiety and post-traumatic stress disorder. In this regard, the clinical heterogeneity exhibited by patients meeting diagnostic criteria for anxious-depressive psychopathology might depend on a number of biological correlates such as HPA axis functioning and inflammation (Lamers et al., 2012). Specifically, the HPA axis is known to be overactivated in a subset of anxious-depressed subjects, such that these patients exhibit increased cortisol levels and impaired stress reactivity mediated by glucocorticoid resistance (Pariante, 2017). Furthermore, excess cortisol has been described to compromise hippocampal integrity, measured in terms of both volume and connectivity, in depression (Campbell et al., 2004; Córdova-Palomera et al., 2016a).

Glucocorticoid resistance refers to the inability of body homeostasis to downregulate HPA axis activity after a stressor has ended; this downregulation occurs after binding of cortisol to the glucocorticoid receptor (GR) by means of a negative feedback mechanism. Glucocorticoid resistance has been suggested to be mediated by epigenetic desensitization of the GR via methylation in the promoter region of the *NR3C1* gene, which codes for the glucocorticoid receptor. In this regard, Weaver and colleagues found robust CpG-specific *Nr3c1* hypermethylation in rat pups reared by mothers exhibiting little maternal behavior (Weaver et al., 2004). The same research group had previously reported that rats reared by low-caring mothers exhibit decreased hippocampal GR expression, decreased glucocorticoid feedback sensitivity, and increased fearfulness under conditions of novelty; resembling an anxious-depressive-like phenotype (Francis et al., 1999). Human studies in postmortem brain tissue similarly described increases in *NR3C1* exon 1_F methylation coupled with decreased GR expression in the hippocampus of abused subjects who had committed suicide (McGowan et al., 2009).

In the light of these results, methylation of the human *NR3C1* gene has been extensively analysed in association with several psychiatric disorders and stressful stimuli (Palma-Gudiel et al., 2015b). Despite the complexity of the *NR3C1* promoter region, that research effort has focused on exon 1_F (the human homologue of exon 1_7 in rats). However, the promoter region of the *NR3C1* gene includes a total of nine alternative non-coding first exons (Turner and Muller, 2005); their tissue specificity and differential function is still under study and limited attention has been paid to methylation at the first exons other than 1_F (Palma-Gudiel et al., 2015b). Notably, exon 1_D is located in the 5′ end of a large CpG island spanning some 3 kb within the promoter region of the *NR3C1* gene (Fig. 1). CpG island shores have previously been highlighted as intermediate regions where methylation is more variable than within CpG islands themselves or in the so-called “open sea” (Ziller et al., 2013). Thus, exon 1_D emerges as a widely unexplored potential target whose methylation may contribute to modulating GR expression and to the physiopathology of stress-related disorders.

As methylation is known to modulate gene expression, differential methylation of the *NR3C1* gene may mediate brain changes, particularly in brain areas enriched in the GR, such as the hippocampus. Alterations in hippocampal functional connectivity have already been associated with major depressive disorder (Cao et al., 2012), anxiety disorder (Cui et al., 2016) and HPA axis reactivity in a healthy sample (Kiem et al., 2013). Nevertheless, few studies to date have analyzed the impact of *NR3C1* methylation on brain circuitry with a substantial scarcity of studies simultaneously assessing methylation, psychopathology and neuroimaging. Specifically, Na and colleagues were the only research group to focus on hippocampus as a target brain area putatively associated with both *NR3C1* methylation and depression in humans, reporting a positive association between *NR3C1* methylation and hippocampal subfields (Na et al., 2014). Hence, we aimed to analyze for the first time hippocampal functional connectivity, rather than

Please cite this article as: Palma-Gudiel, H., et al., Increased methylation at an unexplored glucocorticoid responsive element within exon.... European Neuropsychopharmacology (2018), http://dx.doi.org/10.1016/j.euroneuro.2018.03.015