EPIGENETIC MODIFICATIONS IN TRANSGENIC MOUSE WITH HUMAN POLYMORPHISM (Val66Met) OF BDNF GENE

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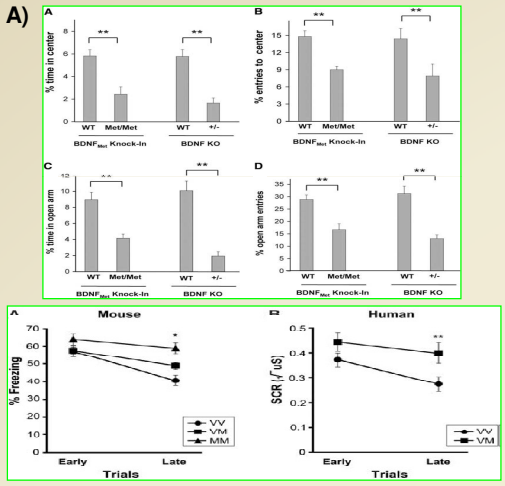
INTRODUCTION

Epigenetic mechanisms involve self-perpetuating changes in chromatin structure and function that have been shown to regulate neuronal differentiation, neurodegeneration, circadian rhythms, seizure, memory, drug addiction, and stress response. Chromatin contains regions highly condensed (heterochromatin), and regions less condensed (euchromatin). The different regions reflect distinct functional states: euchromatin is activately transcribed, while heterochromatin is usually transcriptionally inactive. Post-translational modifications of histone tail residues, such as acetylation or methylation, can remodel the structure of chromatin activating or repressing gene expression. Several lines of evidence suggest an important role for BDNF in the pathogenesis of anxiety and mood disorder. Moreover, a human polymorphism in the BDNF gene (Val66Met) that causes a Met/Val substitution in codon 66 of proBDNF has been associated with major susceptibility to cognitive deficits, neuropsychiatric and neurodegenerative diseases, eating and metabolic disorders. The BDNF Val66Met transgenic mouse is the only existing animal model that recapitulates the phenotypic hallmarks of the human polymorphism. Indeed, both human and mice $BDNF_{Met}$ allele carriers show reduced hippocampal volume and cognitive deficit [1,2]. Moreover the human and the mouse BDNF Val66Met-carrier show alterations in the extinction of fear conditioning, a type of learning involved in phobias and post-traumatic stress disorder. The aim of this work was to study the impact of the BDNF Val66Met polymorphism on the epigenetic mechanisms that regulate expression of genes involved in synaptic plasticity, such as the trascriptional factors CREB and c-Fos, the neurotrophic factor BDNF and the NMDA receptor subunits. To analyse epigenetic modifications at the gene promoters, we employed the chromatin immunoprecipitation (ChIP) technique followed by amplification of immunoprecipitated DNA fragments by quantitative q-PCR (qChIP) and specific primers. Chromatin was immunoprecipitated with specific antibodies that recognize post-translational modifications in the H3 histone protein [anti-acetyl histone H3 (Lys9,14), marker of active gene expression and anti-trimethyl histone H3 (Lys27), marker of silenced genes].

Increased anxiety-related behaviour in $BDNF^{Met/Met}$ mice



Recent studies highlighted an anxiety-like phenotype in $BDNF^{Met/Met}$ mice. $BDNF^{Met/Met}$ mice displayed an increased anxiety-related behaviour in the elevated plus maze and the open field tests (A) (Chen et al. 2006 Science 314, 140). Furthermore, Soliman and colleagues demostrated that humans and mice carrying Met allele showed a reduced fear extinction (B) (Soliman et al. 2010 Science 327, 863). Deficit in fear extinction has been associated with anxiety disorders, such as phobias and post-traumatic stress disorder.

MATERIALS AND METHODS

Animals. BDNF Val66Met founder were kindly donated from Prof. Lee, Cornell University, New York). Three months old male $BDNF^{Val/Val}$ and $BDNF^{Met/Met}$ mice were sacrified and hippocampus were dissected.

Chromatin Immunoprecipitation (ChIP). Hippocampus was incubated in formaldehyde 1% to crosslink DNA to histone proteins, and then homogenated in Homogenization Buffer (PBS 1X + 1% SDS). Chromatin was fragmentated with 13 cycles of sonication (20" ON/40" OFF) and diluted ten-fold with Dilution Buffer 1X (kit Magna ChIP, Upstate). Immunoprecipitation was carried out overnight at 4° C by using anti-acetyl histone H3 (Lys9,14) or anti-trimethyl histone H3 (Lys27) antibodies (both from Millipore) or IgG. Not immunoprecipitated chromatin (Input) was used to normalize qChIP data. Immunoprecipitated DNA was purified by spin columns (kit Magna ChIP, Millipore).

qChIP. Levels of acetylation in Lys9,14 and trimethylation in Lys27 at gene promoter was determined by amplification of immunoprecipitated DNA fragments using quantitative real-time PCR. qPCR was performed on ABI7900HT thermocycler by gene promoter specific primers and SYBR green (10 min at 95° C, 15 sec at 95° C and 1 min at 60° C for 40 cycles).

Real-time PCR. Total RNA was extracted from the hippocampus (RNeasy Mini kit, Qiagen) and single-stranded cDNA was synthesized (QuantiTect Reverse Transcription kit, Qiagen). qRT-PCR amplification was carried out with gene specific TaqMan probes (Bdnf transcript VI: Mm01334042_m1; NMDAR-NR2A mRNA: Mm00433802_m1; b-Actin mRNA: Mm00607939_s1; Ubc mRNA: Mm01201237_m1; Gapdh mRNA: Mm99999915_g1) using the comparative Ct (ΔΔCt) method on ABI Fast 7900HT. Data from qPCR was normalized on geometric mean of three standard housekeeping genes (β-Actin, Gapdh, Ubc).

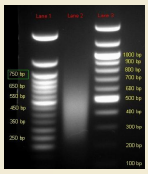
List of the primers used in the qPCR

Gene promoter region	Forward sequence (5'-3')	Reverse sequence (5'-3')
CREB (1)	AGGGAGGACGGTACCCAGTA	TTCTTTGCTGGGAAGAAAGC
c-Fos (1)	TACGACCCCTTCAGGCATAC	TAAAGGACGGCAGCACCTAC
BDNF P1 (2)	TGATCATCACTCACGACCACG	CAGCCTCTCTGAGCCAGTTACG
BDNF P2 (2)	CCGTCTTGTATTCCATCCTTTG	CCCAACTCCACCACTATCCTC
BDNF P3 (2)	GTGAGAACCTGGGGCAAATC	ACGGAAAAGAGGGAGGGAAA
BDNF P4 (2)	CTTCTGTGTGCGTGAATTTGCT	AGTCCACGAGAGGGCTCCA
BDNF P5 (2)	ACTCACACTCGCTTCCTCCT	GCACTGGCTTCTCTCCATTT
NMDA-NR1 (3)	TGTCCCTGGTTCTCTGTATGC	AAAGACAGCTGCCTGGACTG
NMDAR-N2A (4)	GAGTGAGGGGGGTGATGGAG	AATCTCAGGGTGTGGAGAGC
NMDAR-N2B (3)	TCGGGTTTCACATTCGACTC	CCTGTAAGGTGGAGGAGAGC

Forward and Reverse Primers used in the qPCR.
(1)Koshibu et al. 2009,
(2)Tsankova et al. 2006,
(3)Dhar et al. 2009,
(4)Renthal et al. 2007.

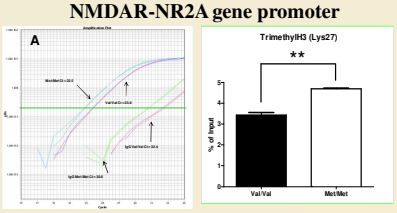
Optimization of chromatin fragmentation

In a ChIP experiment is very important to obtain genomic DNA fragments of about 500 bp. Therefore, sonication procedure has been optimized.



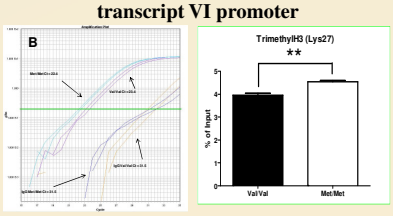
Chromatin fragmentation was evaluated by agarose gel elettrophoresis. Lane 1: DNA Ladder 50 bp, lane 2: sonicated chromatin sample, lane 3: DNA Ladder 100 bp. It is possible to note the enrichment of DNA fragments with a length between 300 and 600 bp.

Trimethylation of histone H3 (Lys27) at the NMDAR-NR2A gene promoter



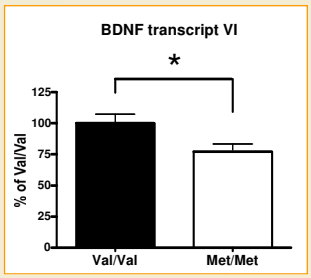
Anti-trimethyl histone H3 (Lys27) immunoprecipitated hippocampal chromatin was analyzed by real-time PCR. (A) Amplification plot of the NMDAR-NR2A gene promoter. (B) Increased trimethylation on the Lys27 residue of histone H3 in Met/Met compared to Val/Val mice. This suggests a reduction of NMDAR-NR2A gene transcription. Results are calculated as "percent input" values (abundance of the DNA fragment of interest in the final immunoprecipitate with respect to the abundance of the DNA fragment in the "Input"). Student t-test, ** $p < 0.005$.

Trimethylation of histone H3 (Lys27) at the BDNF transcript VI promoter



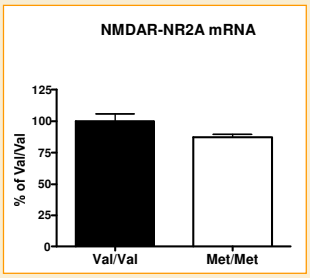
Anti-trimethyl histone H3 (Lys27) immunoprecipitated hippocampal chromatin was analyzed by real-time PCR. (A) Amplification plot of the BDNF transcript VI promoter. (B) Increased trimethylation on the Lys27 residue of histone H3 in Met/Met compared to Val/Val mice. This suggests a reduction of BDNF transcript VI. Results are calculated as "percent input" values (abundance of the DNA fragment of interest in the final immunoprecipitate with respect to the abundance of the DNA fragment in the "Input"). Student t-test, ** $p < 0.005$.

Levels of BDNF transcript VI are significantly lower in Met/Met mice



RNA extracted from the hippocampus was analyzed by RT-PCR. Results are calculated by the ΔΔCt method. Data are presented as % of Val/Val. Student t-test, * $p < 0.05$

Levels of NMDAR-NR2A mRNA are not different in Met/Met mice



RNA extracted from the hippocampus was analyzed by RT-PCR. Result are calculated by the ΔΔCt method. Data are presented as % of Val/Val.

CONCLUSION

1. The comparison between Val/Val and Met/Met mice highlighted significant epigenetic variations in two selected promoters.
2. The reduced transcription suggested by the epigenetic findings has been confirmed for BDNF VI by qPCR measurement. With TaqMan probe.
3. The transcript VI has been shown to be transported to distal dendrites, where it is likely responsible for the local synthesis of BDNF. These results are in line with the reduced dendritic trafficking of BDNF previously observed in Met/Met mice and with reduction of the activity-dependent BDNF protein release [3]. These modifications could be related to characteristic Met/Met phenotypic features (reduced dendritic complexity in the hippocampus and cognitive deficits).

BIBLIOGRAPHY

[1] Chen et al. Science. 2006; 314:140-143.
[2] Soliman et al. Science. 2010; 327:863-865.
[3] Chiaruttini et al. Mol. Cell. Neurosci. 2008; 37:11 – 19.

No potential conflict of interest