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# Control of the cerebral cortex plasticity through the non-cell autonomous function of OTX2 homeoprotein

Jessica Apulei

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# Sorbonne Université

ECOLE DOCTORALE CERVEAU-COGNITION-COMPORTEMENT

*CIRB – Collège de France / Équipe Alain Prochiantz*

## CONTROL OF THE CEREBRAL CORTEX PLASTICITY THROUGH THE NON- CELL AUTONOMOUS FUNCTION OF OTX2 HOMEOPROTEIN

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Thèse de Doctorat de Neurosciences

Dirigée par **Alain PROCHIANTZ**

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“Il lavoro e l'applicazione continui

sono il cibo del mio spirito.

Quando comincerò a cercare il riposo,

allora smetterò di vivere.”

Francesco Petrarca



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## LIST OF ABBREVIATIONS

<b>5caC</b>	5-carboxylcytosine
<b>5fC</b>	5-formylcytosine
<b>5hmC</b>	5-hydroxy methylcytosine
<b>5mC</b>	5-methyl citosine
<b>A1</b>	Primary auditory cortex
<b>ATAC-seq</b>	Assay for Transposase-Accessible Chromatin using sequencing
<b>Bcd</b>	Bicoid
<b>BDNF</b>	Brain-derived neurotrophic factor
<b>BSO</b>	Buthionine sulfoximine
<b>ChABC</b>	Chondroitinase ABC
<b>ChP</b>	Choroid Plexus
<b>CP</b>	Critical period
<b>CSF</b>	Cerebrospinal fluid
<b>DA</b>	Dopamine
<b>DNMT</b>	DNA methyl transferase
<b>DSB</b>	Double stranded break
<b>ECM</b>	Extracellular matrix
<b>EMX2</b>	Empty spiracles homeobox 2
<b>GABA</b>	$\gamma$ -aminobutyric acid
<b>Gad</b>	glutamic acid decarboxylase
<b>GADD45</b>	Growth Arrest and DNA Damage-inducible 45
<b>GAG</b>	Glycosaminoglycans
<b>H3k27me3</b>	Histone 3 lysine 27 trimethylated
<b>H3K9me</b>	Histone 3 lysine 9 trimethylated
<b>HAT</b>	Histone acetyltransferases
<b>HDAC</b>	Histone deacetylases
<b>HOXA9</b>	Homeobox A 9
<b>HP</b>	Homeoprotein
<b>IEG</b>	Immediate early gene

<b>LINE</b>	Long Interspersed Nuclear Elements
<b>MD</b>	Monocular deprivation
<b>MeCP</b>	Methyl binding protein
<b>MILI</b>	Miwi like protein
<b>MIWI</b>	Murine Piwi protein
<b>mPFC</b>	Medial prefrontal cortex
<b>NARP</b>	Neuronal activity-regulated pentraxin
<b>ORF</b>	Open reading frame
<b>OTX</b>	Orthodenticle Homeobox
<b>PAX</b>	Paired box 6
<b>PirB</b>	Paired immunoglobulin-like receptor B complex
<b>PIWI</b>	P-element induced wimpy testes
<b>PNN</b>	Perineuronal net
<b>PRH</b>	Proline-rich homeodomain
<b>PV</b>	Parvalbumin
<b>qPCR</b>	Quantitative polymerase chain reaction
<b>ROS</b>	Reactive oxygen species
<b>RTT</b>	Rett syndrome
<b>SETDB1</b>	SET domain, bifurcated 1
<b>SNpc</b>	Substantia Nigra pars compacta
<b>SUV39H1</b>	Suppressor of variegation 3-9 1
<b>TE</b>	Transposable elements
<b>TET</b>	Ten eleven translocation
<b>V1</b>	Primary visual cortex
<b>VTA</b>	Ventral tegmental area

# INTRODUCTION

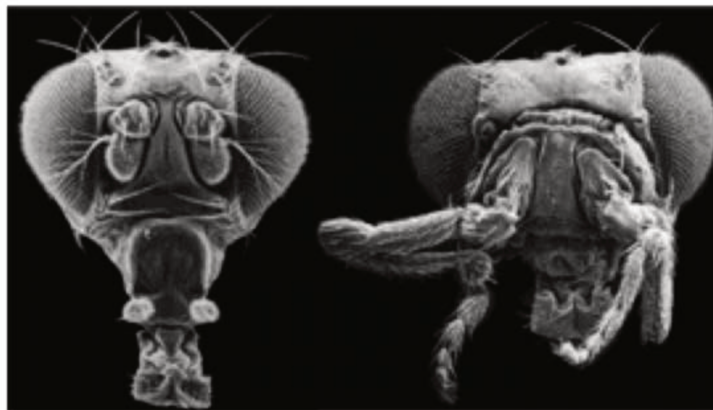




## I. ROLE OF HOMEOPROTEINS DURING DEVELOPMENT

### A. Discovery of homeodomain proteins

Homeoproteins (HPs) are a major class of transcription factors active at all stages of development and in adult life. They are encoded by homeobox genes, a family of genes discovered for their functions during early embryonic development, in particular for their role in the development of the antero-posterior axis of arthropods and vertebrates. In 1967, Gehring described in *Drosophila* the *Antennapedia* homeotic mutation in which antennae are replaced by ectopic legs (Figure 1) (Gehring, 1967). The analysis of the *Antennapedia* gene led to the discovery of a sequence of 180 base pairs, the homeobox, which is highly conserved in all homeobox genes.



*Figure 1. Antennapedia Mutation in Drosophila*

*On the right is shown a Drosophila head with the homeotic Antennapedia mutation: ectopic legs replace the antennae, compared to a normal Drosophila on the left (Photos by F.R. Turner, Indiana University).*

The homeobox encodes a 60 amino acid domain called homeodomain, conserved in eukaryotes (Carrasco et al., 1984; Laughon and Scott, 1984; McGinnis et al., 1984; Shepherd et al., 1984). The homeodomain is the DNA-binding domain of HP transcription factors. The homeodomain structure, first described for Antennapedia (Qian et al., 1995), is a compact globular structure and contains an unstructured N-terminal arm followed by three well-defined  $\alpha$ -helices arranged in a helix-loop-helix-turn-helix manner (Gehring et al., 1994). Helices 1 and 2 are anti-parallel and helix 3 is virtually perpendicular to the axis established by the first two helices (Billeter et

al., 1993, 1990; Wolberger, Liu, and Johnson, 1991). Helix 3 is called "recognition helix" and contacts the major groove of double-stranded DNA (Figure 2). Two other sites of contact include one between helix 1 and the DNA backbone and another one between the flexible N-terminal arm and bases in the minor groove (Otting et al., 1990).

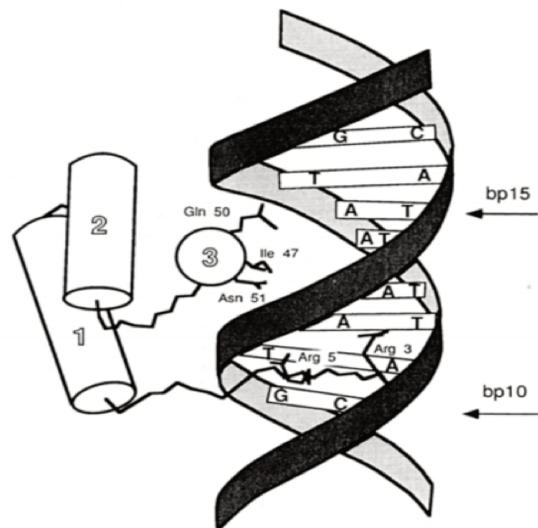


Figure 2. Homeodomain-DNA interaction

The homeodomain contains three well-defined  $\alpha$ -helices (represented by cylinders 1, 2 and 3). The third helix of the homeodomain contacts the major groove of the DNA. There are two additional sites of contact, between the first helix and the DNA backbone, and between the flexible amino-terminal arm and bases in the minor groove (From Kissinger et al., 1990).

While homeoproteins were first described as transcription factors, they can also bind RNAs. As early as 1996, it was demonstrated that BICOID (Bcd) recognizes the mRNA of *Caudal* and controls the translation through binding of the translation factor eIF4E (Dubnau and Struhl, 1996). The interaction site with eIF4E is largely conserved within the HP family and regulation of translation has been extended to other HPs including EMX2, HOXA9, PRH, OTX2 and ENGRAILED (Nedelec et al., 2004; Topisirovic and Borden, 2005; Brunet et al., 2005).

## B. Roles of OTX HPs during mouse development

During mouse embryonic development, *Otx2* is expressed as early as E5.5, before gastrulation, in the visceral endoderm and epiblast. After gastrulation and neural induction at E7.5, *Otx2* remains expressed in the endoderm and mesoderm but is also expressed in the rostral neural

plate which will give rise to the telencephalon, diencephalon, and mesencephalon (Acampora et al., 2001). *Otx1* expression begins at E8.5 in the epithelium that later becomes the anterior brain and mesencephalon (Simeone et al., 1992). During brain development and regionalization, *Otx1* and *Otx2* expression patterns are similar. At later stages, *Otx1* continues to be expressed in the cortical ventricular zone at the beginning of neurogenesis (E10.5) and in the neural progenitors that will later give rise to layers V and VI of the cortex (Simeone et al., 1993; Frantz et al., 1994). By late embryonic and early postnatal stages, *Otx2* expression becomes predominant in the choroid plexus and in several areas of the brain involved in visual information processing: the retina, the lateral geniculate nucleus of the thalamus, and the superior colliculus (Nothias et al., 1998).

The deletion of *Otx1* produces viable mice that suffer from epileptic seizures, have telencephalic abnormalities, and have delayed development of the visual and auditory sensory organs (Acampora et al., 1995). In the adult, the brains of *Otx1*<sup>-/-</sup> mice have a reduced cortical thickness. On the contrary, the total inactivation of *Otx2* is lethal very early in development due to the loss of rostral brain regions. However, heterozygous mice for *Otx2* are viable and fertile (Martinez-Morales, 2001; Koike et al., 2007).

### C. Unconventional transfer of HPs

In the early 1990s, Alain Prochiantz and his team discovered that HPs have the property of transferring between cells, due to the presence in the homeodomain of two short domains respectively allowing HP secretion and internalization. The first observation that led to the finding of this unconventional HP signaling function was the spontaneous internalization of the homeodomain of Antennapedia in live neurons *in vitro*, without alteration of the plasma membrane (Joliot et al., 1991; Le Roux et al., 1993). It was then demonstrated that within the homeodomain, a 16 amino acid sequence in helix 3, also known as penetratin, is necessary and sufficient to allow for homeodomain internalization (Derossi et al., 1994). A key role is played by tryptophan in position 48 (W48) (Figure 3A), which cannot be replaced by any other hydrophobic amino acid without dramatically reducing internalization. Penetratin internalization properties have been extensively studied and are now well described.

Internalization occurs at 4°C as well as at 37°C, supporting an energy-independent capture of the peptide. Penetratin does not bind a chiral receptor, suggesting that internalization is not receptor-dependent (Derossi et al., 1996).

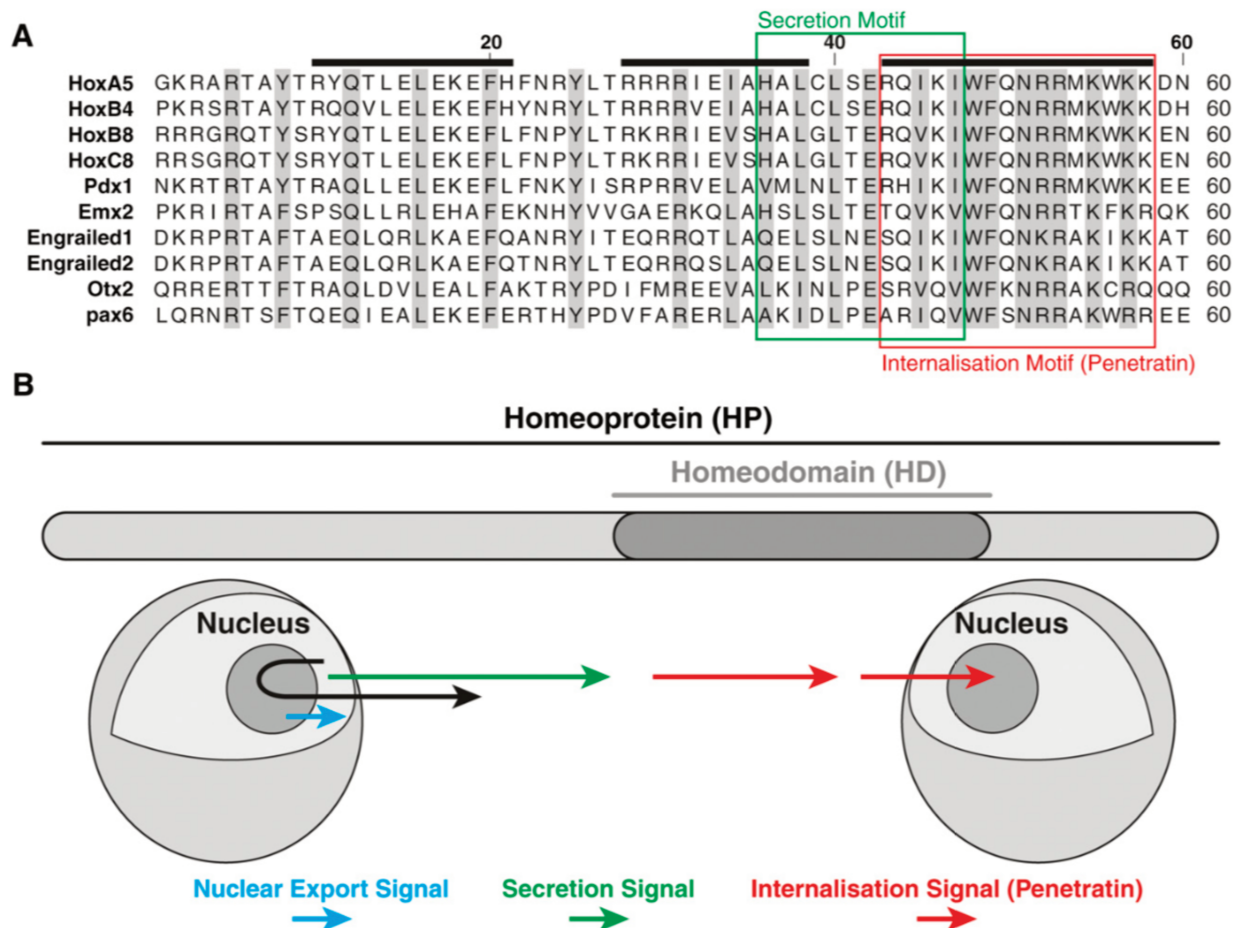
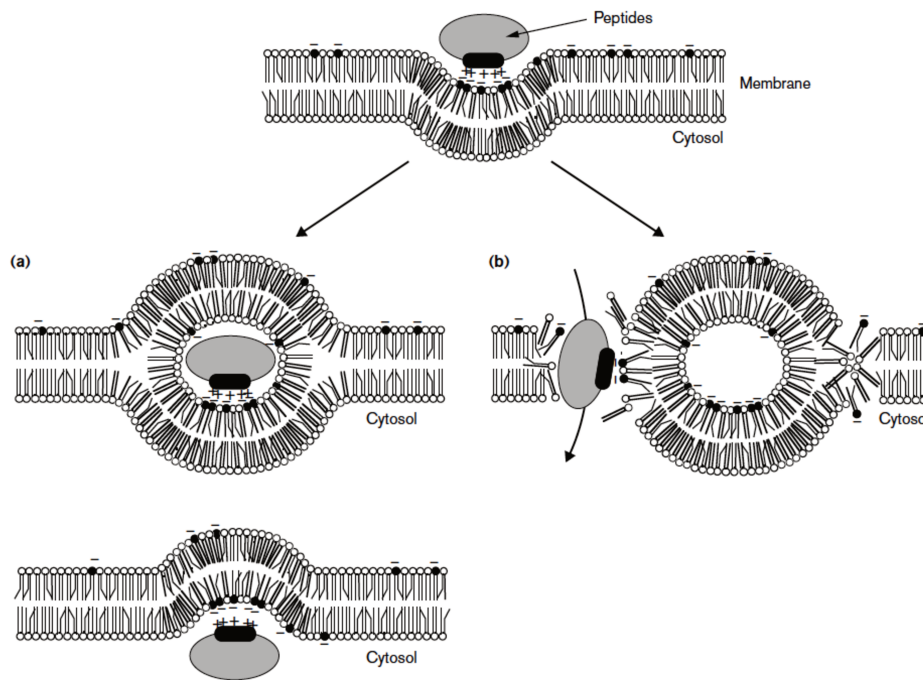


Figure 3. Conserved homeodomains between HPs

A. Homeodomain sequence alignments for 10 HPs verified for intercellular transport. The internalization sequence corresponds to helix 3 of the homeodomain, and the secretion sequence includes the end of helix 2, the beginning of helix 3, and the  $\beta$ -turn between the two helices. The three helices are indicated by bold dark lines. B. Schematic representation of the internalization and secretion sequences of HPs and of their functions in intercellular transport (Spatazza et al., 2013).

Internalization of HPs happens through a non-vesicular and endocytosis-independent mechanisms. Penetratin is poorly structured in water but adopts a helical structure in a hydrophobic environment. A model supported by biophysical experiments proposes that the third helix interacts with charged phospholipids at the outer side of the membrane and that a

destabilization of the bilayer induced by W48 induces the formation of inverted micelles that travel across the membrane and eventually open on its cytoplasmic side (Figure 4) (Berlose et al., 1996; Christiaens et al., 2002; Dupont, Prochiantz, and Joliot, 2007). The same mutations block the internalization of helix 3 and of full-length HPs, suggesting the existence of shared internalization mechanisms despite size differences.



*Figure 4. Homeoproteins internalization model*

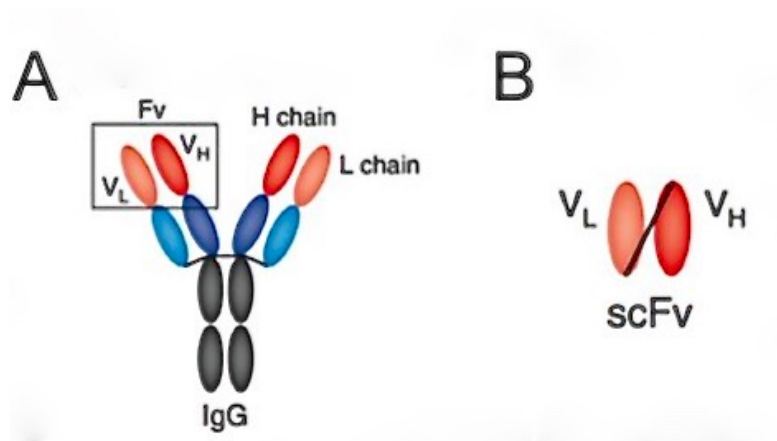
*A correlation between the formation of inverted micelles and Penetratin peptide translocation has been made. The involvement of electrostatic interactions and of a tryptophan residue in position 48 of the homeodomain have been established. It was thus proposed that the tryptophan destabilizes the membrane and induces the formation of inverted micelles. In model (a), the peptides are trapped in the micelles, which can reopen inside the cell. In model (b), the micelles perturb the structure of the bilayer, allowing peptide translocation followed by membrane repair (From Prochiantz, 2000).*

Homeoproteins do not use the same mechanisms for entering and exiting the cell (Joliot and Prochiantz, 2004; Dupont, Prochiantz, and Joliot, 2007; Joliot et al., 1997). Their secretion has been particularly studied for ENGRAILED: when expressed by COS cells, it transfers to co-cultured neurons (Joliot et al., 1997). This secretion is made possible by the  $\Delta 1$  motif, a signal that spans helices 2 and 3 of the homeodomain and is distinct from the internalization domain (Figure 3A). This domain overlaps with a nuclear export sequence, suggesting a link between

secretion and nuclear export (Figure 3B). The secretion is energy-dependent, does not involve the ER-Golgi pathway and likely requires association of HPs to caveolae-like vesicles enriched in cholesterol and glycosphingolipids. However, the exact mechanism of secretion is not yet fully understood. Analytical studies performed *ex vivo* demonstrate that, at least for PAX6 and ENGRAILED, ~5% of the total amount of proteins are present at the cell surface (Di Lullo et al., 2011; Joliot et al., 1997; Wizenmann et al., 2009).

#### D. Homeoprotein as *in vivo* signaling proteins

Homeoproteins exert their non-cell autonomous activities through the secretion and internalization sequences identified within the homeodomain. To study such activity, specific mutations cannot be carried out without affecting DNA-binding, and therefore the cell-autonomous functions of HPs. To discriminate between cell autonomous and non-cell autonomous functions, it was therefore necessary to develop specific tools, such as secreted single-chain antibodies (scFv) against HPs. ScFv antibodies target HPs in the extracellular space and specifically disrupt non-autonomous properties. A scFv is not actually an antibody fragment, but rather a fusion protein of the variable regions of the immunoglobulin heavy and light chains connected with a short linker peptide (Figure 5). The scFv is thus secreted from one cell and neutralizes the targeted antigen (the HP secreted from the same or a different cell) in the extracellular space. The use of secreted scFv has demonstrated that PAX6 regulates the development of the eye anlagen in zebrafish (Lesaffre et al., 2007), the migration of oligodendrocyte precursors in the chicken embryonic neural tube (Di Lullo et al., 2011), and the migration of Cajal-Retzius cells in the mouse neuroepithelium (Kaddour et al., 2019). Similar scFv-based approaches have shown that the transfer of ENGRAILED is involved in the formation of the anterior cross vein in the imaginal disc of the *Drosophila* wing (Layalle et al., 2011) and in the guiding of retinal ganglion cells at the level of the frog and chick optic roof (Brunet et al., 2005; Wizenmann et al., 2009). More recently, the scFv strategy has been applied to OTX2 in order to study its non-cell autonomous roles in regulating critical periods of plasticity during cerebral cortex postnatal mouse development (Bernard et al., 2016).



*Figure 5. scFv technology to block HPs transfer*

*(A) An antibody is composed of two heavy chains (H chain) and two light chains (L chain). These chains are themselves composed of a constant part and a variable part (V<sub>H</sub> and V<sub>L</sub>). Each antibody has two variable fragments (Fv). (B) A scFv antibody is produced by fusion of the variable part of the heavy chain (V<sub>H</sub>) and the variable part of the light chain (V<sub>L</sub>) of a monoclonal antibody. The chains are linked by a peptide chain schematized here by a black line linking the two domains.*



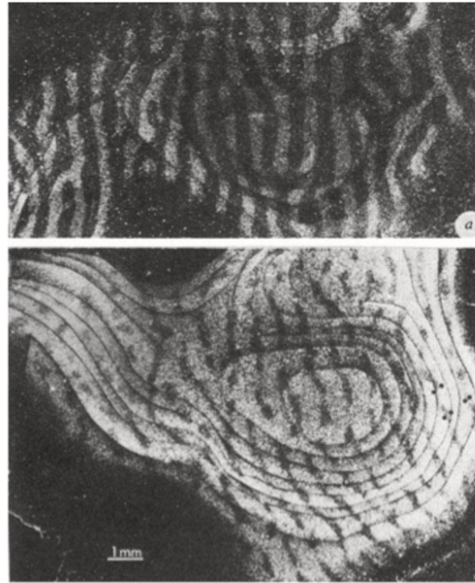


## II. CRITICAL PERIODS OF PLASTICITY

### A. Definition of critical periods

Throughout lifetime, the brain is able to modify and shape its circuits in response to new experiences. This capacity is at its highest during postnatal development and allows the remodeling of cerebral cortical synaptic circuits in response to the flow of sensory input received from the external world. Heightened postnatal plasticity was initially studied in the sensory-motor cortex and led to the definition of specific developmental windows, known as “critical periods” (CPs), during which neuronal circuits adapt with great efficiency. Critical periods are associated with different brain areas and functional compartments of the cerebral cortex but CPs are not necessarily synchronous. In general, CPs for development of primary sensory areas (smell, audition, touch, and vision) come first, followed by those for language, locomotion and finally those involved in higher cognition. Furthermore, plasticity is very limited outside CPs, in particular post-CP when circuits and synapses have matured.

Critical periods have been observed in various systems and across species (Hensch, 2004), but the most widely studied model for CPs is ocular dominance plasticity which takes place in the primary visual cortex (V1). Pioneering studies in cats by Hubel and Wiesel showed that thalamocortical inputs in V1 are organized in patterned columns responding uniquely to either the contralateral or ipsilateral eye (Wiesel and Hubel, 1963). Inputs from the two eyes compete when they first converge in the binocular zone of the V1. When one eyelid is temporarily closed (monocular deprivation (MD)) during CP for binocular vision, the thalamic terminals corresponding to the open-eye take over, leading to an increase in the width of the columns innervated by these “open-eye-terminals” and to a shrinkage of the other “non-functional” columns (Figure 6). If visual input is imbalanced during binocular V1 CP, either through misaligned eyes or congenital cataracts, then this results in amblyopia, a disorder that touches 2–5% of the human population (Holmes and Clarke, 2006). In children 7 to 17-year-old, treatments such as patching the stronger eye are possible. However, this is less effective than patching during 3 months and 7 years of age that corresponds to the CP (Scheiman et al., 2005).



*Figure 6. Plasticity in the visual pathway as a result of sensory information during development*

*The ocular dominance columns are visualized by autoradiography after injection of a radioactive tracer into one eye. The tracer is transported to the lateral geniculate nucleus and then trans-synaptically to the striate cortex. Labeled and unlabeled bands alternate corresponding to the injected and non-injected eye, respectively. UPPER: Normal pattern. BOTTOM: Modified pattern in an animal raised with MD during CP (From Wiesel, 1981).*

After these studies in cats, the CP for ocular dominance plasticity was studied in other species, such as ferrets (Issa et al., 1999), macaques (Horton and Hocking, 1997), and mice (Gordon and Stryker, 1996). In the mouse, the V1 cortex is not organized in columns and, due to the lateral positioning of the eyes, there is a natural ocular dominance for the contralateral eye in the V1 binocular zone. However, as in the cat, MD during the CP of plasticity leads to a shift of the afferences in favor of the open-eye (Figure 7). Sensitivity to MD is restricted to the CP for ocular dominance plasticity. Critical period in mice opens at postnatal day 20 (P20), soon after eye opening (P14), peaks at P30 and closes at P40 (Figure 8). Indeed, MD performed outside of this period provokes little or no remodeling.

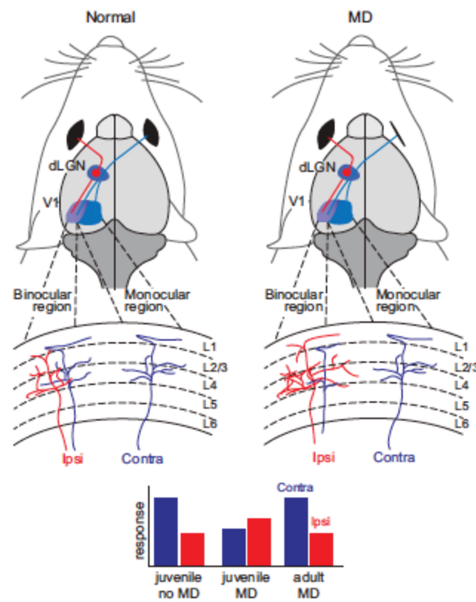


Figure 7. Critical period of ocular dominance and remodeling of connections

The thalamic afferences of the contralateral eye (in blue) and the ipsilateral eye (in red) are in competition in the binocular area (purple). Monocular deprivation (MD) of a juvenile mouse reduces the afferences of the contralateral eye and thus the associated response in the visual cortex, with an opposing effect in the ipsilateral eye. No large-scale changes are observed after MD in the adult (From Di Nardo et al., 2018).

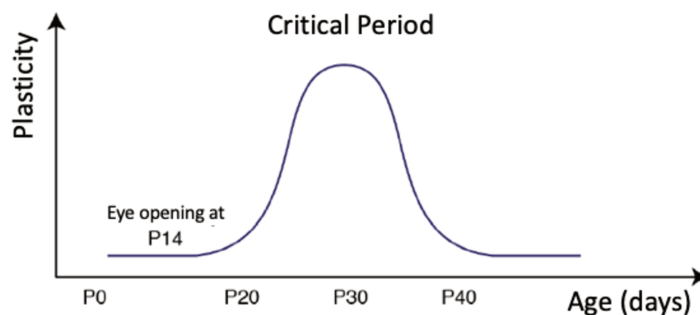


Figure 8. Critical period windows of plasticity in mice

The ocular dominance plasticity timing in mice has been well characterized. Following eye opening 14 days after birth (P14), plasticity opens at around P20, peaks at P30 and closes at around P40.

## B. Cellular and molecular mechanisms of cortical plasticity

Critical period onset is determined by the maturation of local inhibitory circuits and many molecular factors have been implicated in the regulation of the onset and closure of the CP (Fagiolini and Hensch, 2000; Hensch, 2005; Sur et al., 2013). Indeed, the onset of plasticity can be modified by interfering with the maturation of the inhibitory system. The primary inhibitory neurotransmitter in the brain,  $\gamma$ -aminobutyric acid (GABA), is synthesized by glutamic acid decarboxylase (GAD) encoded by two distinct genes, *Gad65* and *Gad67*. Of the two isoforms, GAD67 is responsible for 90% of GABA synthesis and knock-out mice for GAD67 die at birth. In contrast, knock-out mice for GAD65 are viable and have normal GABA concentrations and ocular dominance. Thus, MD in these mice during the CP has no effect (Hensch, 1998), but plasticity can be restored by diazepam, which increases GABA affinity for its receptors. Curiously, this restoration is possible at any age in these mice, which underscores the importance of the level of inhibitory transmission in CP initiation.

GABAergic inhibitory interneurons constitute a very diverse group of neurons that account for nearly 20% of cortical neurons. However, CP onset corresponds closely to the emergence of parvalbumin (PV) inhibitory interneurons (del Rio et al., 1994). Parvalbumin is one of the three calcium-binding proteins, also including calretinin and calbindin, which are expressed in largely non-overlapping groups of most GABA-producing neurons in the cortex. Parvalbumin interneurons are fast-spiking cells that give rise to oscillatory activity in the gamma-frequency range of 30-80 Hz (Marín, 2012), due to a potassium conductance (Kv3 class). The specific blockade of the potassium channel Kv3.1 slows the establishment of ocular dominance plasticity, mimicking the *Gad65* knock-out, in a cell-specific manner (Hensch, 2005). There are two types of PV interneurons: the chandelier cells which form synapses onto the initial segment of excitatory pyramidal cell axons and the large basket cells that mostly contact excitatory pyramidal cell soma (Figure 9). Parvalbumin cell-induced inhibition triggers cortical plasticity through GABA<sub>A</sub> receptors containing  $\alpha 1$  subunit and present on the pyramidal cell soma (Fagiolini, 2004). Thus, the optimal ratio of excitatory and inhibitory circuit activity for CP onset is driven by the maturation of PV large basket cells.

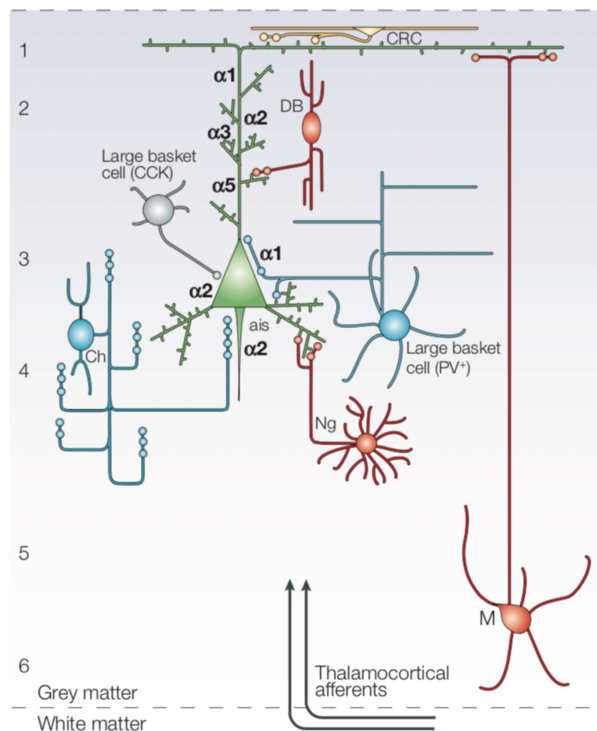


Figure 9. Neuronal architecture in the cerebral cortex

Diversity of interneurons and organization of V1 in six different layers (noted 1 to 6). Parvalbumin cells (PV+) are part of the basket cells (in blue) present in layer IV of V1. They establish synaptic contacts with the soma and proximal dendrites of pyramidal cells (green) and control the excitatory/inhibitory balance that regulates plasticity (From Hensch, 2005).

Critical period onset is also regulated by other factors, such as brain-derived neurotrophic factor (BDNF), which promotes the maturation of the PV cells in an activity-dependent manner. Overexpression of BDNF triggers a premature onset of the CP of V1 by inducing the maturation of PV interneurons (Figure 10) (Huang et al., 1999). On the contrary, blocking BDNF prevents the development of ocular dominance plasticity in the kitten V1 (Cabelli et al., 1997).

Another known factor that controls CP onset is the neuronal pentraxin (NARP), which is an activity-regulated protein that promotes PV cell maturation by controlling glutamate receptor expression (Chang et al., 2010). Interestingly, *Narp* KO mice fail to install ocular dominance plasticity throughout life (Gu et al., 2013). The maturation of PV cells is accompanied by a sequence of structural and molecular events that lead to circuit rewiring and physiological consolidation (Hensch, 2005).

Tissue-type plasminogen activator is a major serine protease, which, upon release, cleaves the physical connections between pre- and postsynaptic partners and induces dendritic spine motility (Mataga et al., 2004, 2002; Oray et al., 2004). The deprivation-induced increase in tissue-type plasminogen activator, along with spine pruning, fails to occur in mice lacking *Gad65* (Mataga et al., 2002, 2004). Multiple protein kinases, such as CaMKII, PKA, ERK (Antonini et al., 1999; Cristo, 2001; Taha and Stryker, 2002; Trachtenberg and Stryker, 2001), and homeostatic regulators, such as TNF (Kaneko et al., 2008), eventually converge on gene transcription programs mediated by CREB to ultimately strengthen open eye connections.

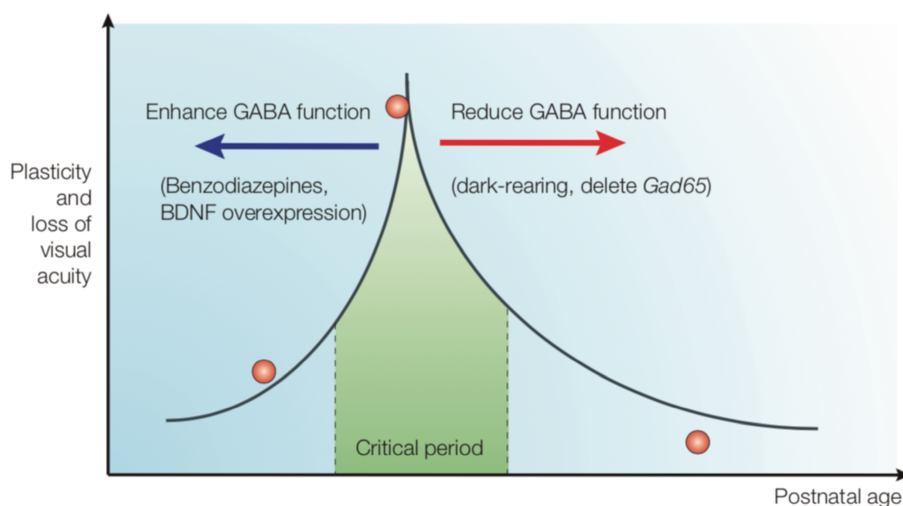


Figure 10. CP is controlled by the GABAergic system

*The CP for ocular dominance can be induced ahead of time by increasing GABA function. A reduction in GABA function retards plasticity opening (From Hensch, 2005).*

The CP closes as molecular brakes gradually emerge to dampen plasticity. These brakes include structural brakes, which physically prevent neurite pruning and outgrowth, and functional brakes acting on neuromodulatory systems. Examples of structural brakes include myelin factors and PNNs. Indeed, myelin and myelin-associated inhibitors have been shown to control CP. Indeed, proteins found in myelin (Nogo, myelin-associated glycoprotein, myelin oligodendrocyte glycoprotein) limit axonal sprouting by their binding to Nogo receptor and paired immunoglobulin-like receptor B complex (PirB) (Atwal et al., 2008). *Nogo* receptor KO mice exhibit ocular dominance plasticity beyond the CP. In mice lacking functional PirB, ocular dominance plasticity remains at all ages (Syken, 2006). During CP, layer IV PV cells in V1 are

gradually enwrapped by a specialized ECM enriched in glycosaminoglycans (GAG) and condensed into perineural nets (PNNs) that can be visualized by the lectin *Wisteria Floribunda* agglutinin (WFA) (Härtig, Brauer, and Brückner, 1992; Köppe et al., 1997). PNNs constitute a highly organized structure, the function of which is only partially understood (Härtig et al., 1992). The deposition of PNNs also intensify after CP closure in other cortical regions such as the mouse barrel cortex (McRae et al., 2007), the amygdala (Gogolla et al., 2009), and the auditory cortex (Lee et al., 2017).

Other factors implicated in the transitions of CP are DNA methylation and histone modifications. DNA methylation is known to generally repress transcription by preventing the binding of transcription factors to the DNA or by recruiting repressor complexes that condense the chromatin structure (Moore et al., 2013). Closure of the CP for ocular dominance is associated with a downregulation of visual experience-induced histone acetylation and phosphorylation (Putignano et al., 2007). Indeed, rapid increases in ERK-dependent phosphorylation of histones is associated with activation of the juvenile V1 and less so in older mice (Putignano et al., 2007). In adult mice, ocular dominance plasticity can be reinstated through treatment with the HDAC inhibitor trichostatin A. Furthermore, the use of conditional deletion mice for methyl-CpG-binding protein 2 (MeCP2) has highlighted the importance of MeCP2 in regulating CP (He et al., 2014). A specific deletion of MeCP2 in PV cells, but not somatostatin interneurons or pyramidal cells, leads to a delay in the initiation of V1 plasticity (He et al., 2014).

### C. Mechanisms of reopening of plasticity in adult

The brain could be seen as an intrinsically plastic organ in which adult plasticity is dampened by molecular and structural brakes that limit rewiring after CP closure (Takesian and Hensch, 2013). Indeed, by targeting the brakes described in the previous paragraph, it is possible to accelerate or delay or even re-open plasticity in adulthood.

For example, cortical infusion of a GAD inhibitor promotes plasticity in adult V1 (Berardi et al., 2003). Adult rats exposed to complete darkness, show reduced expression of GABA<sub>A</sub> receptors, thus altering the excitation/inhibition balance and enhancing V1 plasticity (He et al., 2007). Furthermore, transplantation of juvenile inhibitory neurons induces ocular dominance



plasticity after CP closure in V1 (Southwell et al., 2010). Environmental enrichment in adult amblyopic rats restored normal visual acuity and ocular dominance (Tognini et al., 2012). Some of these effects are explained by a reduction of GABAergic inhibition, accompanied by an increased BDNF expression and were prevented by enhancement of inhibition through benzodiazepine cortical infusion (Sale et al., 2007). When animals are treated with fluoxetine, a selective serotonin reuptake inhibitor, similar results were obtained for the reopening of plasticity (Vetencourt et al., 2008). In a similar manner, this treatment reduces GABAergic inhibition in V1 and is accompanied by an increased expression of BDNF.

Plasticity in adulthood can also be induced by lifting the structural brakes. Chondroitinase ABC (ChABC) is an enzyme that digests the chondroitin sulfate chains contained within PNNs (Prabhakar et al., 2005). Digestion of these chains is able to induce ocular dominance plasticity after CP closure (Pizzorusso, 2002). Furthermore, ChABC treatment can rescue cortical acuity in adult amblyopic rats with a complete recovery of ocular dominance, visual acuity and dendritic spine density (Pizzorusso et al., 2006). In the adult amygdala, ChABC degradation of PNNs is capable of re-opening plasticity allowing for fear memories to be erased by extinction training (Gogolla et al., 2009).

On the epigenetic side, increasing histone acetylation in adulthood can reactivate ocular dominance plasticity (Putignano et al., 2007).

### III. REGULATION OF CEREBRAL CORTEX PLASTICITY BY NON-CELL AUTONOMOUS OTX2

#### A. The transfer of OTX2 into the visual cortex regulates CP

Important functions of HPs are not restricted to embryonic development. During postnatal development and in the adult, *Otx2* expression is ongoing although restricted to specific areas including the retina, the lateral geniculate nucleus, the ventral tegmental area (VTA), the superior colliculus, the cerebellum, and the choroid plexus (ChP). Postnatally, OTX2 protein is found in V1, primarily in PV cells, despite the fact that the *Otx2* locus is silent in these cells (Sugiyama et al., 2008). Thus, OTX2 function in V1 is non-cell autonomous and OTX2 protein must be transported from extracortical sources, which include the ChP and very likely the retina (Spatazza et al., 2013; Sugiyama et al., 2008). To demonstrate that OTX2 can be transported *in vivo* along the visual pathway, a tagged OTX2 recombinant protein was injected into the retina and was detected in PV cells of V1. The accumulation of OTX2 in PV cells following CP opening is activity-dependent given that during dark rearing or after enucleation, OTX2 is reduced in V1 (Sugiyama et al., 2008). Finally, the ChP is a major site of *Otx2* expression and the source of extracortical OTX2 (Figure 11). Indeed, reducing *Otx2* expression in the ChP by conditional knock-down in adult mice results in decreased levels of OTX2 in V1 (Spatazza et al., 2013).

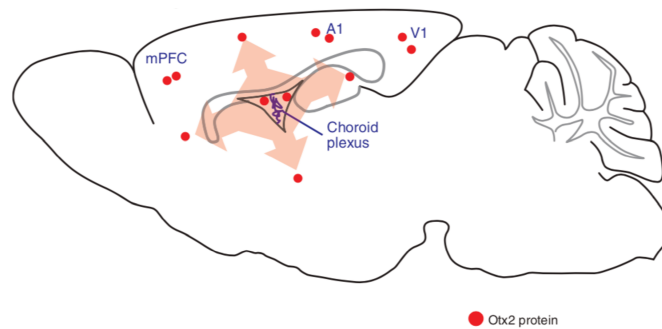


Figure 11. OTX2 distribution in the cortex

*Choroid plexus secretes OTX2 into the cerebrospinal fluid. OTX2, produced in the choroid plexus, is found in the cortex and has been shown to control plasticity of the primary visual cortex (V1), primary auditory cortex (A1) and medial prefrontal cortex (mPFC) (From Di Nardo et al., 2018).*

In V1, the majority of neurons containing OTX2 protein are GABAergic interneurons and over 70% of them are PV cells. OTX2 accumulation in PV cells parallels PV expression. Indeed, the accumulation of OTX2 regulates the maturation of PV cells. OTX2 protein is almost undetectable before CP onset, continues to accumulate during CP and persists in adulthood (Sugiyama et al., 2008). Intracortical infusion of exogenous OTX2 in V1 of P17 mice results in its specific capture by PV cells and accelerates CP opening (Figure 12). At a molecular level, this early opening is paralleled by increased PV, Kv3.1b potassium channel, GAD65 and the  $\alpha 1$ -subunit of GABA<sub>A</sub> receptor expression (Sugiyama et al., 2008). Conversely, down-regulating *Otx2* expression in the retina prevents ocular dominance plasticity. The interception of extracellular OTX2 by antibody injection either in V1 or in the retina has similar effects (Sugiyama et al., 2008). Finally, *Otx2* conditional KO in juvenile mice do not display ocular dominance plasticity during CP and plasticity is rescued by treatment with benzodiazepine, as in the *Gad65* knock-out mice, which confirms a role for OTX2 in inhibitory circuit maturation (Sugiyama et al., 2008). Furthermore, in dark-reared mice where CP onset is delayed, OTX2 levels are reduced by 70 % (Sugiyama et al., 2008). “Normal” PV cell maturation can be restored by the infusion of exogenous OTX2, again confirming that OTX2 is a crucial extra-cortical signal for the maturation of inhibitory circuits and the activation of CP-associated plasticity.

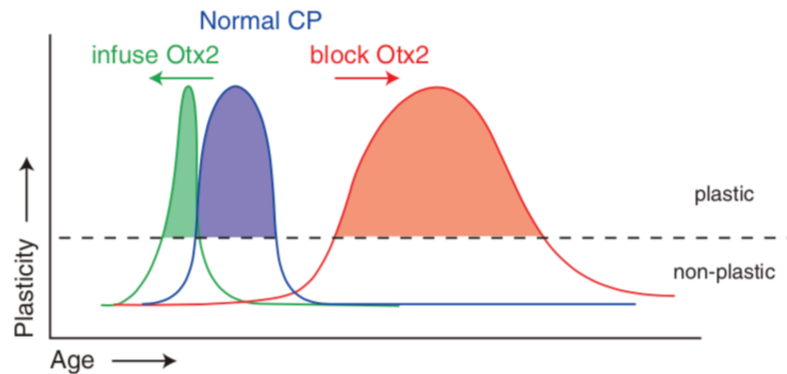


Figure 12. OTX2 signaling regulates CP timing

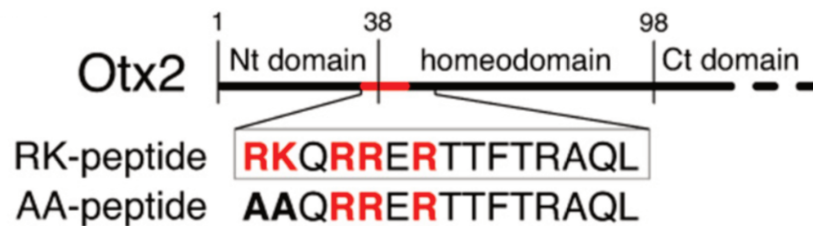
The CP of plasticity (in blue) is accelerated following OTX2 infusion (in green) or delayed by reducing OTX2 levels (in red) (From Di Nardo et al., 2018).

#### B. The transfer Of OTX2 in the adult maintains a non-plastic state

Specific transfer of OTX2 into PV cells is necessary and sufficient to open, then close, the CP of plasticity in V1. Both gain- and loss-of-function experiments (Beurdeley et al., 2012; Sugiyama et al., 2008) indicate that OTX2 internalization enhances several markers of PV cells maturation, including PNN formation. The accumulation of endogenous OTX2 suggests that there are OTX2 specific binding sites on PV cells. Indeed, PV-expressing neurons become enwrapped by PNNs during CP and there is a strong colocalization between OTX2 and PNNs in layer IV of V1 (Beurdeley et al., 2012; Miyata et al., 2012). ChABC injection in adult V1 to disrupt PNNs also decreases OTX2 staining and the number of PV cells, suggesting a role for PNNs in specific recognition of OTX2 before its internalization (Beurdeley et al., 2012).

A short motif within OTX2 sequence (RKQRRERTTFTRAQL), which partially overlaps with the beginning of the homeodomain, possesses traits of a consensus GAG-binding domain (Figure 13) (Cardin et al., 1989). This RK motif is necessary for preferential OTX2 recognition of PNNs. Indeed, while a full-length FITC-OTX2 protein injected in the visual cortex shows a preference towards PNN-enwrapped cells, FITC-OTX2-AA protein, in which the RK doublet is replaced by two alanines, the specificity for PNNs is lost. Indeed, FITC-OTX2-AA is also internalized by other type of cells. When a synthetic peptide (RK-peptide) corresponding to this

GAG-binding motif is infused into the adult V1, it competes with endogenous OTX2 and blocks its transfer into the PV cells (Beurdeley et al., 2012).



*Figure 13. The GAGs recognition domain of OTX2*

*The OTX2 sequence (black line) contains a GAG recognition pattern (red line) rich in arginine (R) and lysine (K). The mutation of the RK sequence into two alanines (A) results in a decrease in interaction with the GAGs of the PNNs (From Beurdeley et al., 2012).*

After RK-peptide infusion, the amount of OTX2 captured by PV-cells is decreased, resulting in a down-regulation of PV expression and a decrease in PNN assembly. The ongoing positive feedback of nascent PNNs attracting OTX2, thus triggering their own continued maintenance throughout life, may serve to prevent adult plasticity (Beurdeley et al., 2012). Thus, controlling PNNs and the internalization of OTX2 open the hope for a therapeutic approach for amblyopia in adult mice (Beurdeley et al., 2012).

Indeed, by blocking the transfer of OTX2 transfer in a scFv antibody mouse model that conditionally expresses scFv-OTX2 (STOP-lox system) re-opens the CP (Figure 14) (Bernard et al., 2016). Crossing these mice with PV::Cre mice results in scFv-OTX2 expression in the V1 PV cells. This prevents the entrance of endogenous extracellular OTX2 in PV cells and delays expression of plasticity genes in the juvenile mouse. Furthermore by inducing the adult expression of scFv-OTX2 in the ChP and its secretion into the CSF where it sequesters endogenous extracellular OTX2, activates adult plasticity (Bernard et al., 2016).

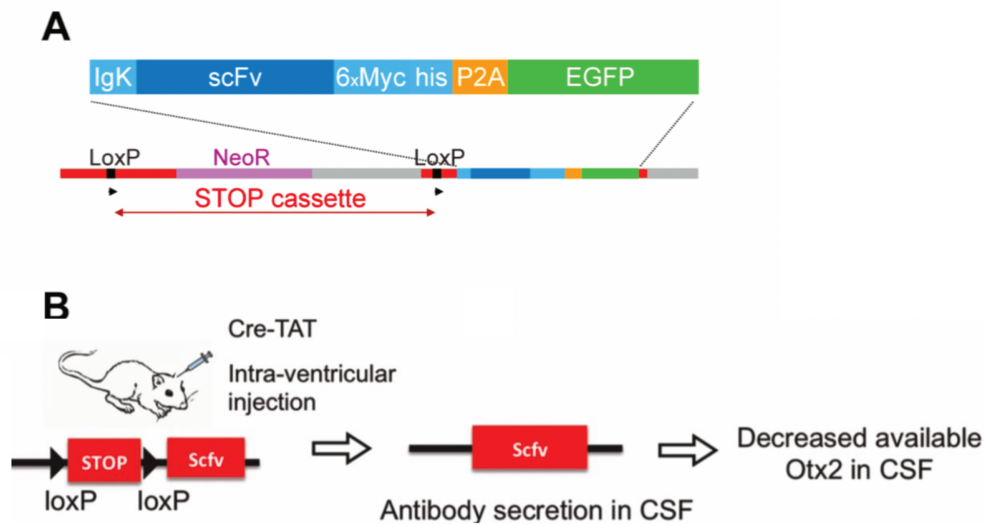


Figure 14. Inducible scFv Knock-In mice

*A. Construct for scFv knock-in mouse. The single chain antibody (scFv) is knocked into the ROSA26 locus of C57Bl6 mice. The scFv plasmid contains an IgK signal peptide for secretion, 6 Myc tags for detection. A tag EGFP after a skipping peptide (P2A) enables the detection of the expression of the scFv. A STOP cassette is inserted before the scFv construct, flanked by two LoxP sites to allow for conditional expression. B. Example of use of the transgenic mouse expressing a scFv antibody against the HP OTX2.*

These studies on OTX2 led to the hypothesis of a two-threshold model of OTX2 accumulation in PV cells for regulating the plasticity window within V1 (Figure 15). Reaching the first threshold of the concentration of OTX2 opens the CP. This period of plasticity closes once OTX2 reaches a second concentration threshold. In adults, constant accumulation maintains a non-plastic state. Thus, reducing OTX2 levels in adult PV cells below the second threshold reopens plasticity.

It is of note that OTX2 protein is present in PV cells not only in V1, but also across various brain regions, including prefrontal, auditory, and somatosensory cortices, as well as the basolateral amygdala (Spatazza et al., 2013). Consequently, one cannot preclude that OTX2 may regulate other types of CPs.

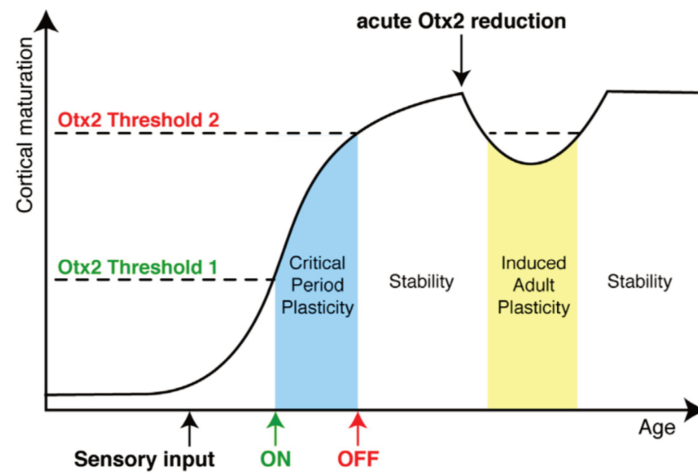


Figure 15. Two-threshold model of OTX2 plasticity control

*OTX2* accumulation in PV cells leads to cortical maturation. At a first *OTX2* concentration threshold, plasticity is opened. Reaching a second threshold closes plasticity and the continuous accumulation of *OTX2* maintains an adult non-plastic state. A reduction of *OTX2* in the adult leads to a transient induction of plasticity (From Prochiantz and Di Nardo, 2015).

## IV. EPIGENETICS MODIFICATION AND PLASTICITY

### A. Epigenetics: the study of chromatin structure

The term “epigenetics” was coined by Waddington in 1942 (Tronick and Hunter, 2016). Epigenetics is the study of heritable changes in gene expression that are not mediated at the level of DNA sequence. In eukaryotic nuclei, DNA is wrapped around an octameric histone core comprising of two copies of each of the canonical histones H2A, H2B, H3, and H4. This basic structure, known as the nucleosome, is repeated along the double-stranded DNA, with a fifth type of histone, the linker histone H1, bridging together consecutive nucleosomes.

Seminal work by Emil Heitz showed that the chromatin of eukaryotic cells can be broadly distinguished into two forms: heterochromatin and euchromatin (Sexton and Cavalli, 2015). Heterochromatin regions are more compact in chromatin conformation, as defined by nuclease accessibility (Weintraub and Groudine, 1976); they contain few actively expressed genes and replicate late in S-phase (Holmquist, 1987). In contrast, euchromatin is more open and accessible to nucleases, is rich in actively transcribed genes, and replicates early during S-phase. Euchromatin regions are highly enriched for acetylated histones whereas heterochromatin contains histones that are predominantly hypoacetylated (Figure 16). Molecular mechanisms that mediate epigenetic regulation include DNA methylation and chromatin/histone modifications.



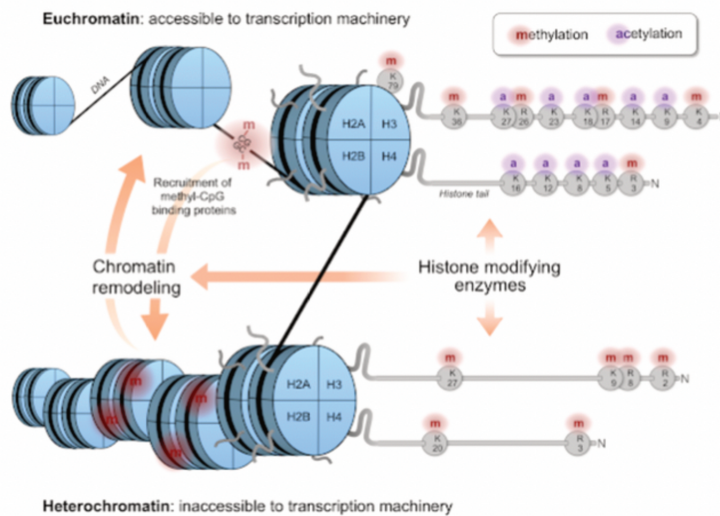


Figure 16. Gene function is primarily regulated by chromatin accessibility

Shown here is a summary of acetyl (purple) and methyl (red) modifications of specific arginine (R) and lysine (K) residues on N-terminal tails of histones, as well as DNA methylation at active and silent regions of chromatin.

### DNA methylation

One level of chromatin modification is the DNA methylation at cytosine residues, which involves a chemical modification of DNA whereby cytosine is converted into 5-methylcytosine. Cytosine methylation in mammals occurs primarily in CpG dinucleotides in most cell types (Cooper, Taggart, and Bird, 1983; Bird, 1986). Cytosine methylation at CpG dinucleotides in promoters is correlated with transcriptional repression, while cytosine methylation in gene bodies appears to be correlated with an active transcriptional state in many cells (Watt and Molloy, 1988; Ben-Hattar and Jiricny, 1988; Iguchi-Arigo and Schaffner, 1989; Ball et al., 2009; Lister et al., 2009).

A notable exception to this trend occurs within neurons, where cytosine methylation within gene bodies is correlated in a negative way with transcription (Lister et al., 2013; Guo et al., 2011). The process of methylation is dependent on the presence of methyl donors and methyltransferases (DNMT) which mediate either maintenance (i.e. DNMT1) or de novo DNA methylation (i.e. DNMT3) (Fagiolini, Jensen, and Champagne, 2009).

Within the context of mature neurons, methylated CpG dinucleotides were thought to be very stable, however, they are now known to be dynamically regulated (Kaas et al., 2013; Roth et al., 2015). This mechanism is very important in synaptic plasticity and memory formation (Ma et al., 2009; Martinowich, 2003). Indeed, it was shown that inhibition of DNMT activity alters DNA methylation in the adult brain and changes the methylation landscape surrounding plasticity genes such as *Bdnf* (Levenson et al., 2006). Furthermore, it was shown that inhibition of DNA methylation abolished memory in rats (Miller et al., 2010) and that the deletion of *Dnmt1* and *Dnmt3a* in post-mitotic neurons induces deficits in learning and memory in mice (Feng et al., 2010). The deletion of both *Dnmt* genes is necessary to induce this phenotype, suggesting that these enzymes have partially-redundant functions in neurons. Thus, DNA methylation must be considered of fundamental importance in mature neurons (Feng et al., 2010).

Like methylation, demethylation also plays a crucial role in chromatin regulation. Demethylation consists in the removal of a methyl group from a cytosine residue. For years, it had been assumed to be a passive process, via loss of methylation marks during cell division, but recently it has been shown to occur also independently of cell division (Oswald et al., 2000). Demethylation of DNA can occur via DNA repair pathways mediated by Growth Arrest and DNA Damage-inducible (GADD) 45 proteins (GADD45  $\alpha$  and GADD45  $\beta$ ), which, in neurons, take part in active DNA demethylation processes implicated in gene activation (Barreto et al., 2007; Ma et al., 2009). The GADD45 proteins are thought to be master coordinators of this process by recruiting deaminases and glycosylases to promoter regions (Rai et al., 2008). GADD45 $\beta$ -mediated activity-dependent demethylation was first shown for the promoter of *Bdnf* (Gavin et al., 2012) and deletion of the gene results in hippocampus-dependent long-term memory deficits (Leach et al., 2012). Moreover, a reduction in expression levels of the immediate early gene *Arc* was also associated with reduced levels of GADD45 $\beta$  and DNA demethylation (Grassi et al., 2017). Interestingly, the *Gadd45 $\beta$*  gene has an altered expression in the mouse visual cortex after MD (Majdan and Shatz, 2006; Tognini et al., 2015).

A second pathway for DNA demethylation employs TET (ten eleven translocation) enzymes. These enzymes carry out sequential reactions by which 5mC can be oxidized to form 5-hydroxy methylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) (Ito et al., 2011; Tahiliani et al., 2009). The 5hmC mark is more enriched in the brain when compared to

other tissues (Kriaucionis and Heintz, 2009; Szulwach et al., 2011) and TET1 is activated by neuronal activity (Kaas et al., 2013). Within the brain, 5hmC is acquired in a developmental dependent manner, occurs exclusively in the CpG rich environment, and is increased in the gene bodies of highly expressed genes (Szulwach et al., 2011; Mellen et al., 2012). This suggests a role for 5hmC in active transcription within neurons. It was shown that a subset of genes associated with learning and memory were positively regulated by TET1 expression (Kaas et al., 2013). In addition, activity related genes, such as *Npas4*, *Fos*, and *Arc*, are downregulated and hypermethylated in *TET1* null mice (Rudenko et al., 2013).

#### *Methyl binding protein: MeCP2*

Cytosine methylation allows for the recruitment of several proteins, including MeCP2 commonly known as a repressor of transcription once bound to 5mC (Martinowich, 2003; Ma et al., 2009). Indeed, *in vitro*, MeCP2 was found to repress the transcription of reporter genes through its association with Sin3a, a transcriptional repressor, and histone deacetylases (HDACs), which facilitate chromatin compaction (Nagy et al., 1997; Hassig et al., 1997; Laherty et al., 1997). Transcriptional repression associated with MeCP2 is relieved by treatment with the HDAC inhibitor trichostatin A, suggesting that MeCP2-mediated HDAC recruitment to methylated DNA is a critical step in MeCP2-mediated repression (Nan et al., 1998).

MeCP2 is ubiquitously expressed but is highly abundant in the brain. Within the central nervous system, MeCP2 is expressed at low levels during neurogenesis but its expression gradually increases during neuronal maturation and synaptogenesis, reaching a maximum in mature neurons. This suggested a potential role for MeCP2 in the maintenance of neuronal maturation, activity and plasticity (Samaco, 2004; Jung et al., 2003; Kishi et al., 2005). The importance of MeCP2 for the proper functioning of neurons and glia is also highlighted by neurological disorders associated with altered expression or loss of function of MeCP2 (Fasolino and Zhou, 2017; Lombardi, Baker, and Zoghbi, 2015; Lyst and Bird, 2015). Loss of function mutations in *MeCP2* are the cause of a neurological disorder called Rett syndrome (RTT) (Amir et al., 1999). As *MeCP2* is an X-linked gene, RTT primarily affects females. Indeed, males with loss of function mutations in *MeCP2* typically die in utero or perinatally (Schüle et al., 2008). Following a period of seemingly normal development during the first 6–18 months of life, RTT patients undergo a period of developmental stagnation followed by rapid, progressive motor

deterioration and neurological regression. Patients also display characteristic hand wringing and autism spectrum-like behavior (Pohodich and Zoghbi, 2015). Interestingly, RTT-associated mutations tend to cluster in the methyl binding domain and transcriptional repressor domains of MeCP2, suggesting that transcriptional repression is inhibited in RTT patients (Lyst and Bird, 2015).

MeCP2 is implicated in the regulation of neuronal chromatin architecture and gene transcription in response to neuronal activity and experience during postnatal life (Zhou et al., 2006). The use of conditional *MeCP2* deletion mice has highlighted the importance of this protein in regulating the CPs in primary sensory systems (He et al., 2014). Interestingly, MeCP2 expression increases in the glutamatergic neurons during this CP and visual deprivation attenuates the vision-associated increase of *MeCP2* expression in the thalamus (Yagasaki, Miyoshi, and Miyata, 2018). Also, mice lacking *MeCP2* expression exhibit deficits in experience-dependent refinement of synapses after P20 leading to an immature state of synaptic connections (Noutel et al., 2011). Furthermore, mice with *MeCP2* deletion show an accelerated maturation of PV neurons with an increase in GABA synthesis enzymes, an increase in PNNs condensation and enhanced GABA transmission (Krishnan et al., 2015; Sigal et al., 2019; Patrizi et al., 2019). These changes correlate with a premature CP onset and closure, suggesting a tight relationship between DNA methylation and molecular mechanisms of CP in the cerebral cortex.

### *Histone modifications*

Epigenetic control of gene expression is also mediated through multiple post-translational modifications of histone proteins at their N-terminal tails, including methylation, acetylation and ubiquitination. Those histone modifications can alter the accessibility to DNA and the density of chromatin structure. Indeed, histone acetylation is associated with increased transcriptional activity whereas histone deacetylation is associated with transcriptional repression. The acetylation state of these nucleosomal proteins is controlled by histone acetyltransferases (HATs), by HDACs, which are recruited by methyl-binding proteins, and by HDAC inhibitors, which effectively increase gene expression through shifting histones to an acetylated state.

Histone acetylation and chromatin remodeling are required to facilitate synaptic plasticity, learning, and long-term memory formation. Indeed, genetic or pharmacological manipulations of histone acetylation and deacetylation result in a large spectrum of learning and memory impairments. Specifically, deletion of *Hdac2* embryonically results in enhanced long-term potentiation and fear conditioning, while overexpression impairs fear conditioning and spatial learning (Guan et al., 2009). Deletion of *Hdac2* postnatally in glutamatergic neurons similarly replicated these findings (Morris et al., 2013). However, mice with a deletion of *Hdac1* have no overt phenotypes suggesting that HDAC2 is sufficient to ensure, at this degree of analysis, normal memory and learning-specific capacity (Guan et al., 2009). The exposure of dark-reared mice to light triggers the acetylation of histones and is more pronounced in mice during CP than in adulthood (Jiang et al., 2010; Kirkwood, Lee, and Bear, 1995; Putignano et al., 2007). Manipulation of histone acetylation by treatment of adult mice with HDAC inhibitors promotes adult plasticity in V1 and is able to rescue visual acuity deficits elicited during early life (Putignano et al., 2007; Silingardi et al., 2010; Yang, Lin, and Hensch, 2012; Gervain et al., 2013). The positive effects of HDAC inhibition on cortical plasticity are not confined to V1 and can be translated into an increased auditory perception in adult mice and in humans (Yang, Lin, and Hensch, 2012; Gervain et al., 2013). Together, these findings suggest that several HDACs act as crucial regulators of learning and memory.

Among other histone modifications, methylation has been implicated in heterochromatin formation and the regulation of promoter activity. The histone residues on which methylation occurs, include the following lysine and arginine residues: H3 (K4, 9, 27, 36, and 79), H4K20, H3 (R2, 8, 17, and 26), and H4R3. Methylation of histone H3 lysine 9 (H3K9me) is associated with both heterochromatin formation and gene silencing in euchromatin (Kouzarides, 2007). Methylation of H3K9 is performed by several methyltransferases, including SUV39H1 and SETDB1 (Schultz et al., 2002). H3K9 is primarily found under di- or tri-methylated forms and, in mammalian genomes, the presence of these forms often coincides with DNA methylation.

## B. Homeoproteins and epigenetic regulation

In the laboratory, it was previously demonstrated that *Engrailed1* heterozygous mice are very sensitive to oxidative stress and display loss of dopaminergic neurons. As *Engrailed1* heterozygous mice display motor and non-motor features of Parkinson disease, they have been

accepted as model for the disease. Furthermore, injection of the HP ENGRAILED protects the dopaminergic neurons from death. One mode of the protective activity of the HP ENGRAILED against oxidative stress is to maintain the perinucleolar and perinuclear heterochromatin (Rekaik et al., 2015). As a result, in the *Engrailed* heterozygote, relaxation of the heterochromatin leads to illegitimate transcription, including that of transposable elements (TE), in particular from the Long Interspersed Nuclear Elements (LINE) family. The ability of ENGRAILED to down-regulate *Line* transcription seems to involve both heterochromatin maintenance and direct repression of *Line* transcription through promoter binding. Given the similarities between OTX2 and ENGRAILED in their ability to protect midbrain dopaminergic neurons (Rekaik et al., 2015), this suggests that OTX2 may regulate chromatin structure and the expression of TEs not only in a cell-autonomous way, as shown for ENGRAILED, but also in a non-cell autonomous one. This novel aspect of HP physiology may open a new angle to better understand the link between epigenetic regulation and cortical plasticity. While HPs have been described as having a role in chromatin remodeling (Hannon et al., 2017; Rekaik et al., 2015), with an action that depends on their concentration (Hannon et al., 2017), their possible epigenetic role in V1 cortex plasticity has not been studied.

### C. LINE-1 in the neural system and plasticity

Transposable elements, discovered by Barbara McClintock in the 1940s, are responsible for mutagenesis by insertion and for transcriptional control of nearby genes (McClintock, 1950). Transposable elements are classified by whether they act via a DNA (DNA transposons) or an RNA (retrotransposons) intermediate (Figure 17). They are further distinguished by whether they encode proteins to mediate their own mobility (autonomous elements) or rely upon proteins encoded by other elements (non- autonomous elements) (Beck et al., 2011).

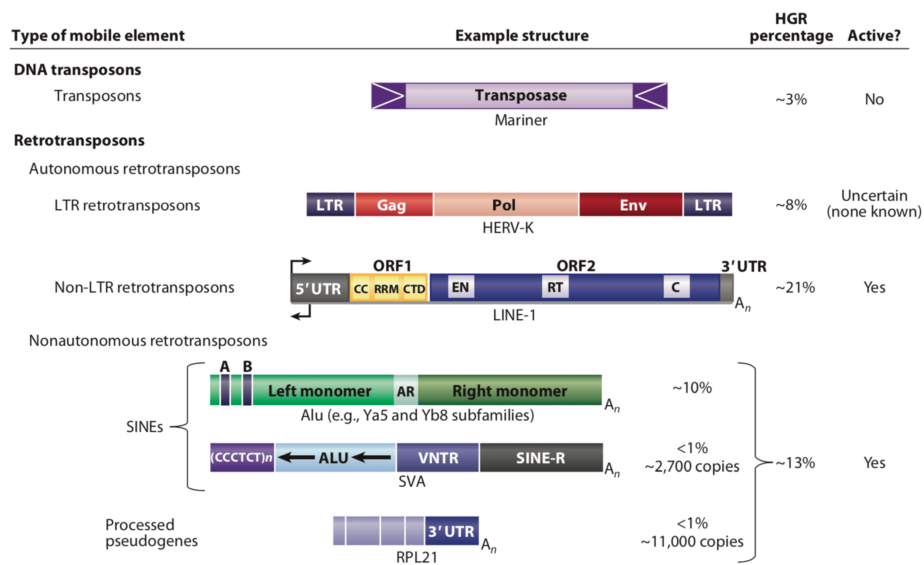


Figure 17. Classes of mobile elements

The classes of mobile genetic elements in the human genome, showing the type of mobile elements, the structure of representative elements, the percentage of each element in the human genome reference sequence (HGR), and whether each class of elements is currently active (From Beck et al., 2011).

With genomic drift during evolution, most TEs have lost, as a result of mutations, their ability to move. In the context of this thesis, I will primarily discuss the Long Interspersed Nuclear Element-1 (LINE-1) family. Many of its members are "fossilized", but some still remain active, and participate in genome modifications. It is estimated that in humans approximately 80 to 100 LINE-1 are active and competent for retrotransposition. Long interspersed nuclear element-1 is a repetitive RNA retrotransposon family that constitutes as much as 20% of the mammalian genome (Beck et al., 2011). They can be classified structurally into 5 sub-families (A, Tf, Gf, Gf, F, and V), which differ from each other in the number and nature of repetitions within the 5' UTR (Sookdeo et al., 2013). The LINE-1 sub-families A, Tf, and Gf are considered to be the only ones capable of retrotransposition. Long interspersed nuclear element-1 contains 2 open reading frames, ORF1 and ORF2. ORF1 encodes a 40-kDa protein with RNA-binding and nucleic acid chaperone activity (Ostertag and Kazazian Jr, 2001), while ORF2 encodes a 150-kDa protein with endonuclease and reverse transcriptase activity.

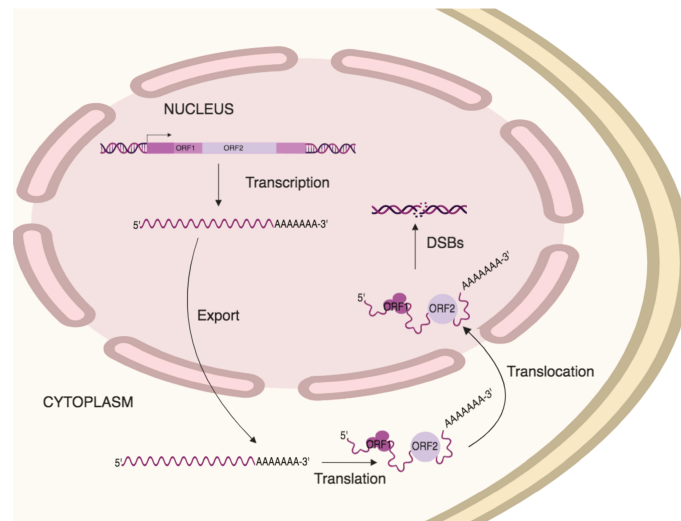


Figure 18. Retrotransposition cycle of LINE-1

The life cycle of LINE-1 begins with polymerase II transcription and mRNA export into the cytoplasm, where *Orf1p* and *Orf2p* are translated. The ORF proteins and the LINE-1 RNA assemble to form ribonucleoprotein (RNP) complexes. To reinsert back into the genome, the RNPs must introduce a break in genomic DNA; however, it is not clear if the RNPs reenter the nucleus via a transport mechanism or if nuclear envelope breakdown is required.

Because reverse transcription does not often go all the way to the 5' end of the RNA, most inserted LINE-1 reverse transcripts are mutated or truncated in 5' (Gilbert et al., 2005). These newly inserted sequences, whether complete (active) or not, induce genomic changes. Long interspersed nuclear element-1 can insert in exons, leading to gene mutations with potential functional consequences (Kazazian et al., 1988). Similarly, insertion into introns can induce splicing errors and exon jumps (Belancio, Roy-Engel, and Deininger, 2008). Finally, the endonuclease activity of ORF2p can cause breaks in DNA (Beck et al., 2011), suggesting a deleterious role for LINE-1 independent of their insertion (Gasior et al., 2006; Lin et al., 2009).

Long considered specific to germ cells, expression and retrotransposition of LINE-1 have since been observed in the brain (Muotri et al., 2005). In a study on human post-mortem tissues it was found that the level of retrotransposition is more important in the hippocampus than in the liver or heart (Coufal et al., 2009). The question of whether retrotransposition is of biological, physiological or pathological significance, remains open. Some studies suggest that it can contribute to somatic mosaicism in the brain (Baillie et al., 2011). Long interspersed nuclear element-1 appears to be involved in neurological diseases such as RTT (Muotri et al., 2005), Ataxia telangiectasia (Coufal et al., 2011) or Schizophrenia (Bundo et al., 2014).



Several mechanisms have evolved to counteract the harmful effects of LINE-1. Strong methylation of the CpG-rich 5' UTR region of *Line-1* inhibits expression of LINE-1 in a variety of cells (Yoder et al., 1997). Another level of repression involves heterochromatin (Garcia-Perez et al., 2010). There are two types of heterochromatin that contain specific methylations at the level of histones H3, either H3k9me3 for chromocenters, or H3k27me3 for perinuclear and perinuclear heterochromatin (Bártová et al., 2008). Repetitive elements are often found in heterochromatin labeled with H3k9me3 (Pauler et al., 2008). Furthermore, it was shown, that *Line-1* neuronal transcription and retrotransposition are increased in the absence of MeCP2 (Coufal et al., 2011; Muotri et al., 2010). In neural stem cells, MeCP2 forms a repressive complex with HDAC1 to inhibit *Line-1* expression (Coufal et al., 2009). Indeed, *MeCP2* knockout mice express an increased amount of LINE-1 elements when compared to littermates (Coufal et al., 2009).

Another mechanism involves P-element induced wimpy testes (PIWI) proteins that belong to the family of argonaut proteins, capable of associating with small interfering RNAs. These RNAs, which bind to PIWI, are 24-31 nucleotide-long and are called RNAs interacting with PIWI (RNA<sub>pi</sub> or piRNA) (Siomi et al., 2011). In the *Drosophila*, the piRNAs, initially generated from transposons, bind to PIWI and serve as a guide for cleaving the transcripts of active transposons in germ cells (Malone et al., 2009). This mechanism provides a robust system to fight transposons and seems to be present in all mammals. In mice, two murine PIWI proteins (MIWI2) and MIWI-like (MILI) are involved in the defense against transposons in germ cells. MIWI2, guided by the piRNA, appears to be involved in the methylation of retrotransposon sequences (Aravin et al., 2008; Carmell et al., 2007).

Taken together, these data challenge the initial view that *Line-1* are useless selfish genes constituting junk DNA. In marked contrast, a growing body of evidence suggests that LINEs may play a role in the physiology and the pathology of several organs and particularly of the brain. This new avenue of research promises to be very stimulating, and much work remains in order to achieve full understanding of the role of retrotransposon transcription or retrotransposition in post-mitotic neurons.

## V. Objectives of the thesis

OTX2 is an essential protein in the establishment of plasticity windows in the postnatal developing cerebral cortex. Intercellular transfer, from an extracortical source to PV cells, plays a key role in determining its activity once internalized at the source. It is likely that OTX2, once internalized, regulates gene transcription without precluding translation regulation and epigenetic control. Yet very little is known about the mechanisms underlying the non-cell autonomous activity of OTX2 once internalized.

The first objective of my thesis was to identify the targets of OTX2 in PV cells for the control of the ocular dominance CP within V1. To answer this question, we sought direct transcription targets of OTX2 through *in vivo* and biochemical approaches, and we discovered that OTX2 controls the *Gadd45 $\beta$ / $\gamma$*  promoter. Epigenetic and functional analyses were then used to evaluate how *Gadd45 $\beta$*  expression impacts CP and adult plasticity.

A second objective was to explore whether OTX2 could control the maturation of PV cells through epigenetic modifications. To answer this question, I focused my attention on the study of different epigenetic marks and I found that some of them are modified by OTX2 during post-natal development and in the re-opening of adult plasticity. Furthermore, some of these epigenetic changes are associated with mobile elements expression, raising the interesting possibility that CPs of plasticity might be periods of genomic instability. I thus evaluated TE expression in V1 during juvenile development and adult plasticity and found an increase in ORF1p expression during plasticity periods. This opens the possibility, not yet fully explored, of an interaction between OTX2 and LINE expression active in the regulation of cerebral cortex plasticity.

My thesis will be presented in two main articles:

**Article 1: Non-cell Autonomous OTX2 Homeoprotein Regulates Visual Cortex Plasticity Through GADD45 $\beta$ / $\gamma$**

**Article 2: Non-cell autonomous OTX2 homeoprotein controls the epigenetic state of parvalbumin cells in visual cortex plasticity periods**



# RESULTS



## Article 1: Non-cell Autonomous OTX2 Homeoprotein Regulates Visual Cortex Plasticity Through *GADD45 $\beta/\gamma$* .

OTX2 HP controls V1 plasticity through its non-cell autonomous activity which leads to its preferential accumulation in PV cells where it regulates their maturation. However, the molecular mechanisms (signaling, transcription, translation, etc.) that allow for OTX2 to promote plasticity during juvenile development while suppressing it in adulthood have not been fully identified. The objective of this study is to identify the direct transcriptional targets of OTX2 in PV cells as well as the possible epigenetic modifications induced by OTX2.



A list of candidate genes was obtained by bulk RNA sequencing from micro-dissected layer IV of the V1 of heterozygous *Otx2<sup>+/-GFP</sup>* and wild type control littermates, at both plastic and non-plastic stages. The use of cycloheximide, a translation inhibitor, allowed for the separation of indirect from direct OTX2 transcriptional targets.

Among the 22 candidate genes in layer IV of the V1, OTX2 directly controls the transcription of *Gadd45 $\beta/\gamma$*  by physically binding to the *Gadd45 $\beta/\gamma$*  promoter. Accordingly, OTX2 enhances *Gadd45 $\beta$*  expression during postnatal development but represses its expression in the adult. The physical interaction between OTX2 and the promoter of *GADD45 $\beta/\gamma$*  was validated by gel shift and immunoprecipitation of OTX2-bound chromatin. Viral overexpression of *GADD45 $\beta$*  in V1 of adult mice is sufficient to increase the expression of plasticity genes and to reopen a window of ocular dominance plasticity. Finally, *GADD45 $\beta$*  is able to modify the epigenetic status of PV cells, in particular by varying the methylations of certain CpG islets of *Egr2*, *Fos* and *Gadd45 $\beta$*  promoter regions.




## ORIGINAL ARTICLE

# Non-cell Autonomous OTX2 Homeoprotein Regulates Visual Cortex Plasticity Through *Gadd45b/g*

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## Abstract

The non-cell autonomous transfer of OTX2 homeoprotein transcription factor into juvenile mouse cerebral cortex regulates parvalbumin interneuron maturation and critical period timing. By analyzing gene expression in primary visual cortex of wild-type and *Otx2*<sup>+/GFP</sup> mice at plastic and nonplastic ages, we identified several putative genes implicated in *Otx2*-dependent visual cortex plasticity for ocular dominance. Cortical OTX2 infusion in juvenile mice induced *Gadd45b/g* expression through direct regulation of transcription. Intriguingly, a reverse effect was found in the adult, where reducing cortical OTX2 resulted in *Gadd45b/g* upregulation. Viral expression of *Gadd45b* in adult visual cortex directly induced ocular dominance plasticity with concomitant changes in MeCP2 foci within parvalbumin interneurons and in methylation states of several plasticity gene promoters, suggesting epigenetic regulation. This interaction provides a molecular mechanism for OTX2 to trigger critical period plasticity yet suppress adult plasticity.

**Key words:** critical period, epigenetics, ocular dominance, optical imaging, parvalbumin interneuron

## Introduction

Experience-dependent plasticity shapes neural circuits in postnatal life during defined transient windows of heightened plasticity, or critical periods (CP), which generally begin in primary sensory areas and move up to more integrated brain regions, ending with plasticity in cortices involved with higher cognition. The developmental importance of such plasticity is exemplified by the loss of visual acuity following monocular deprivation (MD) during the CP for ocular dominance (OD). Imbalanced visual input reduces visual acuity of the “lazy eye” during infancy and leads to amblyopia, which afflicts 2–5% of the human population (Holmes and Clarke 2006). After CP closure, intrinsic potential for plasticity is

actively dampened resulting in the stabilization of brain circuits (Takesian and Hensch 2013).

CP are influenced by the non-cell autonomous transfer of OTX2 homeoprotein transcription factor, which controls the maturation of GABAergic fast-spiking parvalbumin interneurons (PV cells) and is required in adult mice for the maintenance of a nonplastic state (Sugiyama et al. 2008; Beurdeley et al. 2012; Spatzza et al. 2013; Bernard et al. 2016). The OTX2 homeoprotein possesses conserved sequence features within the homeodomain that permit translocation between cells (Joliot et al. 1991). In the brain, OTX2 is secreted by the choroid plexus, transported to the cortex, and then internalized by PV cells enwrapped by extracellular perineuronal



nets (PNNs) (Beurdeley et al. 2012; Spatazza et al. 2013; Kim et al. 2014). Therefore, non-cell autonomous OTX2 has access to the entire cortex and can indeed control the timing of multiple CPs (Lee et al. 2017). Importantly, OTX2 begins to accumulate prior to CP onset and continues to accumulate after CP closure. Thus, OTX2-regulated plasticity can be explained by a 2-threshold model for increased OTX2 accumulation in PV cells: a first concentration threshold triggers CP onset while crossing a second threshold induces and maintains CP closure (Prochiantz and Di Nardo 2015). Furthermore, OTX2 is concentrated by the PNNs that specifically enwrap PV cells, thus, permitting a constant accumulation of OTX2 in certain PV cells throughout the cortex (Bernard and Prochiantz 2016). However, the molecular mechanisms that allow OTX2 to promote cortex plasticity in juvenile mice yet suppress plasticity in adult mice have not been elucidated.

Epigenetic changes are also implicated in cortical plasticity, as CP is regulated by chromatin reorganization through DNA methylation and histone modifications (Putignano et al. 2007; Krishnan et al. 2015, 2017; Lennartsson et al. 2015; Nott et al. 2015; Tognini et al. 2015). DNA methylation can repress transcription by blocking DNA binding of transcription factors or by recruiting repressor complexes containing histone deacetylases to condense chromatin structures (Fagiolini et al. 2009). While several studies have identified differential gene expression implicated in visual cortex plasticity (Majdan and Shatz 2006; Rietman et al. 2012; Tiraboschi et al. 2013; Benoit et al. 2015), we sought targets of OTX2 in plastic and nonplastic cortex. Here, we identify *Gadd45b/g* as direct targets of OTX2 in visual cortex. *Gadd45*-dependent DNA demethylation has been implicated in neurogenesis, long-term memory, and genomic stability (Hollander et al. 1999; Rai et al. 2008; Leach et al. 2012; Niehrs and Schäfer 2012; Sultan et al. 2012). We provide molecular evidence that OTX2 regulates *Gadd45b* expression in either direction, which permits OTX2 to stimulate CP plasticity yet dampen adult plasticity. Increasing expression of *Gadd45b* in adult mouse visual cortex modifies the epigenetic state of PV cells and induces OD plasticity.

## Materials and Methods

### Ethics Statement

All animal procedures were carried out in accordance with the guidelines of the European Economic Community (2010/63/UE) and the French National Committee (2013/118). For surgical procedures, animals were anesthetized with Xylazine (Rompun 2%, 5 mg/kg) and Ketamine (Imalgene 1000, 80 mg/kg) by intraperitoneal injection. This project (no. 00704.02) obtained approval from Ethics committee no. 59 of the French Ministry for Research and Higher Education.

### Animals

Conventionally raised C57Bl/6J mice were purchased from Janvier Laboratories. *Otx2<sup>+/GFP</sup>* knock-in mice were kindly provided by Drs D. Acampora and A. Simeone (IGB, Naples, Italy). The scFv*Otx2<sup>tg/o</sup>* and scFv*Pax6<sup>tg/o</sup>* mouse lines were generated through a knock-in approach, as described previously (Bernard et al. 2016).

### Brain Injections and Infusions

Stereotaxic injections into visual cortex ( $\lambda$ :  $x = 1.7$  mm,  $y = 0$  mm,  $z = 0.5$  mm) of 300 ng of OTX2 protein (Beurdeley et al. 2012) or vehicle (0.9% NaCl), with or without 0.1  $\mu$ g/ $\mu$ L cycloheximide (Sigma C4859) in 2  $\mu$ L were performed with Hamilton syringe

at 0.2  $\mu$ L/min. For conditional choroid plexus expression in scFv mouse lines, intracerebroventricular stereotaxic injections (bregma:  $x = -0.58$  mm,  $y = \pm 1.28$  mm,  $z = 2$  mm) of 40  $\mu$ g of Cre-TAT protein (Spatazza et al. 2013) in 15% DMSO (Sigma D2650), 1.8% NaCl in 2  $\mu$ L were performed with a Hamilton syringe at 0.2  $\mu$ L/min. Two weeks after injection, mice were processed for layer IV dissection or in situ hybridization. For adeno-associated virus (AAV) infection, 2  $\mu$ L ( $3.4 \times 10^{11}$  GC/mL) of either AAV8-Syn-mCherry-2A-mGadd45b (Vector BioLabs) or AAV8-Syn-mCherry (Vector BioLabs) were injected in visual cortex as described above. Two weeks after injection, mice were processed for layer IV dissection, histology or MD.

Stereotaxic infusions into visual cortex (above coordinates) with 3-day osmotic mini pump (Alzet 1003D, Charles River Laboratories) were used to deliver 100  $\mu$ L of either cycloheximide (0.1  $\mu$ g/ $\mu$ L) and/or actinomycin D (0.2  $\mu$ g/ $\mu$ L, Sigma A9415) in 0.9% NaCl.

### Layer IV Dissection

Layer IV dissections were performed as previously described (Bernard et al. 2016). Briefly, visual cortex areas were excised with microscalpels in ice-cold PBS and cut into thin rectangular strips. The strips were laid on their sides to expose the cortical layers and were cut lengthwise in 2 equal parts twice to provide enriched layer IV.

### Analysis of Global Protein Synthesis

L-azidohomoalanine (L-AHA, 50  $\mu$ M, Invitrogen C10102) or vehicle (50% DMSO, 0.9% NaCl) with or without cycloheximide (0.1  $\mu$ g/ $\mu$ L) in 2  $\mu$ L were injected into visual cortex at P17. Mice were sacrificed 6 h after injection and layer IV was dissected for processing and western blot analysis. Biotinylation of L-AHA incorporated in nascent protein was performed by Click-iT Protein Reaction Buffer kit (Invitrogen C10276). Eluted biotinylated proteins were processed for western blot detection with horseradish peroxidase streptavidin (Vector Laboratories SA-5004) and total lane densities were quantified (ImageJ).

### cDNA Libraries and RNA Sequencing

For each genotype and age, samples were processed in duplicate, with each sample containing layer IV dissections pooled from 4 different mice (male and female). Library preparation and Illumina sequencing were performed at the Ecole normale supérieure Genomic Core Facility (Paris, France). Ribosomal RNA depletion was performed with the Ribo-Zero kit (Epicentre MRZH1124), using 2  $\mu$ g of total RNA. Libraries were prepared using the strand specific RNA-Seq library preparation ScriptSeq V2 kit (Epicentre SSV21124). Libraries were multiplexed by 4 on 2 flow cell lane(s). A 50 bp read sequencing was performed on a HiSeq 1500 device (Illumina). A mean of  $47 \pm 6$  million passing Illumina quality filter reads was obtained for each of the 8 samples.

### RNASeq Bioinformatics Analysis

The analyses were performed using the Eoulsan pipeline (Jourden et al. 2012), including read filtering, mapping, alignment filtering, read quantification, normalization and differential analysis. Before mapping, poly N read tails were trimmed, reads  $\leq 11$  bases were removed, and reads with mean quality  $\leq 12$  were discarded. Reads were then aligned against the *Mus musculus* genome (UCSC) using Bowtie (version 0.12.9) (Langmead et al. 2009). Alignments from reads matching more than once on the reference genome were

removed using Java version of SAMtools (Li et al. 2009). To compute gene expression, *Mus musculus* GFF3 genome annotation from UCSC database was used. All overlapping regions between alignments and referenced exons were counted using HTSeq-count 0.5.3 (Anders et al. 2015). DESeq 1.8.3 (Anders and Huber 2010) was used to normalize sample counts and to perform statistical treatments and differential analyses. The RNASeq gene expression analysis excel files and raw fastq data files are available on the GEO repository ([www.ncbi.nlm.nih.gov/geo/](http://www.ncbi.nlm.nih.gov/geo/)) under accession number GSE98258.

### Quantitative RT-PCR

Total RNA was extracted from dissected tissue by using the AllPrep DNA/RNA Mini Kit (Qiagen 80204). cDNA was synthesized from 200 ng of total RNA with QuantiTect Reverse Transcription kit (Qiagen 205313). Quantitative PCR reactions were carried out in triplicate with SYBR Green I Master Mix (Roche S-7563) on a LightCycler 480 system (Roche). Expression was calculated by using the  $2^{-\Delta\Delta Ct}$  method with *Gapdh* as a reference. All primers used are listed in Suppl. Figure S1C.

### In Situ Hybridization

In situ hybridization was performed as previously described (Di Nardo et al. 2007). Briefly, cryosections (20  $\mu$ m) were hybridized overnight at 70 °C with a digoxigenin (DIG)-labeled RNA probes (DIG RNA labeling kit, Roche 11277073910) for either *Gadd45b* or *Gadd45g* mRNA or *Gadd45b* pre-mRNA. After washing, sections were incubated with alkaline phosphatase-conjugated anti-DIG (1/2000; Roche 11633716001) overnight at 4 °C. DAB staining (Vector Laboratories SK-4100) was carried out according to manufacturer's instructions.

### Gel Shift Assay

OTX2 protein (in-house; 0.1  $\mu$ g) was incubated at room temperature for 30 min with 40 fmol of biotinylated DNA oligonucleotide (S1, S2, S3, S4, or S5 for *Gadd45b* and S1, S2, or S3 for *Gadd45g*) and 8 pmol of IRBP unbiotinylated probe (Chatelain et al. 2006) in the presence of 50 ng/ $\mu$ L dIdC in PBS. Complexes were separated on prerun 6% native polyacrylamide gels at 100 V in TBE. Samples were transferred onto a nylon membrane at 380 mA for 45 min, crosslinked with UV and detected using the LightShift Chemiluminescent EMSA Kit (ThermoFisher Scientific P120148), according to manufacturer's instructions. Membranes were imaged using a LAS-4000 gel imager (FUJIFILM).

### Chromatin Immunoprecipitation

Layer IV of primary visual cortices were pooled from 5 P100 WT mice and washed in PBS followed by wash buffer (20 mM HEPES pH 7.4; 150 mM NaCl; 125 mM Glycine, 1 mM PMSF). Nuclei were fixed in 1% formaldehyde (Sigma 252549) in PBS for 15 min at RT, isolated by dounce (pestle B) in lysis buffer (20 mM HEPES pH 7.4; 1 mM EDTA; 150 mM NaCl; 1% SDS; 125 mM Glycine; 1 mM PMSF), and then resuspended in wash buffer. Chromatin was purified by using modified Farnham protocol (<http://farnham.genomecenter.ucdavis.edu/protocols/tissues.html>). Chromatin immunoprecipitation (ChIP) were performed by using a mix of 3  $\mu$ g of anti-Otx2 (goat, R&D AF1979) and 3  $\mu$ g of anti-Otx2 (rabbit, abcam 21990). For control ChIP, 6  $\mu$ g of anti-IgG (rabbit, abcam ab27478) were used. Immunoprecipitated DNA was analyzed by qPCR. Primers corresponding to the S2 probes were used for *Gadd45b* and *Gadd45g*, while primers for actin targeted intron-exon junctions.

### Immunohistochemistry

Mice were perfused transcardially with PBS followed by 4% paraformaldehyde prepared in PBS. Brains were postfixed 1 h at 4 °C and immunohistochemistry was performed on cryosections (20  $\mu$ m) encompassing the entire visual cortex. Heat-induced antigen retrieval in 10 mM sodium citrate was performed only for anti-MeCP2 experiments prior to overnight primary antibody incubation at 4 °C. Primary antibodies included anti-MeCP2 (rabbit, 1/200, Millipore MABE328), anti-mCherry (mouse, 1/200, Clontech 632543), anti-parvalbumin (rabbit, 1/200, SWANT PV235), and biotinylated WFA (1/100, Sigma L1516). Secondary antibodies were Alexa Fluor-conjugated (Molecular Probes). Images were acquired with a Leica SP5 confocal microscope and analyzed with ImageJ.

### MD and Surgery for Optical Imaging

For MD, mice were anesthetized prior to suturing of the left eye as previously described (Gordon and Stryker 1996). Animals were checked daily to ensure sutures remained intact. The eye was opened immediately before recording.

For optical imaging, mice were anesthetized with urethane (1.2 g/kg, intraperitoneal) and sedated with chlorprothixene (8 mg/kg, intramuscular). Atropine (0.1 mg/kg) and dexamethasone (2 mg/kg) were injected subcutaneously with body temperature maintained at 37 °C.

### Visual Stimulation and Optical Imaging Recording

Visual cortical responses were recorded using imaging methods based on Fourier transform following periodic stimulation (Kalatsky and Stryker 2003; Cang et al. 2005). A high refresh rate monitor was placed 20 cm in front of stereotaxically restrained mouse. Stimulation consisted of a horizontal bar drifting downwards periodically at 1/8 Hz in the binocular visual field of the recorded hemisphere (from +5° ipsilateral to +15° contralateral). Each eye was stimulated 5 times alternately for 4 min. Intrinsic signals were recorded using a 1M60 CCD camera (Dalsa) with a 135  $\times$  50 mm<sup>2</sup> tandem lens (Nikon) configuration. After acquisition of the surface vascular pattern, the focus of the camera was lowered by 400  $\mu$ m deeper. Intrinsic signals were acquired with a 700 nm illumination wavelength and frames stored at a rate of 10 Hz, after a 2  $\times$  2 pixels spatial binning.

### Data Analysis

Functional maps for each eye were calculated offline. Prior to Fourier transform, slow varying components independent of the stimulation were subtracted by the generalized indicator function (Yokoo et al. 2001). Retinotopic organization and intensity were computed from the phase and magnitude components of the signal at the frequency of stimulation. For each eye, the 5 activity maps were averaged, filtered with a Gaussian kernel low-pass filter (3 pixels s.d.) and set with a cut-off threshold at 50% of the maximum response. The binocular zone was defined as the intersection between the response regions of each eye. For each session, an OD value at each pixel was defined as (C-I)/(C+I), calculated from the response amplitude from the contralateral (C) eye and the ipsilateral (I) eyes. The OD index was then computed for each session as the average of the OD values in the binocular zone. Consequently, OD index ranged from -1 to 1, with negative values representing an ipsilateral bias, and positive values a contralateral bias.

## Bisulfite Sequencing Analysis

Genomic DNA was processed for bisulfite conversion by using the EpiTect Bisulfite Kit (Qiagen 59104). Converted DNA was amplified with the HotStar Taq Plus Kit (Qiagen 203646) by primers designed with MethPrimer (Li and Dahiya 2002). Primers are listed in Suppl. Fig. S3E. PCR was run with 50 ng converted DNA and 20  $\mu$ M of each primer in 40  $\mu$ L total reaction volume: 14  $\times$  (30 s 94  $^{\circ}$ C; 30 s 62–55  $^{\circ}$ C; 60 s 72  $^{\circ}$ C), 36  $\times$  (30 s 94  $^{\circ}$ C; 30 s 55  $^{\circ}$ C; 60 s 72  $^{\circ}$ C), 1  $\times$  17 min 72  $^{\circ}$ C. Gel-purified products were ligated in the pCR2.1-TOPO vector by using the TOPO-TA cloning kit (Thermo Fischer K4600-01) and then transformed into DH5 $\alpha$  cells. Positive clones were sequenced and percent methylation was analyzed with QUMA (Kumaki et al. 2008).

## Statistical Analysis

Statistical analysis was performed with Prism 6 (GraphPad). Pairwise comparison was performed with Student t-test (with Sidak-Bonferroni correction), while multiple group analyses were done with ANOVA (one- or two-way) followed by Bonferroni correction. To assess the methylation frequency, two-way ANOVA with Fisher's LSD test was applied at individual CpG sites.

## Results

### Non-cell Autonomous Regulation of Gene Expression by OTX2 in the Visual Cortex

A better understanding of how OTX2 regulates plasticity in the visual cortex requires that its non-cell autonomous transcription targets be identified. We dissected layer IV of the visual cortex and used RNA-sequencing to analyze gene expression at postnatal day 30 (P30) and P100 in wild-type (WT) and *Otx2*<sup>+GFP</sup> heterozygous mice. Layer IV dissections provide lysates enriched in PV cells that drive CP plasticity and capture OTX2. These ages were chosen because CP plasticity is opened at P30 in WT but not in *Otx2*<sup>+GFP</sup> mice, given that *Otx2* genetic deletion delays CP opening (Sugiyama et al. 2008), and that the CP is closed at P100 in WT but not yet in *Otx2*<sup>+GFP</sup> mice. Thus, genes with similar expression at P30 in *Otx2*<sup>+GFP</sup> and at P100 in WT mice but with a different level of expression during the CP (P30 in WT or P100 *Otx2*<sup>+GFP</sup> mice) were considered as potential genes involved in plasticity. After applying cut-offs for significance (adjusted  $P < 0.05$ ) and fold change ( $>1.5$ ) in expression between P30 and P100 for both genotypes, we identified 20 candidate genes, all of them upregulated during CP (Fig. 1A,B). No significantly downregulated genes were identified. This small list contains several immediate early genes, including *Arc*, *Fos*, *Egr1/2/4*, and *Nr4a*, already implicated in cerebral cortex plasticity (Kaczmarek and Chaudhuri 1997; Andreasson and Kaufmann 2002; Li et al. 2005; Shepherd and Bear 2011; Vallès et al. 2011; Tognini et al. 2015; Bernard et al. 2016).

However, while this approach revealed potential “plasticity genes” within layer IV, it did not identify genes directly regulated by non-cell autonomous OTX2 in PV cells. In the mouse visual cortex, CP for binocular vision opens at P20 once internalized OTX2 has reached a first concentration threshold (Spatazza et al. 2013). We reasoned that direct OTX2 transcriptional targets are not dependent on protein synthesis (do not require an intermediate target) and thus that their transcripts might still be upregulated in the presence of cycloheximide (CHX), a blocking agent for protein translation in vivo (Sharma et al. 2012). The activity of CHX was validated by the decrease of the nascent protein measured by L-azidohomoalanine incorporation (Suppl. Fig. S1A, B).

Low-dose CHX, or vehicle, was infused in the visual cortex between P20 and P23. Upon CHX infusion, only *Gadd45b/g*, and *Pvr* were still significantly upregulated (Fig. 1C), suggesting that the transcription of all the other candidate genes between P20 and P23 was either not direct or required translation of a cofactor. Because CHX has been reported to stabilize some transcripts (Ooi et al. 1993), we verified whether the upregulation of these 3 genes is indeed transcription-dependent by coinfusion of CHX and actinomycin D, a transcription inhibitor. This cocktail eliminated *Pvr* (Fig. 1C), suggesting that *Gadd45b/g* are the best candidates for direct regulation by OTX2. To exclude OTX2-independent effects, CHX was infused between P20 and P23 in the visual cortex of WT and *Otx2*<sup>+GFP</sup> mice, which have low endogenous OTX2 levels. Indeed, *Gadd45b* was significantly upregulated only in WT mice (Fig. 1D).

We have previously shown that cortical injection or infusion of OTX2 protein result in its specific uptake by PV cells (Sugiyama et al. 2008; Beurdeley et al. 2012) and anticipates CP opening (Sugiyama et al. 2008). To confirm direct *Gadd45b/g* regulation by non-cell autonomous OTX2, we injected recombinant OTX2 protein at P17 with or without CHX. Six hours after injection, exogenous OTX2 increased the expression of *Gadd45b/g* and *Pvr*, but only that of *Gadd45b/g* in the presence of CHX (Fig. 1E). This finding further suggests that *Gadd45b/g* are direct OTX2 targets, given that the effect requires only OTX2 and is translation-independent. *Gadd45b/g* may therefore directly relay OTX2 activity to regulate plasticity in the visual cortex.

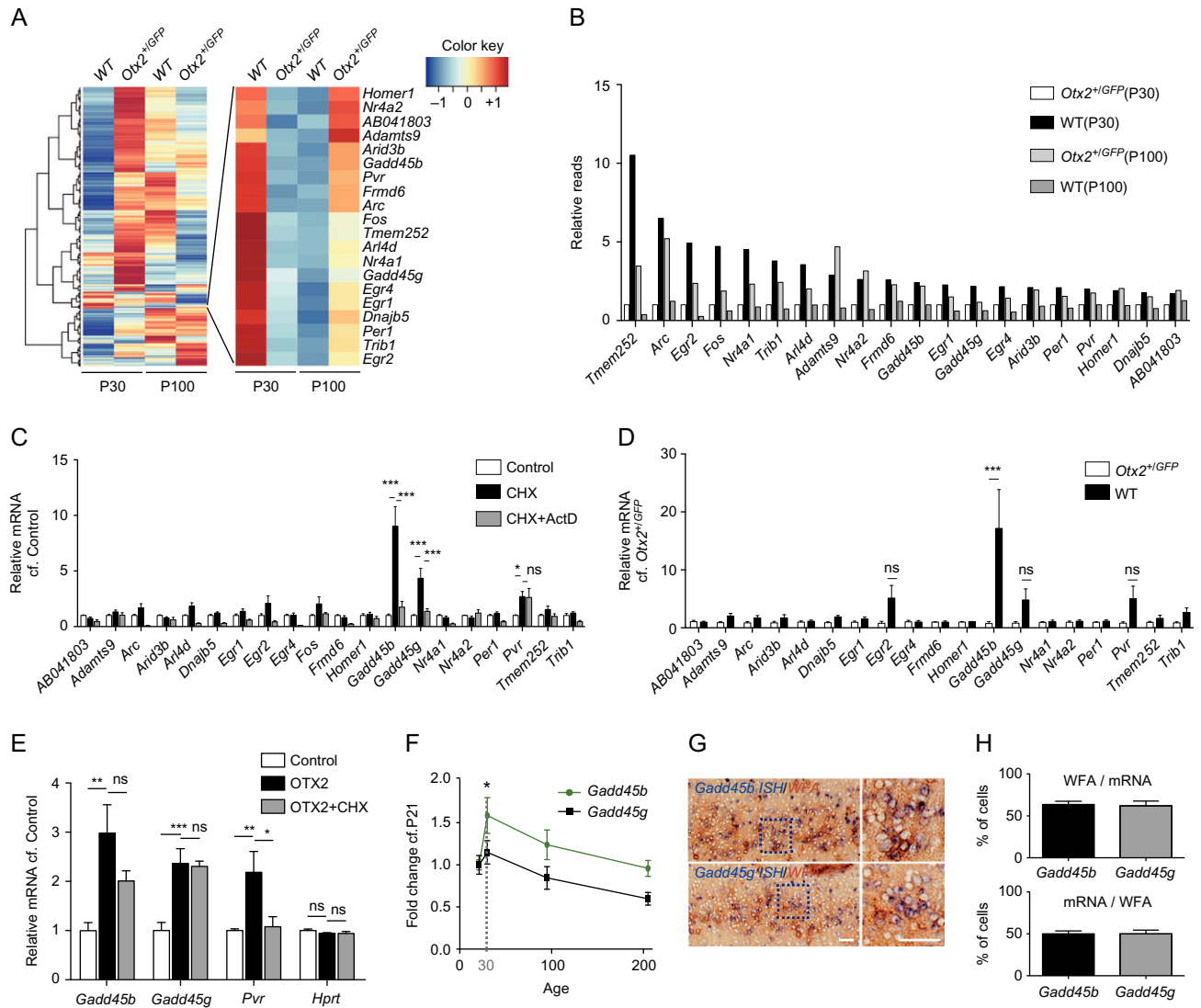
Before exploring this hypothesis further, we confirmed that *Gadd45b/g* cortical expression not only increases between P21 and P30, but also declines as plasticity is turned off (Fig. 1F) and that the 2 genes are expressed in the cells that internalize OTX2 (Fig. 1G). Extracortical OTX2 is primarily internalized by PV cells through their PNNs, which can be stained by the lectin *Wisteria Floribunda Agglutinin* (WFA). Both *Gadd45b/g* are expressed within WFA-stained neurons (Fig. 1G), even though the overlap is only 50% (Fig. 1H), demonstrating expression in other cell types.

### *Gadd45b/g* Genes Contains OTX2 Binding Sequences

Direct regulation of *Gadd45b/g* expression should involve binding of OTX2 to elements within their genes. Based on known *Otx2* consensus sequence (Samuel et al. 2014; Hoch et al. 2015), we identified 5 potential sites in *Gadd45b* and 3 in *Gadd45g* for which probes were synthesized (Fig. 2A and Suppl. Fig. S2A). Gel shift experiments identified at least 2 probes per gene as most promising (Fig. 2B and Suppl. Fig. S2B), and these probes were chased by IRBP (Fig. 2C and Suppl. Fig. S2C), which is known to interact with the DNA-binding site in OTX2 (Chatelain et al. 2006). We then performed ChIP-qPCR analysis with primers for the *Gadd45b* and *Gadd45g* S2 motifs and for actin negative control and detected enrichment for both *Gadd45b* and *Gadd45g* from lysates of P100 mouse V1 layer IV (Fig. 2D). The use of layer IV dissection avoids interference from OTX1, as ChIP antibodies are typically pan-OTX (Kim et al. 2014).

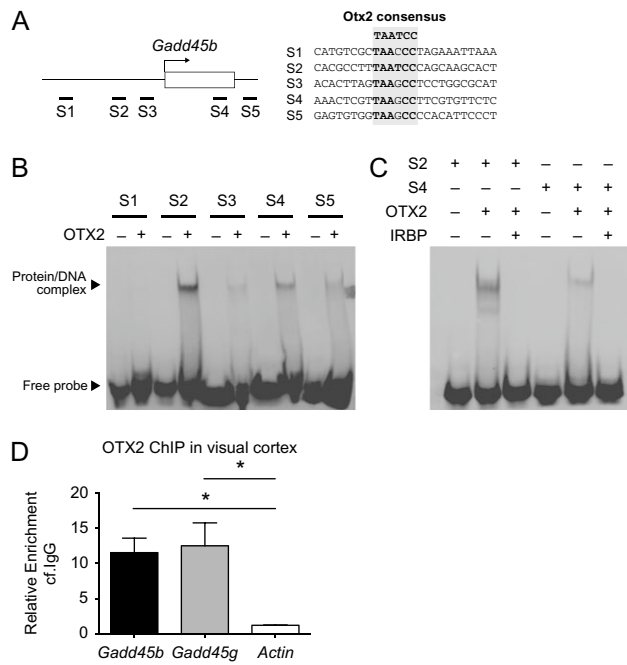
### *Gadd45b* Regulates the Expression of Plasticity Genes in the Mouse Visual Cortex

To further verify the correlation between OTX2 internalization and the expression of *Gadd45b/g*, we took advantage of 2 conditional mouse lines carrying anti-*Otx2* or anti-*Pax6* single chain antibodies (scFv): *scFvOtx2*<sup>tg/o</sup> and *scFvPax6*<sup>tg/o</sup>. The expression and secretion of these antibodies (*scFv-Otx2* and *scFv-Pax6*)



can be induced specifically in the adult choroid plexus by injecting a cell-permeable Cre bacterial recombinase Cre-TAT in the lateral ventricles (Bernard et al. 2016). Secreted scFv-Otx2 neutralizes OTX2 in the cerebrospinal fluid (CSF), reduces the amount of OTX2 internalized by PV cells, induces a strong expression of *Arc*, *Fos*, *Egr4*, and *Nr4a1* in supragranular layers of the visual cortex, and reopens a period of physiological plasticity in the adult (Bernard et al. 2016). We now show that induction of scFv-Otx2 but not scFv-Pax6 increased *Gadd45b* and *Gadd45g* expression in the adult visual cortex (Fig. 3A). This

increase of *Gadd45b* and *Gadd45g* is correlated with the increase of plasticity genes (Fig. 3B). Given its stronger correlation with plasticity gene expression, we privileged analysis of *Gadd45b* for our subsequent experiments. To visualize the increase of the *Gadd45b* expression, we performed in situ hybridization with *Gadd45b* intronic probe to recognize the newly synthesized precursor mRNA (pre-mRNA) (Fig. 3C). Interestingly, induction of scFv-Otx2 but not scFv-Pax6 leads to a significant increase in newly synthesized *Gadd45b* specifically in layer IV (Fig. 3D). These findings reinforce the correlation between *Gadd45b/g*



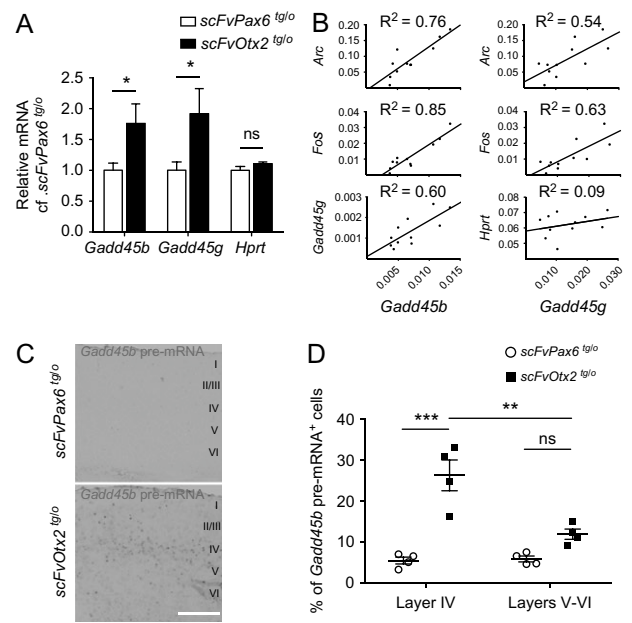
**Figure 2.** *Gadd45b* gene has a sequence recognized by OTX2. (A) Oligonucleotides (S1, S2, S3, S4, S5) mapping on *Gadd45b* gene (left) were chosen based on the Otx2 consensus motif (right). Identical bases are highlighted in grey. (B, C) Gel shift assays with biotinylated DNA probes (S1, S2, S3, S4, S5) in the presence (+) or absence (-) of OTX2 protein (B). Competition assay of the DNA/protein complex is obtained with IRBP unbiotinylated probe (C). (D) qRT-qPCR analysis of OTX2 chromatin-immunoprecipitation performed on chromatin extracted from layer IV of V1 lysates with endogenous OTX2. Error bars,  $\pm$ SEM; \* $P$  < 0.05 by one-way ANOVA with Bonferroni post hoc test;  $n$  = 3.

expression and plasticity, not only during juvenile development but also in the adult mouse.

A consequence of *Gadd45b/g* being direct targets of OTX2, in contrast with *bona fide* “plasticity genes” (Fig. 1C), is that some of these genes may be controlled by GADD45b. To verify this possibility, their expression was measured after low-titer viral expression of *Gadd45b* (AAV8-Syn-mCherry-2A-*Gadd45b*) in the visual cortex of adult (3-month-old) mice, which results in overexpression mainly in PV cells (Fig. 4A,B). Similar to what was observed during juvenile development (Fig. 1F) and after induced adult plasticity (Fig. 3A), *Gadd45b* expression was increased 2-fold on average, which resulted in upregulation of *Gadd45g* and other “plasticity genes” including *Egr2*, *Arc*, *Fos*, and *Egr4* (Fig. 4C). Furthermore, the expression level of these genes was directly correlated with that of *Gadd45b* (Fig. 4D).

### Gadd45b Gain of Function Reopens Physiological Plasticity in the Adult Visual Cortex

The ability of *Gadd45b* to reactivate the expression of “plasticity genes” in the adult suggested that it may also reopen physiological plasticity. OD bias index was evaluated with intrinsic optical imaging by comparing retinotopic map amplitude of contralateral and ipsilateral V1 after sequential activation of contralateral and ipsilateral eyes (Fig. 5). Control adult mice (injected with AAV8-Syn-mCherry) did not reopen physiological plasticity as no difference in OD index was observed after 4-day MD of the contralateral eye (Fig. 5B,C,F). However, viral overexpression of *Gadd45b* (injected with AAV8-Syn-mCherry-2A-*Gadd45b*) induced a general decrease in response strength for both eyes (Fig. 5G)



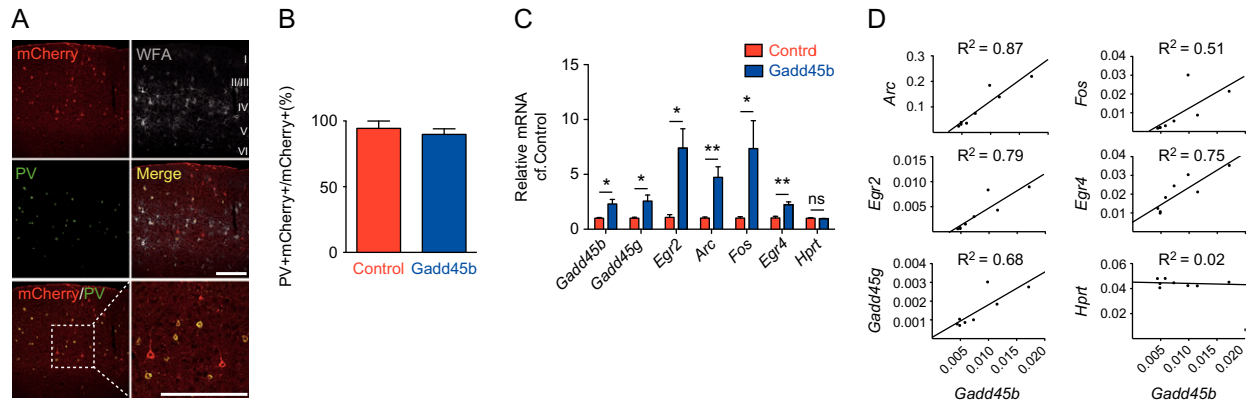
**Figure 3.** Reopening plasticity in the adulthood by blocking OTX2 increases *Gadd45b/g* expression. (A) *Gadd45b* and *Gadd45g* expression in visual cortex layer IV of scFvPax6<sup>tg/o</sup> and scFvOtx2<sup>tg/o</sup> adult mice 2 weeks after intraventricular Cre-TAT protein injection. Error bars,  $\pm$ SEM; \* $P$  < 0.05 by Student *t*-test; 2 independent experiments; mice, scFvPax6<sup>tg/o</sup>  $n$  = 6, scFvOtx2<sup>tg/o</sup>  $n$  = 5. (B) Correlation between expression levels of *Gadd45b*, *Gadd45g* and plasticity genes in the scFv-Otx2 paradigm of reopening plasticity. (C, D) In situ hybridization (C) of *Gadd45b* intronic probes (pre-mRNA) on scFvOtx2<sup>tg/o</sup> or scFvPax6<sup>tg/o</sup> adult mice 2 weeks after intraventricular Cre-TAT protein injection. Quantification of the percentage of *Gadd45b* pre-mRNA positive cells in layer IV or layers V-VI (D). Scale bar, 500  $\mu$ m; Error bars,  $\pm$ SEM; \*\* $P$  < 0.01, \*\*\* $P$  < 0.001 by two-way ANOVA with Bonferroni post hoc test; mice, scFvPax6<sup>tg/o</sup>  $n$  = 4, scFvOtx2<sup>tg/o</sup>  $n$  = 4.

and led to a reopening of physiological plasticity (Fig. 5D–F). The reduction in OD index is mediated by an increase of the open eye response (Fig. 5G), which is in accordance with induced adult V1 plasticity in scFvOtx2<sup>tg/o</sup> mice (Bernard et al. 2016).

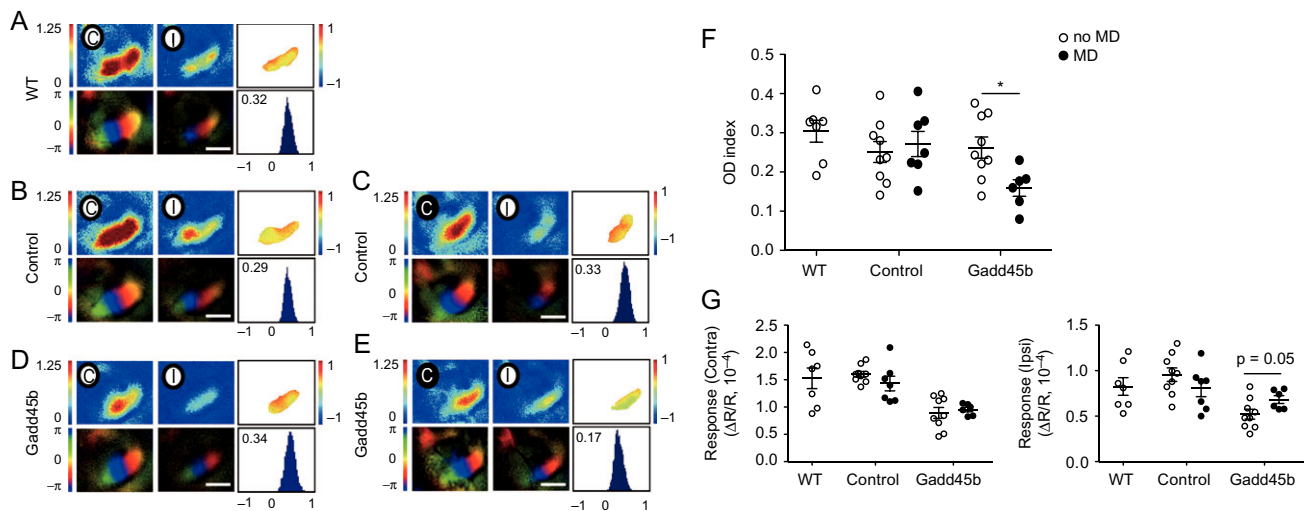
### Gadd45b Controls the Methylation State of Visual Cortex

Given that *Gadd45b* actively participates in DNA demethylation (directly or indirectly), we assessed global methylation patterns of visual cortex layer IV by evaluating the localization of Methyl CpG Binding Protein 2 (MeCP2), a marker for DNA methylation, at different ages (P20, P30, P60, P100). Quantification of the number of MeCP2 foci revealed a decrease during postnatal development specifically in WFA-positive cells in V1 (Fig. 6A,B). To assess whether changes occur in other sensory cortices that undergo CP, we measured foci number in primary auditory cortex and found a similar decrease (Fig. 6C). The observed changes in expression of plasticity genes could happen through rearrangement of the chromatin structure towards a heterochromatic state, or by accumulation of MeCP2 protein within promoters of these genes. We observed the same intensity for MeCP2 protein staining but an increase in the area of foci in V1 of adult mice (Fig. 6D,E), suggesting changes in methylation pattern (and not necessarily total DNA methylation) that lead to a more compact chromatin.

The reduction of MeCP2 foci specifically in V1 WFA-positive cells during postnatal development follows the kinetics of WFA intensity (Lee et al. 2017), suggesting it reflects a consolidated state of mature PV cells. While PNN assembly takes several



**Figure 4.** Overexpression of Gadd45b/g in visual cortex induces plasticity gene expression in the adult. (A) Immunohistochemistry of mCherry (red), PV (green), WFA (grey) in visual cortex of adult mice injected with AAV8-Syn-mCherry-2A-Gadd45b. Scale bars, 200  $\mu$ m. Enlargement is shown in bottom right panel. (B) Proportion of infected cells (mCherry+) that also express PV (PV+) in visual cortex of adult mice 2 weeks after injection of AAV8-Syn-mCherry (Control) or AAV8-Syn-mCherry-2A-Gadd45b (Gadd45b). Error bars,  $\pm$ SEM; mice, Control  $n = 3$ , Gadd45b  $n = 3$ . (C) Expression of plasticity genes measured by RT-qPCR following viral overexpression of Gadd45b in adult mouse visual cortex. Values are averages of fold ratios compared with AAV8-Syn-mCherry injected mice (control). Error bars,  $\pm$ SEM; \* $P < 0.05$ , \*\* $P < 0.01$  by Student t-test; mice,  $n = 4$  per condition. (D) Correlation between expression levels of Gadd45b and plasticity genes shown in (C).

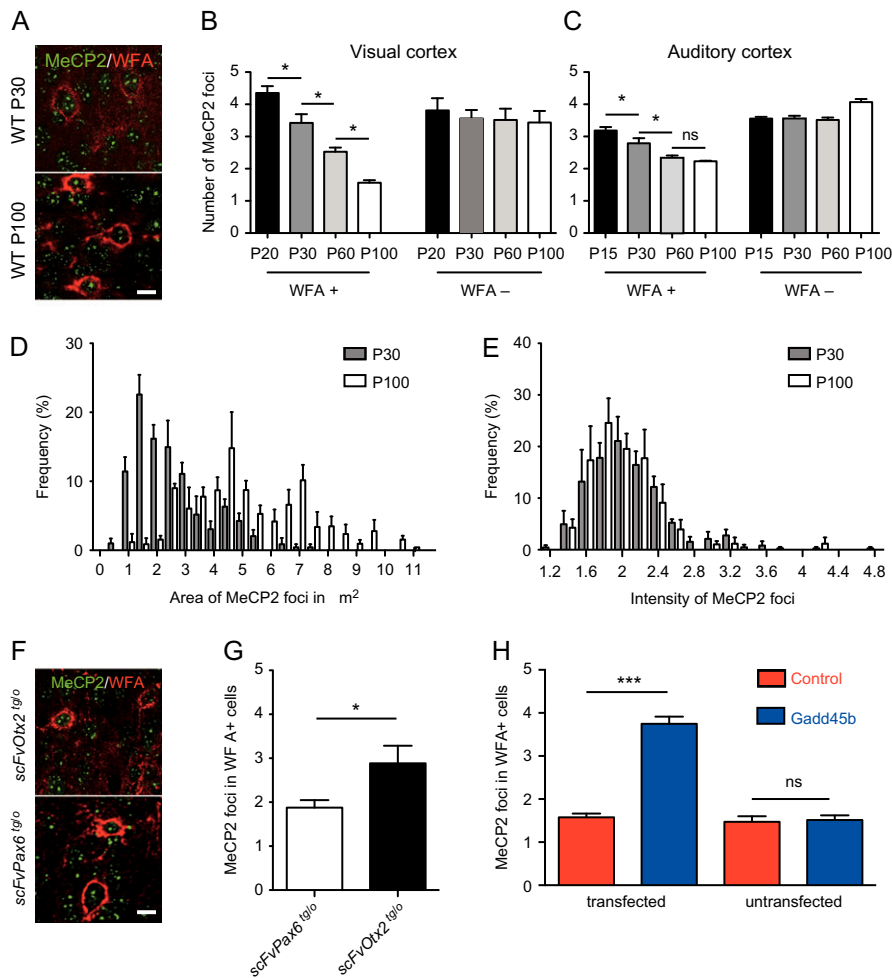


**Figure 5.** Overexpression of Gadd45b in visual cortex reopens the ocular dominance plasticity in the adult. (A-E) Optical imaging maps of responses to the ipsilateral (I) and contralateral (C) eye in the binocular region of V1 in adult wild-type (WT) mice, either (A) un-injected, (B, C) injected with AAV8-Syn-mCherry (Control), and (D, E) injected with AAV8-Syn-mCherry-2A-Gadd45b (Gadd45b). Monocular deprivation (MD) was performed 2 weeks after virus injection (C, E). Stimulated contralateral and ipsilateral eyes are indicated with a white (open) or black (closed) circle. Top left and middle panels represent the signal magnitude for the retinotopic maps ( $\Delta R/R$ ,  $\times 10^{-4}$ ) for each eye; bottom left and middle panels indicate corresponding map regularity. Top right panel shows the normalized ocular dominance (OD) map, and bottom right panel shows the OD histogram, with average value inset. Scale bars, 1 mm. (F) Average OD indices determined in WT mice, AAV8-Syn-mCherry (Control) or AAV8-Syn-mCherry-2A-Gadd45b (Gadd45b) injected mice without MD (white circle) and after 4 days of MD (black circle). Error bars,  $\pm$ SEM; \* $P < 0.05$  by multiple t-tests corrected with Sidak-Bonferroni method; mice,  $n = 6-9$  for each group. (G) Average V1-activation of the contralateral (Contra) response (top panel) and of the ipsilateral (Psi) response (bottom panel). Error bars,  $\pm$ SEM; multiple t-tests corrected with Sidak-Bonferroni method; mice,  $n = 6-9$  for each group.

weeks to reach maximum, it remains surprisingly dynamic in adult V1 when OTX2 levels are reduced (Beurdeley et al. 2012; Spatzza et al. 2013; Bernard et al. 2016). We find MeCP2 foci show similar dynamics, as reopening adult plasticity by blocking OTX2 in CSF of scFvOtx2<sup>tg/o</sup> mice at P90 led to an increase of the number of MeCP2 foci in visual cortex WFA-positive PV cells compared with scFvPax6<sup>tg/o</sup> mice (Fig. 6F,G). This increase is similar to the number of foci observed in WT mice at the peak of plasticity (Fig. 6B). We also observed an increase in MeCP2 foci in visual cortex WFA-positive PV cells of adult mice when Gadd45b was overexpressed by viral injection (Fig. 6H). This effect was cell-autonomous given that WFA-positive cells not over-expressing Gadd45b (i.e., mCherry negative) showed

no change in MeCP2 foci number (Fig. 6H). Thus, both OTX2 and GADD45b levels impact chromatin structure in PV cells.

To determine whether Gadd45b regulates plasticity gene expression via DNA demethylation, we performed bisulfite assays on V1 layer IV after viral Gadd45b overexpression. We targeted for analysis promoter regions enriched in CpG islands of known plasticity genes (Arc, Egr2, and Fos), along with Gadd45b (Fig. 7 and Suppl. Fig. S3). We observed a significant change in at least one methylation site in Egr2, Fos and Gadd45b, but no changes in Arc (Fig. 7A-D). Interestingly, while PV cells express Egr2, Fos, and Gadd45b, they do not express Arc. Given that changes in MeCP2 foci are specific to PV cells (Fig. 6H) and that viral Gadd45b overexpression occurs mainly in PV cells



**Figure 6.** Gadd45b affects the epigenetic state of PNN-enwrapped neurons of the visual cortex. (A) Immunohistochemistry (IHC) of MeCP2 (green) and WFA (red) in visual cortex layer IV of WT mice at P30 and P100. Scale bar, 10  $\mu$ m. (B, C) Average number of MeCP2 foci in WFA-positive (WFA+) and WFA-negative (WFA-) cells during postnatal development in visual cortex layer IV (B) and in auditory cortex layer IV (C). Error bars,  $\pm$ SEM; \* $P$  < 0.05 by one-way ANOVA with Bonferroni post hoc test; mice,  $n$  = 3–7 per age group. (D, E) Distribution of MeCP2 foci area (D) and intensity (E) of WFA+ cells of visual cortex in WT mice at P30 (grey) and P100 (white). Error bars,  $\pm$ SEM; mice,  $n$  = 4 per age group. (F) IHC of MeCP2 (green) and WFA (red) in visual cortex of scFvPax6<sup>tg/tg</sup> and scFvOtx2<sup>tg/tg</sup> mice 2 weeks after intraventricular Cre-TAT protein injection. Scale bar, 10  $\mu$ m. (G) Average number of MeCP2 foci in WFA+ cells in scFvPax6<sup>tg/tg</sup> and scFvOtx2<sup>tg/tg</sup> mice measured by IHC 2 weeks after intraventricular Cre-TAT protein injection. Error bars,  $\pm$ SEM; \* $P$  < 0.05 by Student t-test; mice,  $n$  = 3 for each genotype. (H) Average number of MeCP2 foci in WFA+ cells 2 weeks after injection of either AAV8-Syn-mCherry (Control) or AAV8-Syn-mCherry-2A-Gadd45b (Gadd45b) in adult visual cortex. MeCP2 foci number are compared for transfected and untransfected cells. Error bars,  $\pm$ SEM; \*\*\* $P$  < 0.001 by one-way ANOVA with Bonferroni post hoc test; mice,  $n$  = 3 for each group.

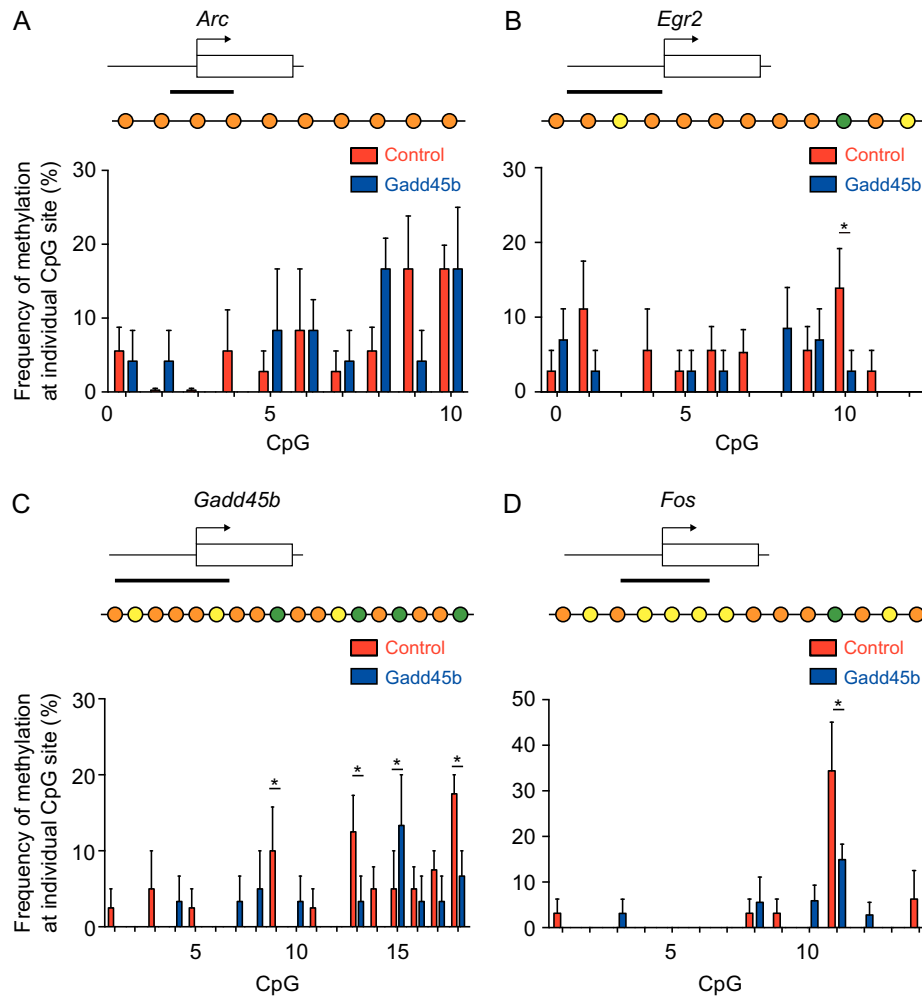
(Fig. 4A), changes in DNA methylation are likely triggered by increasing GADD45b in PV cells.

## Discussion

The Gadd45 family has been implicated in epigenetic gene activation (Barreto et al. 2007; Ma et al. 2009; Gavin et al. 2012), which can impact synaptic plasticity, long-term memory and ultimately animal behavior (Leach et al. 2012; Sultan et al. 2012). Furthermore, Gadd45 genes have altered expression in mouse visual cortex after MD (Majdan and Shatz 2006; Tognini et al. 2015). Thus, changes in Gadd45b/g activity could have a broad impact on gene regulation during PV cell maturation for CP timing, consistent with expression that is highest during CP, as we show here and as has been previously shown in mouse somatosensory cortex (Matsunaga et al. 2015). We find that when OTX2 protein levels are transiently increased in PV cells prior to CP onset, Gadd45b/g levels are rapidly increased in a translation-independent manner. Furthermore,

when Gadd45b expression is increased in PV cells of adult cortex, we find juvenile-like MeCP2 foci along with increased layer IV plasticity gene expression and reactivated OD plasticity. This suggests that OTX2 signaling impacts PV cell maturation and function through GADD45b/g, which can control the expression of a swath of genes and leave epigenetic marks that would eventually stabilize during CP closure and be maintained into adulthood.

It is intriguing that OTX2 regulates Gadd45b/g oppositely in the juvenile and adult cortex. MeCP2 foci number and size gradually change in PV cells over the course of the CP and through to adulthood. In comparison, the state of MeCP2 foci in PV cells rapidly reverses when OTX2 signaling is compromised in adult, a paradigm that reactivates cortical plasticity (Bernard et al. 2016), suggesting that OTX2 impacts the epigenetic state of PV cells by regulating chromatin structure. Increasing levels of OTX2 may affect chromatin accessibility differently in juvenile and adult mice via a cohort of different cofactors and subtly change PV cell activity. We hypothesize that the OTX2 target sites on Gadd45b/g



**Figure 7.** Gadd45b modifies methylation of CpG islands of plasticity genes. (A–D) Schematic representation of *Arc* (A), *Egr2* (B), *Gadd45b* (C), and *Fos* (D) genes showing the transcription initiation site (arrow) and the CpG island (black bar) targeted for bisulfite PCR assay. Methylation frequency is calculated at individual CpG sites for each gene, 2 weeks after viral injection of AAV8-Syn-mCherry (Control) or AAV8-Syn-mCherry-2A-Gadd45b (Gadd45b) in adult visual cortex (Suppl. Fig. S3). Bead representations of the CpG sites are shown above each plot and are color-coded for correlation type: yellow, no methylation; orange, similar methylation; green, differential methylation. Error bars,  $\pm$ SEM; \* $P < 0.05$  by two-way ANOVA with Fisher's LSD test; mice,  $n = 4$  for each group.

are differentially accessible based on OTX2 concentration, whereby certain levels activate while other levels repress transcription. Concentration-dependent accessibility has been recently reported for transcription targets of bicoid (Bcd) homeoprotein in *Drosophila* development (Hannon et al. 2017). Bcd was found to influence chromatin structure to gain access to concentration-sensitive targets at high concentration while insensitive targets are bound at lower Bcd concentrations. While the impact of Otx2 on chromatin structure has yet to be investigated, other homeoproteins including engrailed and Cdx have been implicated in DNA damage response and chromatin remodeling (Rekaik et al. 2015; Amin et al. 2016). The critical importance of homeoprotein concentration is also revealed by their concentration-dependent morphogenic activity for the precise guidance of axons or the formation of regional boundaries during brain development (Prochiantz and Di Nardo 2015). The correlation between *Gadd45b* and plasticity gene expression even at low levels of *Gadd45b* (Fig. 4D) suggests that without fully reopening plasticity, small fluctuations of adult OTX2 and GADD45b/g within PV cells may affect physiological activity.

It must be kept in mind that the mechanisms or circuits of OD plasticity are different between juvenile and adult (Sato and

Stryker 2008; Takesian and Hensch 2013; Hübener and Bonhoeffer 2014; Hattori et al. 2017). For example, short term MD transiently reduces PV firing rates in juvenile mice V1 but not in adult V1 even though reducing PV-specific inhibition restores OD plasticity in adult (Ranson et al. 2012; van Versendaal et al. 2012; Kuhlman et al. 2013). Adult plasticity takes longer to induce and can involve different disinhibitory circuits through experience-dependent myelination, acetylcholine or serotonin pathways, including other cortical areas (Kruglikov and Rudy 2008; Sato and Stryker 2008; Pi et al. 2013; Fu et al. 2014; Yotsumoto et al. 2014; Mount and Monje 2017). Indeed, there are significant differences in gene expression, either when cortical plasticity is induced (Rietman et al. 2012; Tiraboschi et al. 2013), or between juvenile and adult cortex (Majdan and Shatz 2006; Benoit et al. 2015).

Similar to bicoid (Hannon et al. 2017), we hypothesize that OTX2 determines PV activity through chromatin state changes in a concentration-sensitive manner leading to differential gene expression and neuronal responses between juvenile and adult. Thus, it is possible that OTX2-induced alterations in chromatin structure are proportional to the amount of non-cell autonomous OTX2 protein. To investigate this hypothesis, it will be necessary to compare the chromatin structure of PV cells before, during,



and after the CP, and after inducing adult plasticity. Another nonexclusive possibility is that OTX2 interacts with other factors that may differ during the CP and post-CP. Such physiological interactions between homeoprotein signal transduction and classical signaling pathways has been demonstrated for engrailed and PAX6 (Wizenmann et al. 2009; Di Lullo et al. 2011; Stettler et al. 2012). This does not preclude that some genes are induced (or repressed) continuously by OTX2 activity in juvenile and adult such as PNN regulating genes (Lee et al. 2017). Regardless, the change in epigenetic context between juvenile and adult PV cells, as revealed by MeCP2 foci, provides a compelling explanation for differential *Gadd45b/g* regulation by OTX2 activity. As *Gadd45b/g* are epigenetic modifiers (Niehrs and Schäfer 2012), their gradual decrease in expression from CP to adulthood may also be directly implicated in changing chromatin structure within PV cells.

When adult non-cell autonomous OTX2 levels are reduced or when *Gadd45b* is overexpressed in PV cells, the induction of plasticity genes occurs across V1 layer IV. Some of these changes occur in PV cells, while others are more global. Indeed, *Arc* is not transcribed in PV cells yet its expression is increased, and *Gadd45b* expression is increased in layer IV cells though not restricted to PV cells. This suggests that changes in PV cell gene expression impact other layer IV cells. While demethylation is not widespread on the promoters of *Egr2*, *Fos* and *Gadd45b* upon *Gadd45b* overexpression in PV cells, it could be sufficient to induce changes in the expression of these genes. Demethylation is more widespread on the *Gadd45b* promoter itself, which could account for the increase in endogenous *Gadd45b* expression and suggests *Gadd45b* self-regulates. Increased *Gadd45b* expression in adult V1 leads to subsequent DNA demethylation of PV cell plasticity genes and their increased transcription.

The differential kinetics in MeCP2 foci number we observed during postnatal visual and auditory primary cortex development suggest differential recruitment in pyramidal cells and PV cells. Indeed, distinct phenotypes are observed in mice with either general or cell-specific removal of MeCP2 expression. In MeCP2-null mice, PV cell maturation is premature and CP timing is also accelerated (Durand et al. 2012; Tomassy et al. 2014; Krishnan et al. 2015, 2017), which could be explained by the fact that MeCP2 normally represses brain-derived neurotrophic factor expressed in pyramidal cells (Chen et al. 2003; Sampathkumar et al. 2016). Mice lacking MeCP2 only in excitatory neurons experience tremors and anxiety-like behaviors (Meng et al. 2016), whereas mice lacking MeCP2 only in PV cells do not show such phenotypes yet have completely abolished CP plasticity (He et al. 2014). Clearly, MeCP2 recruitment is dependent on the type of neuron and likely its context, impacting a wide variety of mouse phenotypes (Gogolla et al. 2014; He et al. 2014; Krishnan et al. 2015; Meng et al. 2016; Sampathkumar et al. 2016). Interestingly, MeCP2 binds not only methylated CpG but also methylated CpH (H = A/C/T) (Guo et al. 2014; Chen et al. 2015; Gabel et al. 2015), and PV cell specific enhancement of methyl-CpH content (nearly 50% of total methyl-C) has been reported (Mo et al. 2015). In this context, it is rather noteworthy that we find age-dependent changes in MeCP2 foci only in PV cells (in both A1 and V1). Reducing OTX2 levels in adult V1 directly impacted MeCP2 foci number in PNN-enwrapped cells, suggesting that OTX2 plays a role in differential MeCP2 recruitment. It will be important to determine the foci identity and the epigenetic fingerprint of PV cells, since it has been suggested that MeCP2 is implicated in cell-specific epigenetic mechanisms for regulation of gene expression and chromatin structure (Mellén et al. 2012).

While homeoproteins are well-characterized as transcription factors, they also actively control DNA replication and repair,

mRNA export and translation, and protein degradation (Prochiantz and Di Nardo 2015). As non-cell autonomous signaling factors, they have direct access to cytoplasm and nucleus with the potential to broadly impact function of the target cell. By regulating *Gadd45b/g* activity, OTX2 in PV cells could engage epigenetic programs that control the balance between plastic and nonplastic states.

## Supplementary Material

Supplementary material is available at *Cerebral Cortex* online.

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## Notes

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## Article 2: Non-cell autonomous OTX2 homeoprotein controls the epigenetic state of parvalbumin cells in visual cortex plasticity periods

This paper is in preparation and needs to be finalized but the results presented below will constitute its core.

ENGRAILED is able to control the heterochromatin state, whereby its down-regulation or hypomorphism leads to heterochromatin relaxation and to the activation of TEs, in particular from the LINE-1 family (Blaudin de Thé et al., 2018). Given the similarities between OTX2 and ENGRAILED in the protection of midbrain neurons against oxidative stress (Rekaik et al., 2015), we began investigating whether OTX2 regulates chromatin structure and the expression of TEs in a non-cell autonomous way. OTX2 is able to modify the epigenetic state of the PV cells through the control of *Gadd45 $\beta$*  expression but the epigenetic mechanisms by which OTX2 controls plasticity genes transcription are unknown. The objective of this study is to identify some epigenetic modifications by which OTX2 might regulate V1 cortex plasticity.

The results demonstrate that developmental and adult plasticity periods are associated with changes in the staining pattern of H3K9me3 chromatin mark, an increase in DNA DSBs, and an up-regulation of ORF1p expression in PV cells. They confirm that OTX2 physically binds to the *Line-1* promoter and demonstrate that antagonizing LINE-1 upregulation at CP onset abolishes the increase in DNA DSBs in PV interneurons.



# **Non-cell autonomous OTX2 homeoprotein controls the epigenetic state of parvalbumin cells in visual cortex plasticity periods**

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**Keywords:** H3K9me3,  $\gamma$ H2AX, ORF1p, development, FSPV.

## **ABSTRACT**

The non-cell autonomous transfer of OTX2 homeoprotein transcription factor into cerebral visual cortex fast-spiking parvalbumin interneurons regulates their maturation through direct transcriptional regulation of *Gadd45 $\beta/\gamma$*  expression. This mechanism controls the opening and closure of a critical period of plasticity during postnatal development and controls plasticity reopening in the adult. Here, we show that these developmental and adult plasticity periods are associated with changes in the nuclear distribution of the H3K9me3 chromatin mark, an increase in DNA double-strand breaks, and an up-regulation of ORF1p expression in parvalbumin interneurons enwrapped by perineural nets. We show that antagonizing LINE-1 upregulation at critical period onset abolishes the increase in DNA breaks in parvalbumin interneurons, and that OTX2 binds directly to *Line-1* promoter. Accordingly, we propose that plasticity is associated with a transient period of genome instability regulated by OTX2 levels in a subclass of visual cortex inhibitory interneurons.

## **INTRODUCTION**

During early postnatal brain development, sensory experience allows for the activity-dependent refinement of neuronal circuits at the level of cerebral cortex layers III/IV. This was first established by the seminal work of Hubel and Wiesel in monocular deprivation experiments (Wiesel and Hubel, 1963). These authors demonstrated that this plasticity is restricted to a transient critical period (CP) of post-natal development. Indeed, monocular deprivation during this period leads to an enhanced innervation of cerebral cortex layer IV cells by thalamic terminals corresponding to the open eye. This increase is at the expense of the closed eye, leading to amblyopia. In human infancy, misaligned eyes or congenital cataracts can result in amblyopia or “lazy eye”, a pathology affecting 3% of the population (Holmes and Clarke, 2006). It was later shown that CPs exist in several sensory areas and are controlled by the maturation of layer IV fast-spiking parvalbumin (FSPV) inhibitory interneurons which shifts the inhibitory/excitatory (I/E) balance in layers III/IV (Hensch, 2005). During CP, these

cells become increasingly enwrapped by a specific extracellular matrix structure termed perineuronal nets (PNNs), which are typically detected by the *Wisteria floribunda* agglutinin (WFA) lectin (Härtig et al., 1992; Köppe et al., 1997).

During early brain development, an interplay exists between sensory experience and genetic programs leading to the refinement of neuronal circuits. Some studies suggest that the regulation of gene expression through epigenetic mechanisms is at the interface between environmental stimuli and long-lasting molecular, cellular and complex behavioral phenotypes acquired during periods of developmental plasticity (Cholewa-Waclaw et al., 2016; Fagiolini et al., 2009). Epigenetic mechanisms have been proposed as having key functions in the control of plasticity, allowing environmental exposure to shape gene expression (Duncan et al., 2014).

In recent studies, it was shown that CP opening at postnatal day 20 (P20) is triggered by the import of the transcription factor OTX2 traveling from the choroid plexus (ChP) and specifically internalized by FSPV cells owing to OTX2 binding sites present in the PNNs (Beurdeley et al., 2012; Miyata et al., 2012; Spatazza et al., 2013a; Sugiyama et al., 2008). Interestingly, the accumulation of OTX2 is also responsible for CP closure at P40, leading to a two-threshold model in which a first OTX2 concentration threshold allows for CP opening and a second one for CP closure (Prochiantz and Di Nardo, 2015). In further studies, it was established that decreasing OTX2 import by FSPV cells in the adult reopens plasticity and allows for the full recovery of binocular vision in adult amblyopic mice (Beurdeley et al., 2012). At a molecular level, OTX2 regulatory activity during CP and in the adult is due to the direct non-cell autonomous control of *Gadd45 $\beta$*  expression, associated with changes in chromatin methylation specifically within FSPV cells (Apulei et al., 2018).

Plasticity, either during CPs or in the adult, is associated with the GADD45 $\beta$ -controlled expression of several plasticity genes, including immediate early genes (IEGs), such as *c-Fos* and *Egr1/2* in layer III/IV FSPV cells, and *Arc* in layer III pyramidal cells (Apulei et al., 2018). To further explore plasticity-dependent epigenetic variations, we have now investigated whether plasticity is paralleled by changes in the H3K9me3 chromatin mark and by the occurrence of DNA double strand breaks (DSBs), since such breaks facilitate IEGs expression (Madabhushi et al., 2015). We find that developmental CPs and adult plasticity induced by changes in OTX2 internalization are associated with changes in H3K9me3 chromatin patterns and an increase in DSBs. We also report that plasticity is paralleled by the upregulation of long interspersed nuclear element type 1 (LINE-1) open reading frame 1 (ORF1p) expression and that DSBs formation is blocked by stavudine, a reverse transcriptase inhibitor that disrupts LINE-1 activity. Based on chromatin immunoprecipitation studies, we propose

that OTX2 directly regulates *Line-1* expression and the ensuing DSBs formation to provide an additional mechanism participating in the control of visual cortex plasticity.

## **MATERIALS AND METHODS**

### **Ethics Statement**

All animal procedures were carried out in accordance with the guidelines of the European Economic Community (2010/63/UE) and the French National Committee (2013/118). For surgical procedures, animals were anesthetized with Xylazine (Rompun 2%, 5 mg/kg) and Ketamine (Imalgene 1000, 80 mg/kg) by intraperitoneal injection. This project (no. 00704.02) obtained approval from Ethics committee no. 59 of the French Ministry for Research and Higher Education.

### **Animals**

Conventionally raised C57Bl/6 J mice were purchased from Janvier Laboratories. The *scFvOtx2<sup>tg/o</sup>* and *scFvPax6<sup>tg/o</sup>* mouse lines were generated through a knock-in approach, as described previously (Bernard et al., 2016).

### **Brain Infusions**

For conditional choroid plexus expression in scFv mouse lines, intracerebroventricular stereotaxic injections (bregma: x = -0.58 mm, y = ±1.28 mm, z = 2mm) of 40 µg of Cre-TAT protein (Spatazza et al. 2013) in 15% DMSO (Sigma D2650), 1.8% NaCl in 2 µL were performed with Hamilton syringe at 0.2 µL/min. Two weeks after injection, mice were processed for immunohistochemistry. Stereotaxic infusions into visual cortex (lambda: x = 1.7 mm, y = 0 mm, z = 0.5 mm) with 3-day osmotic mini pump (Alzet 1003D, Charles River Laboratories) were used to deliver stavudine (d4T, 10 µM, Sigma Y0000408) and 0.9% NaCl.

### **Chromatin Immunoprecipitation**

Layer IV of primary visual cortices were pooled from 5 postnatal day 30 (P30) WT mice and washed in PBS followed by wash buffer (20 mM HEPES pH 7.4; 150 mM NaCl; 125 mM Glycine, 1 mM PMSF). Nuclei were fixed in 1% formaldehyde (Sigma 252549) in PBS for 15 min at RT, isolated by dounce (pestle B) in lysis buffer (20 mM HEPES pH 7.4; 1 mM EDTA; 150 mM NaCl; 1% SDS; 125 mM Glycine; 1 mM PMSF), and then resuspended in wash buffer. Chromatin was purified by using modified Farnham protocol (<http://farnham.genomecenter.ucdavis.edu/protocols/tissues.html>).



Chromatin immunoprecipitation (ChIP) were performed by using a mix of 3 µg of anti-OTX2 (goat, R&D AF1979) and 3 µg of anti-OTX2 (rabbit, abcam 21990). For control ChIP, 6 µg of anti-IgG (rabbit, abcam ab27478) were used. Immunoprecipitated DNA was analyzed by qPCR.

### **Quantitative RT-PCR**

Total DNA was extracted from dissected tissue by using the AllPrep DNA/RNA Mini Kit (Qiagen 80204). Quantitative PCR reactions were carried out in triplicate with SYBR Green I Master Mix (Roche S-7563) on a LightCycler 480 system (Roche). Expression was calculated by using the  $2^{-\Delta Ct}$  method. The oligonucleotide primers used are as follows: *Gadd45b* forward 5'-CTCGAACCCTCCATCCTGTG-3'; reverse 5' - AAATGTCCCTGGCGTGAGAG-3'; *LINE-1Tf* forward 5'- CCGCCGAACTTAGGAAATTA-3'; reverse 5' -GTCGAATTGCTCTGGCTAATAC-3'; *LINE-1A* forward 5'- GTCGAATTGCTCTGGCTAATAC-3'; reverse 5'-GTCGAATTGCTCTGGCTAATAC-3'; *Actin beta* forward 5'-TTCTACAAATGTGGCTGAGGAC-3'; reverse 5' - TGAGGGACTTCCTGTAACCACT-3'.

### **Immunohistochemistry**

Mice were perfused transcardially with PBS followed by 4% paraformaldehyde prepared in PBS. Brains were postfixed 1 h at 4 °C and immunohistochemistry was performed on cryosections (20 µm) encompassing the entire visual cortex. Heat-induced antigen retrieval in 10 mM sodium citrate was performed prior to overnight primary antibody incubation at 4 °C. Primary antibodies included anti-γH2AX (mouse, 1/200, millipore clone JBW301), anti-Orf1p (guinea pig, 1/ 200, in house), anti-H3K9me3 (rabbit, 1/200, abcam ab8898), and biotinylated WFA (1/100, Sigma L1516). Secondary antibodies were Alexa Fluor-conjugated (Molecular Probes). Images were acquired with a Leica SP5 confocal microscope and analyzed with ImageJ.

### **Statistical Analysis**

Statistical analysis was performed on Prism 6 (GraphPad). Pairwise comparison was performed with Student t-test, while multiple group analyses were done with ANOVA (one- or two-way) followed by Bonferroni correction. Changes in frequency distribution were assessed by Kolmogorov–Smirnov test.

## RESULTS

### H3K9me3 level changes specifically in WFA-positive cells during development

We previously demonstrated that there is a decrease in the number of MeCP2 foci during the CP (Apulei et al., 2018), which prompted us to investigate the staining pattern of H3K9me3, an epigenetic mark of pericentromeric regions often enriched in repetitive sequences such as mobile elements, in particular of the LINE-1 family (Saksouk et al., 2015). The number of H3K9me3 foci was counted in layer III/IV of V1 at different ages starting at P20 (CP opening in the mouse) throughout adulthood (P100) and comparing WFA-stained (WFA+) and non-stained neurons (WFA-). In V1 layers III/IV, WFA- cells are primarily pyramidal neurons while WFA+ cells are primarily FSPV neurons, which have been shown to capture OTX2 from the ChP extra-cortical source (Spatazza et al., 2013). As shown in Figure 1A, B, the number of H3K9me3 foci is not modified with age in the WFA- neurons, whereas it decreases with age in WFA+ FSPV neurons, suggesting that a high number of H3K9me3 foci correlates with plasticity.

To verify this point, we reopened plasticity in the adult by using the *scFvOtx2<sup>tg/o</sup>* mouse model in which the extracellular expression of a single chain antibody directed against OTX2 (scFvOTX2) can be induced at P90 specifically at the level of ChP. Secreted scFvOTX2 decreases the amount of OTX2 imported by FSPV neurons and reopens plasticity at P100 (Bernard et al., 2016). As a control, we used the *scFvPax6<sup>tg/o</sup>* mouse to induce the ChP expression of secreted scFvPAX6 that does not reopen plasticity in the adult (Bernard et al., 2016). We find that scFvOTX2, but not scFvPAX6, increases the number of H3K9me3 foci in WFA+ cells to a level close to that observed at P30, which is the peak of CP-associated plasticity (Fig. 1C). Our findings suggest that H3K9me3 foci number reflects FSPV cell maturation state, whereby foci number is inversely proportional to PV expression, which increases significantly from P20 to P60 (Lee et al., 2017). Given the rapid increase in H3K9me3 foci within WFA+ FSPV cells when adult plasticity is induced by disrupting OTX2 signaling in adult, this observation also suggests that OTX2 regulates the epigenetic state and chromatin structure of FSPV, well in line with a previous analysis of MeCP2 foci distribution (Apulei et al., 2018).

### ORF1p expression increases during the critical cortical period of plasticity

A change in H3K9me3 pattern may have consequences on mobile element expression and genomic stability (He et al., 2019; Pezic et al., 2014). We thus investigated whether LINE-1 expression varies in FSPV neurons during plasticity periods by using an antibody raised against ORF1p (an RNA-binding protein encoded by the LINE-1 bicistronic mRNA) (Blaudin de Thé et al., 2018).

Quantification of expression before (P17), during (P30) and after the plasticity period (P100), reveals that ORF1p staining intensity is significantly higher at P30 than at P17 and P100 within WFA+ cells (Fig. 2A). Because the number of H3K9me3 foci is increased following plasticity reopening in the adult, we compared the intensity of ORF1p immunostaining in the adult following scFvOTX2 or scFvPAX6 induction at P100. Neutralizing OTX2 in the extracellular space has a significant effect on ORF1p expression (Fig. 2B) but this increase in expression is much lower than during normal CP, suggesting differences between developmental and adult plasticity.

To verify if LINE-1 expression can be regulated by the direct binding of OTX2 to *Line-1* genes we performed ChIP-qPCR analysis with primers for promoter regions of *Gadd45 $\beta$*  (as a positive control), *Line 1A*, *Line 1Tf* and for *Actin* (as a negative control) and detected enrichment for both *Line-1A* and *Line-1Tf* from lysates of P30 mouse V1 layer IV (Fig. 2C). Thus, OTX2 can bind to the *Line-1* promoter.

### **DSBs increase specifically in WFA-positive cells during plasticity periods**

Given that DSBs are important for the expression of plasticity genes (Madabhushi et al., 2015), we assessed whether they are associated with our plasticity paradigms. We quantified the number of DSBs in WFA+ or WFA- cells in juvenile and adult V1 by staining for the nuclear protein  $\gamma$ H2AX, a marker for activity-dependent DSBs classically mediated by the type II topoisomerase (Fig. 3A-B). The number of foci increased at CP onset between P17 and P20 yet decreased with CP closure between P30 and P60. These variations were again absent in WFA- cells which notably maintain a rather high level of DSBs throughout life. In addition, reopening plasticity in the adult by inducing scFvOTX2 ChP expression increased the number of DSBs, while scFvPAX6 showed no effect (Fig. 3C).

The previous results show a correlation between the number  $\gamma$ H2AX foci during plasticity periods and the higher intensity of ORF1p staining, thus LINE-1 expression. LINE-1 ORF2p, the second product of the bicistronic LINE-1 mRNA, has both DNase and reverse-transcriptase activities and it was shown before that stavudine (2',3'-didehydro-2',3'-dideoxythymidine, d4T), a nucleoside analogue and strong LINE-1 reverse transcriptase inhibitor (Jones et al., 2008), prevents the formation of DSBs in midbrain neurons (Blaudin de Thé et al., 2018). We thus evaluated if this was also the case in FSPV neurons by infusing either stavudine or vehicle in mouse cortex. Figure 3 D, E illustrates that stavudine infusion in V1 between P17 and P20 antagonizes DSBs specifically in WFA+ cells (Fig. 3 D, E).

## DISCUSSION

Homeoprotein transcription factors are expressed throughout life, and their sites of expression vary considerably between developmental and adult stages (Prochiantz and Di Nardo, 2015). Adult functions are poorly understood, and previous studies from our laboratory have demonstrated that ENGRAILED and OTX2 are respectively involved in the regulation of midbrain dopaminergic neuron survival and cerebral cortex plasticity (Alvarez-Fischer et al., 2011; Apulei et al., 2018; Bernard et al., 2014; Beurdeley et al., 2012; Ibad et al., 2011; Rekaik et al., 2015; Sonnier et al., 2007; Spatazza et al., 2013). A case of particular interest is provided by OTX2 transcriptional activity and epigenetic regulation which occurs specifically in FSPV (Apulei et al., 2018), and the present study was aimed at further deciphering related mechanisms.

ENGRAILED is able to control the heterochromatin state and the activation of LINE-1 family (Blaudin de Thé et al., 2018). In a similar way, other factors in other cellular contexts have been shown to exert a regulation on LINE-1 activity. A first level of regulation is through the modification of chromatin domains. Many genetic LINE-1 sequences are compacted in heterochromatin regions, and their expression is thus repressed. An interesting recent example is provided by a cocaine-induced change in heterochromatic H3K9me3 and the ensuing unsilencing of repetitive elements in the nucleus accumbens (Maze et al., 2011). The same correlation was reported at the level of the hippocampus (Hunter et al., 2012). The present study adds to the concept by showing that H3K9 expression is affected during juvenile development and in the plasticity-induced adult, suggesting that OTX2 could have a role in defining the heterochromatin state of FSPV cells and thus indirectly controlling *Line-1* expression.

A first hint is the observation that ORF1p protein expression is increased at the peak of plasticity and then decreased throughout life. Indeed, LINE-1 expression in the nervous system has been reported before (Thomas et al., 2012), and a striking finding is their activity during development and in adult neural stem cells, providing a basis for neuronal genetic mosaicism (Singer et al., 2010). Indeed, not all the LINE-1 RNA species are present in neuronal cells, but it is of note that FSPV neurons in the V1 layer IV that co-stained with the ORF1p antibodies showed a higher ORF1p intensity at the peak of plasticity. Furthermore, in these neurons, *Line-1* expression is increased at the peak of plasticity leading to the formation of DSBs. Some factors can act on LINE-1 gene expression, both by modifying chromatin structure and by direct transcriptional regulation. Direct regulation was shown for p53 and SIRT6, two proteins involved in the regulation of aging (Van Meter et al., 2014; Wylie et al., 2016). The present study identifies the homeoprotein OTX2 as a putative regulator of *Line-1*

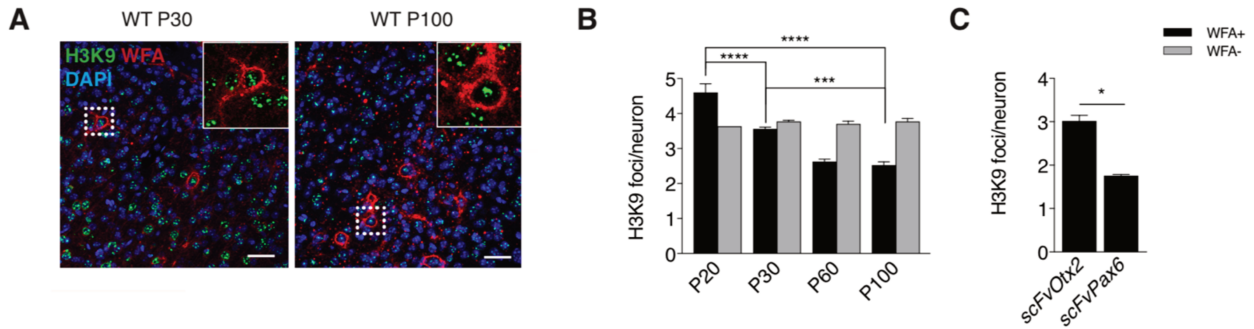
transcription by its binding to the promoter as shown in our ChIP experiment. It can thus be proposed that OTX2 controls the expression of *Line-1* only in part through heterochromatin maintenance similarly to PAX3 and PAX6 homeoproteins that regulate chromatin states through their binding to intergenic major satellite repeats (Bulut-Karslioglu et al., 2012) without excluding direct regulation.

A role for OTX2 in the control of LINE-1 expression is supported by the finding that stavudine blocks DSBs at the CP onset. Interestingly, it is known that the occurrence of DNA DSBs facilitate IEG expression (Madabhushi et al., 2015). We speculate that OTX2 could induce plasticity gene expression also by directly controlling *Line-1* expression. We can only hypothesize on how stavudine works and future studies are necessary to show that blocking reverse transcription allows better access of OTX2 at the chromatin level for inducing IEGs transcription. Retrotransposon expression does not mean retrotransposition and ORF2p can act as an endonuclease without mandatory retrotransposition, In fact retrotransposition in post-mitotic cells is considered unlikely because of the integrity of the nuclear membrane in the absence of mitosis, thus the impossibility for the ribonucleoparticle composed of the *Line-1* mRNA, ORF2p, and ORF1p to gain access to the nucleus. However, one cannot preclude that ORF2p could be individually transported to the nucleus thanks to its nuclear localization signal (Goodier, 2004) and thus not only introduce a nick in the DNA but also reverse transcribe nuclear resident LINE-1 transcripts. Given that we demonstrated that LINE-1 expression is increased at the peak of plasticity, it is conceivable that LINE-1-mediated generation of DSBs is a general driver of gene transcription. It was already demonstrated that binding sites for several other transcription factors are embedded in distinct families of retrotransposon elements (Bourque et al., 2008; Wang et al., 2007).

Repetitive elements not only serve as promoters, but may also be important regulators of nuclear structure, and different retrotransposon elements may function as regulatory elements in different tissues. Retrotransposition has recently been suggested to contribute to neuronal plasticity with a prevalence of neuronal genes (Muotri et al., 2005, 2009). Retrotransposition also takes place in embryonic stem cells and was suggested to be important in early development (Garcia-Perez et al., 2007). Thus, transcription factors other than OTX2 might operate to control *Line-1* expression in different regions of the central nervous system. Together, these findings reinforce the concept that specific retrotransposon elements can control specific cellular states or response through gene regulation. Our findings uncover a potential role for OTX2 in regulating chromatin structure and LINE-1 mobile element expression in FSPV cells during plasticity. We speculate that other homeoprotein transcription factors could also control the expression of mobile elements in the adult and have important epigenetic roles.

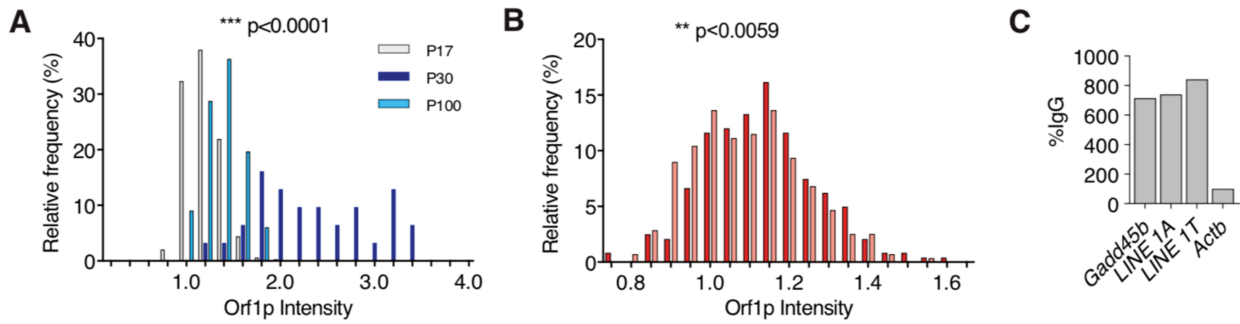
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**Fig. 1. H3K9me3 distribution is affected in PNN-enwrapped neurons of the visual cortex**

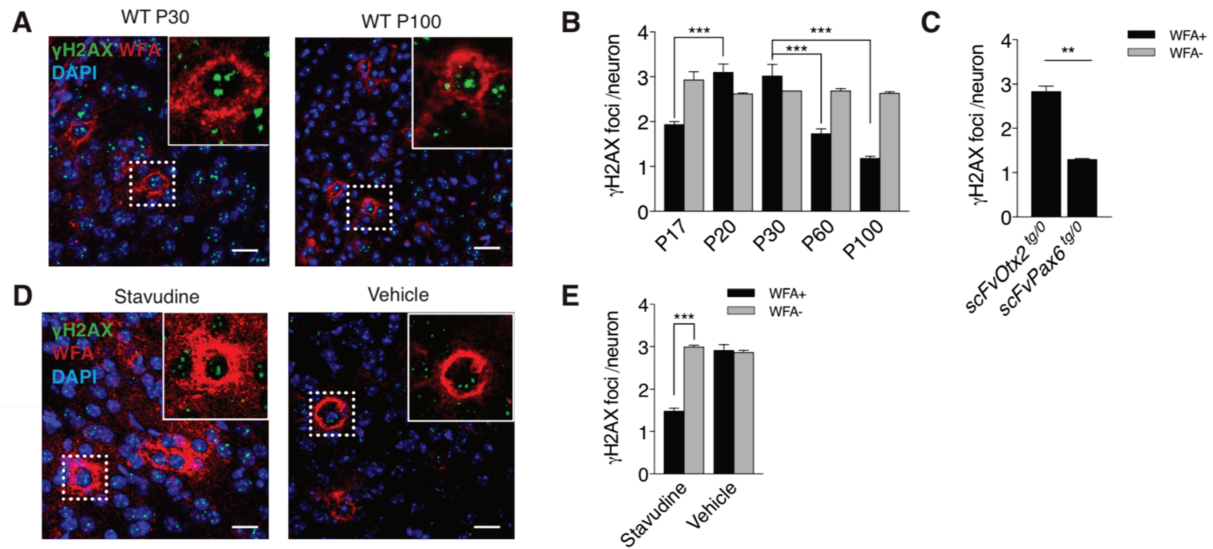
(A) Immunohistochemistry (IHC) of H3K9 (green), WFA (red) and DAPI (blue) in visual cortex layer IV of WT mice at P30 and P100. Scale bar, 40  $\mu$ m. (B) Average number of H3K9 foci in WFA-positive (WFA+) and WFA-negative (WFA-) cells during postnatal development in visual cortex layer IV. Error bars,  $\pm$ SEM; \*\*\* $P < 0.0001$  by two-way ANOVA; mice,  $n = 2-4$  per age group. (C) Average number of H3K9 foci in WFA+ cells in P100 *scFvPax6*<sup>tg/o</sup> and *scFvOtx2*<sup>tg/o</sup> mice measured by IHC 2 weeks after intraventricular Cre-TAT protein injection. Error bars,  $\pm$ SEM; \* $P < 0.01$  by Student t-test; mice,  $n = 2$  for each genotype.



**Fig. 2. ORF1p expression increases during the CP of plasticity**

(A) Distribution of ORF1p intensity of WFA+ cells of visual cortex in WT mice at P17 (grey), P30 (dark blue) and P100 (light blue);  $***P < 0.001$ ; 337 (P17), 31 (P30) and 66 (P100) neurons were quantified in  $n = 2-5$  per age group; Kolmogorov–Smirnov test. (B) Distribution of ORF1p intensity of WFA+ cells of visual cortex in *scFvPax6<sup>tg/o</sup>* (pink) and *scFvOtx2<sup>tg/o</sup>* mice (red).  $**P < 0.005$ ; 241 (*scFvOtx2<sup>tg/o</sup>*) and 278 (*scFvPax6<sup>tg/o</sup>*) neurons were quantified in  $n = 3$  mice. (C) qRT-qPCR analysis of OTX2 chromatin-immunoprecipitation performed on chromatin extracted from layer IV of V1 lysates with endogenous OTX2.





**Fig. 3.  $\gamma$ H2AX distribution changes specifically in WFA positive cells**

(A) Immunohistochemistry (IHC) of  $\gamma$ H2AX (green), WFA (red) and DAPI (blue) in visual cortex layer IV of WT mice at P30 and P100. Scale bar, 40  $\mu$ m. (B) Average number of  $\gamma$ H2AX foci in WFA-positive (WFA+) and WFA-negative (WFA-) cells during postnatal development in visual cortex layer IV. Error bars,  $\pm$ SEM; \*\*\* $P < 0.0001$  by two-way ANOVA; mice,  $n = 2-5$  per age group. (C) Average number of  $\gamma$ H2AX foci in WFA+ cells in P100 *scFvPax6<sup>tg/o</sup>* and *scFvOtx2<sup>tg/o</sup>* mice measured by IHC 2 weeks after intraventricular Cre-TAT protein injection. Error bars,  $\pm$ SEM; \* $P < 0.005$  by Student t-test; mice,  $n = 2$  for each genotype. (D) IHC of  $\gamma$ H2AX (green), WFA (red) and DAPI (blue) in visual cortex layer IV of Stavudine and Vehicle 3d infused mice. Scale bar, 40  $\mu$ m. (E) Average number of  $\gamma$ H2AX 2 foci in WFA-positive (WFA+) and WFA-negative (WFA-) cells in Stavudine and Vehicle 3d infused mice. Error bars,  $\pm$ SEM; \*\*\* $P < 0.0001$  by two-way ANOVA; mice,  $n = 3$  per age group.

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# DISCUSSION



In this discussion, I will first focus on the transcriptional targets and epigenetic modifications regulated by the transfer of OTX2 into cortical PV interneurons. I will then further discuss the potential role of OTX2 as a key regulator of plasticity in more complex systems, as well as the hypothesis that OTX2 regulates cognitive functions, possibly through a transfer from the VTA to PV cells in higher order cortices.

## I. OTX2 as a regulator of transcription through GADD45 $\beta$ activity

### A. Model for chromatin OTX2 accessibility thresholds

We find that when OTX2 protein levels are transiently increased in PV cells prior to CP onset, *Gadd45 $\beta$ / $\gamma$*  mRNA levels are rapidly increased in a translation-independent manner. This suggests that OTX2 signaling impacts PV cell maturation and function through direct *Gadd45 $\beta$ / $\gamma$*  transcription regulation. In turn, GADD45 $\beta$ / $\gamma$  can control the expression of a swath of genes. The GADD45 family has been implicated in epigenetic gene activation (Barreto et al., 2007; Ma et al., 2009; Gavin et al., 2012), which can impact synaptic plasticity, long-term memory and ultimately animal behavior (Leach et al. 2012; Sultan et al., 2012). Furthermore, *Gadd45* genes have altered expression in mouse V1 after MD (Majdan and Shatz, 2006; Tognini et al., 2015). Thus, changes in GADD45 $\beta$ / $\gamma$  activity could have a broad impact on gene regulation during PV cell maturation essential for CP timing. This is consistent with an expression of plasticity genes at its highest during CP.

One intriguing finding deserving further studies is that OTX2 regulates *Gadd45 $\beta$*  in opposite directions in juvenile and adult mice. Understanding how OTX2 activates *Gadd45 $\beta$*  yet represses it in the adult will likely reveal mechanistic details that might also provide an insight into OTX2 activity in other contexts, such as embryonic development. One possibility is that accessibility of DNA targets of OTX2 changes with OTX2 concentration levels. This possibility is in accordance with the Bicoid (Bcd) HP, which can influence chromatin structure in a concentration-dependent manner. Indeed, the occupancy by Bcd of target sequences is not determined by the binding site but by chromatin accessibility (Hannon, Blythe, and Wieschaus, 2017). In this model, Bcd influences chromatin structure and thus gains access to distinct targets depending on its concentration along the anteroposterior axis of the *Drosophila* embryo at the blastula syncytial stage. This may be a common property of developmental transcription factors that must gain access to their target enhancers during the chromatin remodeling resulting in large-scale transitions in the gene regulatory landscape.



Different concentrations of OTX2 during development (before and during CP) and adulthood could therefore influence its access to promoters and enhancers. Thus, we hypothesize that the OTX2 binding sites on *Gadd45 $\beta$*  are differentially accessible based on OTX2 concentration, whereby certain levels activate while other levels repress transcription. In accordance with chromatin structure changes, our experiments show that specific modifications in the number and size of MeCP2 foci occur specifically in PV cells during plasticity periods. It will be important to determine the foci identity and the epigenetic fingerprint of PV cells since it has been suggested that MeCP2 is implicated in cell-specific epigenetic mechanisms for regulation of gene expression and chromatin structure (Mellen et al., 2012).

This OTX2 concentration-dependent mechanism does not eliminate a more classical mechanism based on the association of the transcription factors with combinations of co-factors that differ before, during and after CPs. For example, during embryonic stem cell differentiation, OTX2 induces enhancer activation by recruiting p300 histone acetyltransferase (Yang et al., 2014). It also mediates transcription inhibition via binding to Groucho/TLE (Agoston and Schulte, 2009; Yasuoka et al., 2014).

## B. GADD45 $\beta$ -mediated regulation of gene expression

DNA modifications provide an important epigenetic layer of regulatory control in gene expression (Baker-Andresen, Ratnu, and Bredy, 2013), and are at work in plasticity-related genes. Recently, Savell and colleagues have shown that changes in DNA methylation regulate *c-Fos* expression by directing DNA methyltransferase (DNMT) activity to the *c-Fos* promoter (Savell et al., 2016). In addition, DNA hydroxymethylation regulates both *c-Fos* and *Arc* expression in the hippocampus (Rudenko et al., 2013), and chronic drug exposure increases DNA methylation at the *c-Fos* and *Arc* promoters, with a profound effect on learning and memory (Massart et al., 2015; Wright et al., 2015). Several signaling pathways that regulate DNA demethylation have also been found to influence gene expression in relation with physiological plasticity (Li et al., 2013, 2014; Kaas et al., 2013; Rudenko et al., 2013; Ratnu, Wei, and Bredy, 2014). The most direct pathway for DNA demethylation involves independent members of the GADD45 family of DNA repair proteins. GADD45 $\alpha$ ,  $\beta$ , and  $\gamma$  proteins have been shown to form complexes with DNA repair enzymes and to guide the removal of 5-mC by either base or nucleotide excision repair (Barreto et al., 2007; Rai et al., 2008; Ma et al., 2009). Previous studies demonstrated that this family is active in different brain areas but with varying

targets and outcomes. For example, global *Gadd45 $\beta$*  KO affects contextual but not cued fear conditioning (Leach et al., 2012). On the other hand GADD45 $\gamma$ , but not GADD45 $\alpha$  nor GADD45 $\beta$ , is required in the prelimbic prefrontal cortex for cued fear conditioning (Li et al., 2019). Interestingly, as already mentioned, *Gadd45* genes have altered expression in mouse V1 after MD (Majdan and Shatz, 2006). Furthermore, P25-old mice subjected to 3 days of MD show an upregulation of *Dnmts* and a downregulation of *Gadd45 $\alpha$* ,  *$\beta$* , and  *$\gamma$*  in V1 contralaterally to the deprived eye (Tognini et al., 2015). The reduction of GADD45 combined with DNMT increase results in an enhancement of DNA methylation in the deprived cortex.

Demethylation of some genes is likely implicated for the establishment of plasticity and we show that GADD45 $\beta$  is involved in this process. In contrast, little is known about the upstream plasticity-mediated mechanisms of its activation. One of these mechanisms is though OTX2 as a direct regulator for *Gadd45 $\beta$*  transcription, which in turn intervenes in the control of plasticity genes. As other studies implicate GADD45 in neuronal plasticity, memory formation, and fear conditioning, it remains possible that OTX2 also regulates *Gadd45 $\beta$*  similarly in regions controlling these other brain functions. Research on GADD45 $\beta$  activity in different brain areas will have to identify in which cell types the expression of members of the GADD45 family is necessary in the context of the latter physiological functions. Our own research demonstrates a specificity of action of GADD45 $\beta/\gamma$  in PV interneurons of V1 layer IV, fundamental for CP regulation. All in all, our data and those of the literature suggest an important role for mechanisms controlling DNA methylation in the experience-dependent refinement of cortical circuits during postnatal development.

### C. LINE-1 the missing link for plasticity

In the first paper (Apulei et al., 2018), we discovered that GADD45 $\beta$  demethylates its own promoter and likely influences the transcription of other plasticity genes, such as *Fos*, *Egr4* and *Nr4a1*. However, a detailed understanding of the molecular mechanisms by which these immediate early genes (IEGs) are regulated during CPs is lacking. My second study shows an increase in *Line-1* transcription at the peak of plasticity, specifically in PV cells, paralleled by an increase in ORF1p. Neuronal activity can also trigger the formation of DNA DSBs in the promoters of a subset of IEG, including *Fos*, *Npas4*, and *Egr1*. The generation of targeted DSBs within *Fos* and *Npas4* promoters is sufficient to induce their expression (Madabhushi et al., 2015). It was demonstrated that activity-

dependent DSBs formation is likely mediated by the type II topoisomerase, Topoisomerase II $\beta$  (Topo II $\beta$ ), and that the knockdown of *TopoII $\beta$*  attenuates both DSB formation and IEG expression following neuronal stimulation (Madabhushi et al., 2015). This phenomenon may be used to control the rapid induction and temporal extent of learning-associated IEGs expression (O'Hagan, Mohammad, and Baylin, 2008; Wossidlo et al., 2010; Morano et al., 2014). Common motifs within promoters of the IEGs suggest the existence of common DNA DSBs and repair targets. Given the fact that, during the peak of plasticity, there is an increase of GADD45 $\beta$  transcription regulated by OTX2 following its internalization, it cannot be excluded that GADD45 $\beta$  demethylates some LINEs inserted in the proximity of plasticity genes. Together, these findings suggest two possibilities. A first one is that GADD45 $\beta$  demethylates several key CpGs in IEG promoters, thus lifting the transcription inhibition by giving access to RNA-PolIII. A second one is that GADD45 $\beta$  demethylates *Line-1* promoter CpGs, enhancing LINE-1 expression and allowing for ORF2p endonuclease activity to introduce nicks in the IEG promoters in a topoisomerase-like activity, thus facilitating their transcription. The latter possibility is illustrated in Figure 19, yet it is clear that further studies are necessary to fully understand how the several levels of regulation might intermingle.

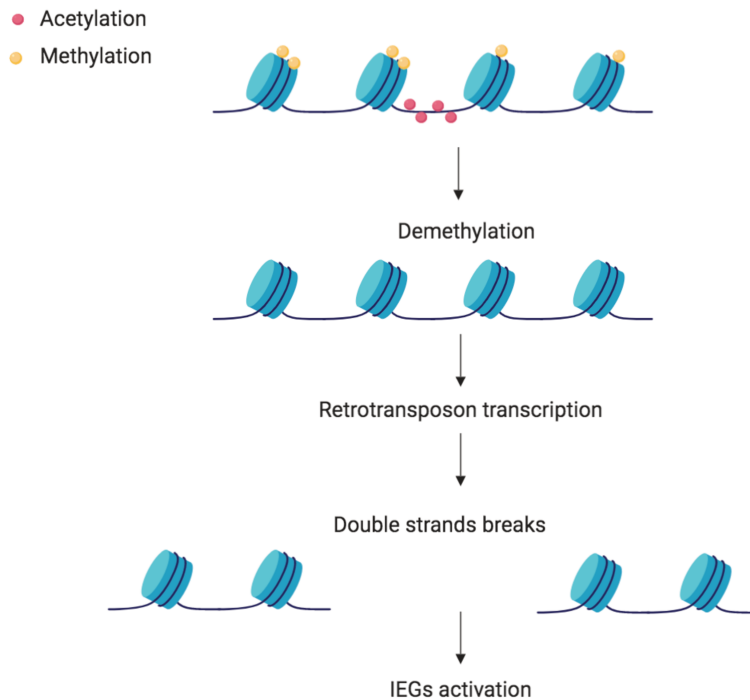


Figure 19 IEGs activation by epigenetic modifications

The induction of expression of IEGs is dependent of *GADD45β*-mediated DNA demethylation, DSB formation though *Line-1* expression, followed by DNA repair (not represented).

#### D. The necessary junk: new functions for TEs

If our hypothesis in which LINE-1 could regulate gene expression is true, this overturns the vision of LINE-1 elements as purely negative. Indeed, LINE-1 were long seen as junk DNA or spurious elements with negative outcomes and only serving as emergency-activated drivers of evolution in the gametes. In support with our hypothesis, recent evidence points to a somatic function for *Line-1* transcripts including cell proliferation (Kuo et al., 1998), differentiation (Mangiacasale et al., 2003), and early embryonic development (Pittoggi et al., 2003). However, it is still unclear how these different retrotransposons are acting and whether TE expression is followed or not by actual retrotransposition. Indeed, TE transcripts might also serve as long non-coding RNA with regulatory functions.

The importance of TEs for brain development has been recently demonstrated (Baillie et al., 2011; Coufal et al., 2009). Controlled retrotransposition during development might provide a selective advantage in defined contexts like pluripotency maintenance or neuronal plasticity (Mita and Boeke, 2016). In the adult, LINE-1 retrotransposition was mainly found in progenitor cells of the hippocampus dentate gyrus (Muotri et al., 2009). This creates genetic diversity among new neurons, providing a possible basis for somatic Darwinian selection in response to environmental challenges. In this context, it was also demonstrated that exercise has a positive effect on LINE-1 retrotransposition *in vivo* (Muotri et al., 2009).

## II. OTX2, a master regulator of cortical plasticity?

Non-cell autonomous OTX2 is present throughout the adult cortex (Spatazza et al., 2013) and its transfer regulates the timing of CPs in other cortices. The CPs for various cortices are spread during postnatal development and OTX2 transfer has been proposed as a general regulator of cortical plasticity (Lee et al., 2017). It will therefore be interesting to examine whether OTX2 targets in V1 identified by our work are also regulated by OTX2 in other cortices. Indeed, some of our RNA-seq data overlap with genome-wide studies in other models of cortical plasticity.

### A. OTX2 non-cell autonomous roles in other cortices

Critical periods for cerebral cortex development, first discovered in V1 by Hubel and Wiesel, also operate in the sensorimotor and auditory areas (Lee et al., 2017; Papale and Hooks, 2018). It is plausible that CPs not only exist for sensory stimuli but also for social behaviors. While the neurobiological mechanisms that govern early development of sensory systems are relatively well-understood, the mechanisms that drive developmental plasticity of higher-order cortical areas require further studies (Larsen and Luna, 2018). For instance, in the rat barrel cortex, induction of adult plasticity through an enriched environment reveals differential expression of genes such as *Arc*, *Egr1-4*, *Nr4a1-2*, *Gadd45b-g*, *Ari4d*, and *Cyr61* (Valles et al., 2011).

As previously explained, the maturation of PV cells leads to an excitatory/inhibitory (E/I) balance shift necessary to open plasticity and to consolidate a non-plastic state after plasticity closure. The E/I shift observed in the prefrontal cortex can be seen as a sign of maturation (CP and transient plasticity) which takes place between early and late adolescence in humans (Insel, 2010).

Accordingly, Wolf Singer's laboratory has reported that the strength of the oscillations driven by a cognitive task (recognition of Moody figures) decreases between early and late adolescence followed by consolidation in the adult (Uhlhaas and Singer, 2010). This recovery after late adolescence does not fully take place in schizophrenic (SZ) patients, suggesting that they have experienced a dysfunction during a CP involving the dorso-lateral prefrontal cortex and possibly other structures (Uhlhaas and Singer, 2010, 2012). Indeed, several reports propose that some psychiatric diseases may find their origin, at least in part, in cortical dysfunctions that occur in a period that precedes the onset of puberty (Dobbs, 2010; Fagiolini, Jensen, and Champagne, 2009; Insel, 2010; Rubenstein, 2010). Defective maturation of PV cells has been reported in the cortex of subjects with SZ (Akbarian, 1995; Hashimoto et al., 2003) and has been proposed as one of the causes of psychiatric phenotypes (Caballero et al., 2014; Fine, Zhang, and Stevens, 2014; Gandal et al., 2012; Marín, 2012; Volk et al., 2012). Perineuronal nets assembly is a mark for layer IV PV cell maturation and/or physiological status throughout the cortex, supporting the idea that similar mechanisms might be at work in the regulation of all CPs. Thus, studying these periods in easily accessible models, such as the establishment of binocular vision, may help to understand basic events leading to higher brain functions and disorders.

As already mentioned, OTX2 protein is specifically internalized in PV during CP. OTX2 has been found not only in PV cells of sensory cortices but also in structures governing more complex systems, such as the amygdala, cingulate and limbic cortices (Spatazza et al., 2013). Critical periods have been proposed for these regions in mice: for fear extinction in the amygdala (Gogolla et al., 2009), for acoustic preference (Yang, Lin, and Hensch, 2012) and for SZ-related symptoms (Belforte et al., 2010). All of these regions are linked to the emotional and anxiety state of the animal. Several findings involving OTX2 in phenotypes related to depression and anxiety are of particular interest in this context. The Nestler laboratory revealed a CP for adult mouse depression regulated at the level of the Ventral Tegmental Area (VTA). Indeed, maternal separation between P10 and P20, but not during P2 to P12 nor after P20 (end of weaning), renders mice more susceptible to depression-like behaviors (social avoidance, anhedonia, immobility upon forced swimming) after social defeat. This imprinting is due to a transient decrease in the expression of *Otx2* in the VTA dopaminergic neurons (Peña et al., 2017).

In addition, preliminary experiments comparing wild type and *Otx2*<sup>+/*GFP*</sup> mice (heterozygous for *Otx2*) have revealed a strong hypo-anxious behavior of the mutant in the elevated plus-maze and light-dark box tests (Figure 20, C. Vincent, J. Gilabert-Juan and colleagues, unpublished observations). This hypo-anxious behavior does not reflect midbrain dopaminergic (mDA) neuron loss in the VTA. It is also not due to concentration changes in DA and DA metabolites (DOPAC, HVA) nor in 5HT and its metabolite (5HIAA) in the cortex (anterior and posterior), hippocampus or amygdala. Strikingly, viral overexpression of OTX2 in the ChP reverses the phenotype, suggesting that anxiety can be regulated by OTX2 coming from the choroid plexus and internalized by specific cells in the cerebral cortex. Accordingly, neutralizing OTX2 secreted from the ChP into the CSF with the scFv strategy (Bernard et al) induces a hypo-anxious behavior in the adult (Vincent and colleagues, unpublished observation).

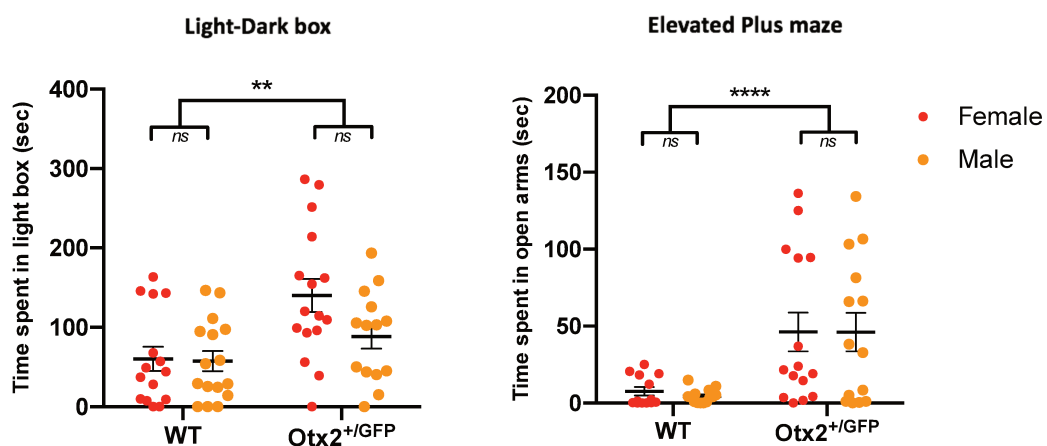


Figure 20. Behavioral analysis revealing alterations of anxiety-related behavior in *Otx2*<sup>+/*GFP*</sup> mice

Adult *Otx2* heterozygote mice show a hypo-anxiety behavior in the Light-Dark and Elevated Plus-maze tests.

An exciting hypothesis is that the VTA could impact cerebral structures controlling mood and cognition in a similar way than OTX2 coming from the eye controls the CP for the V1. This means that a signal originating from the VTA could trigger PNNs assembly, thus allowing for the internalization of OTX2 coming from the ChP, fostering further PV cells maturation. This signal could either be VTA-derived OTX2 or other activity-dependent molecular or physiological changes. Based on studies in V1, the model proposed is that, upon stimulation, a signal at the level of the VTA induces the transfer of information to target structures allowing for initial assembly of PNNs with the

consequence that OTX2 coming from the ChP will recognize OTX2-binding sites within the PNNs and start accumulating into the PV cells.

## B. OTX2 regulating mobile elements in the VTA

Oxidative stress on mDA neurons in the SNpc enhances the expression of full-length mobile elements of the LINE-1 family (Blaudin de Thé et al., 2018). ORF2p endonuclease activity is responsible for the formation of DNA DSBs that accompany oxidative stress. The HP ENGRAILED expressed in the adult SNpc represses *Line-1* expression, thus protecting mDA neurons from oxidative stress-induced damage and death. For this reason, we are interested in studying mobile elements with a focus on OTX2, the VTA and cortical PV cells. Mobile elements such as LINE-1 are increasingly studied in the context of the nervous system where they are expressed in adult neural stem cells and differentiated neurons (Erwin, Marchetto, and Gage, 2014), and where their overexpression has been noted during aging and in psychiatric diseases (Maxwell, Burhans, and Curcio, 2011; Tan et al., 2012; Li et al., 2013; Richardson, Morell, and Faulkner, 2014). The laboratory of Fred Gage recently reported that low maternal care induces *Line-1* expression in the hippocampus and that this increase is correlated with a decrease in the methylation of specific CpG sites in the *Line-1* promoter. While they did not extend their study to other brain regions, our own preliminary experiments demonstrate that LINE-1 expression is increased in the VTA of *Otx2<sup>+/-GFP</sup>* mice at P30 and P100 similarly to what was observed in the SNpc of *En1<sup>+/-</sup>* animals (Figure 21).

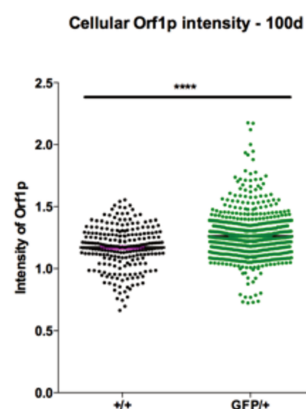


Figure 21. *ORF1p* expression in the VTA

*ORF1p* LINE-1- encoded protein is upregulated in the VTA mDA neurons from the *Otx2<sup>+/-GFP</sup>* mouse



Given that OTX2 protects mDA neurons from oxidative stress similarly to ENGRAILED, and that protection by ENGRAILED involves LINE-1 repression (Rekaik et al., 2015), it is possible that OTX2 represses *Line-1* expression in the VTA, and perhaps in cortical PV cells. Since our preliminary experiments suggest that OTX2 represses *Line-1* expression, it would be interesting to test the hypothesis that some epigenetic changes, primarily DNA methylation, are induced by transient *Line-1* expression in VTA mDA cells. Those epigenetic changes could lead to a permanent depressive state in the adult even after OTX2 expression has resumed normal levels (Nagy and Turecki, 2012). In addition to the repression of LINE-1 by OTX2, PIWIL1 and PIWIL2 (PIWIL4 to a lesser degree) are expressed in the SNpc, VTA, hippocampus, and cortex (Figure 22) (Blaudin de Thé et al, 2018) and could participate in the repression of LINE-1. The upregulation of LINE expression under oxidative stress together with the ability of ENGRAILED and OTX2 to repress LINE expression suggests that OTX2 in the VTA and in PV cells may repress oxidative stress-induced mobile element overexpression and thus protect the cells against genome instability.

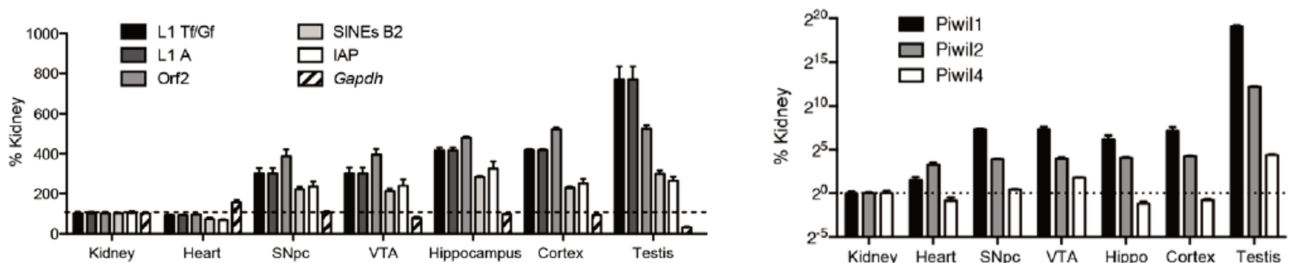


Figure 22. *Line-1* and PIWI expression in different tissues

Left: Expression of the three *Line-1* families, SINE B2 and IAP in different brain regions (compared to kidney). Right: Expression of *Piwil1* (*Miwi*), *Piwil2* and *Piwil4* in the same structures (From Blaudin de Thé et al, 2018).

# PERSPECTIVES AND CONCLUSION

In the cortex, OTX2 controls the maturation of PV cells and the condensation of PNNs, which are two processes essential for the CP temporal onset and closure. My thesis highlights some of the mechanisms of OTX2 for regulating V1 plasticity. OTX2 regulates *Gadd45 $\beta$*  expression by binding

to its promoter and *Gadd45β* expression is strongly correlated with plasticity genes (*Arc*, *Egr2*, *Egr4*, *Fos*). Indeed, GADD45β expression in adults is sufficient to reactivate plasticity. The methylation of plasticity gene promoters seemed regulated by GADD45β and PV cell MeCP2 foci number is correlated with plasticity, which strongly suggests that epigenetic changes regulate plasticity status. Variations in chromatin compaction could explain the dual action of OTX2, as an activator of plasticity at P20 and a repressor at P40. One limitation of our approach is that we only quantified methylation within promoters of selected genes and did not consider the spatial conformation of the chromatin. In order to study the global effect of OTX2 on PV cells chromatin structure, it will be necessary to perform whole-genome sequencing after bisulfite conversion. Chromosome conformation capture (3C) experiments will further map changes in long-range contacts. Obtaining the epigenetic signature of PV cells at different stages during developmental stages will be a key step in our understanding of OTX2 mechanisms in the broader context of cerebral cortex plasticity.

Interestingly, the various epigenetic modifications identified occur specifically in cortical PV interneurons. In order to study these specific changes, I started developing a tool to isolate PV cells by FACS. Based on the observation that infusion of OTX2 in the brain results in the specific capture by PV cells, we made a recombinant protein containing OTX2 N-terminus and homeodomain fused to GFP. This allows for specific labeling of PV cells after injection in the brain parenchyma (Figure 23).

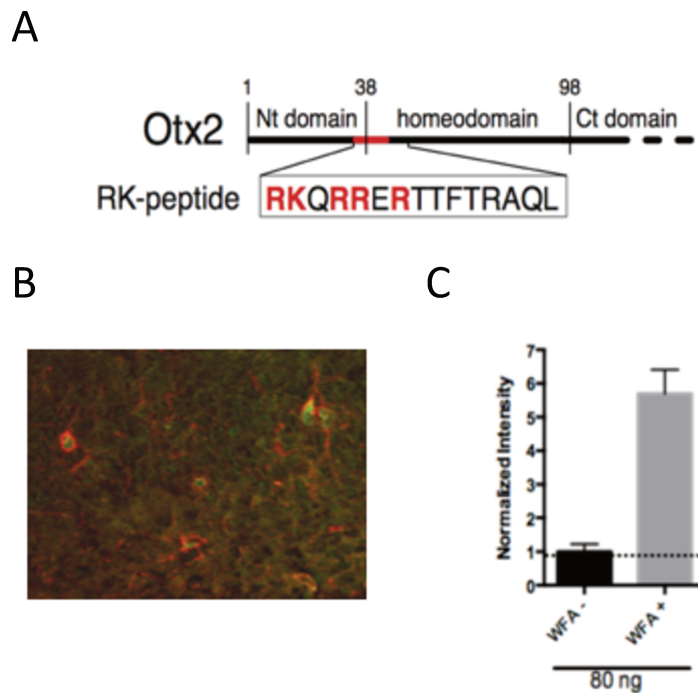


Figure 23. Gaining access to PV cells

The RK peptide (A) and homeodomain-linked to GFP allow specific addressing to PNNs expressing (WFA<sup>+</sup>) cells (B). The homeodomain can be mutated in position 50, thus made inactive but still allowing internalization. (C) Fluorescence activated cell sorting shows a 6-fold increase in WFA<sup>+</sup> cells.

This fusion protein also has an engineered mutation within the DNA-binding domain that disrupts OTX2 activity in order to provide a tool to label PV cells. The advantage of this technique is that it can be used in any mouse model as long as PNNs are formed around PV cells. Another possibility is to cross PV::Cre mice with NuTRAP mice. The NuTRAP technique allows a simultaneous isolation of cell-type specific mRNA in translation and the chromatin from any cell type for which a Cre line exist. This technique combines the TRAP (Translating Ribosome Affinity Purification) method, which allows biochemical isolation of ribosome-bound mRNA through a fluorescent protein-tagged ribosomal protein expressed in a specific cell type, and INTACT (Isolation of nuclei tagged in specific cell types) method, which allows the affinity purification of tagged nuclei through a labeled nuclear membrane protein (Roh et al., 2017). The advantage of this method is that it is robust even with low input samples, minimizing the number of animals required for such studies.

In the second article, I have shown that in PV cells there is a transient increase in DSBs during CP. Cortical injection of a retrotransposition inhibitor confirmed that retrotransposon activity participates in DSB formation specifically in PV cells. I also discovered that OTX2 is able to bind to the promoter of *Line-1* and might thus directly control their expression. Indeed, it has already been demonstrated by Lennartsson and colleagues that TE expression can occur in V1 (Lennartsson et al., 2015). Our use of a general retrotransposition inhibitor does not tell us anything about the type of TEs expressed in PV cells and the control of LINE-1 expression by OTX2 needs further investigation. Whatsoever, direct or indirect OTX2-controlled LINE-1 expression might allow the transcription of plasticity genes through DNA break formation in plasticity gene promoters. Another possibility is that OTX2 controls the opening of the chromatin via GADD45 $\beta$  which in turn allows LINE-1 expression with the ensuing production of DSBs. It will be necessary in the future to verify these hypotheses and how they cohabitate, or not.

If OTX2 is a general regulator of CP plasticity across the cerebral cortex, these genes/pathways may also be modified at other time points in other cortices. In order to study the role of OTX2 in other cortices, it would be interesting to block OTX2 transfer in the adult and examine changes in gene expression and epigenetics. Several studies propose that some psychiatric diseases may find their origin, at least in part, in cortical dysfunctions occurring during the period that precedes puberty onset. Interestingly, defective maturation of PV cells has been reported in the cortex of subjects with SZ and has been proposed as one of the causes of psychiatric phenotypes (Caballero et al., 2014; Fine et al., 2014; Gandal et al., 2012; Marín, 2012; Volk et al., 2012). Furthermore, PNNs assembly has also been linked to SZ: PNNs density is reduced in the amygdala and the entorhinal and prefrontal cortices of subjects with schizophrenia (Paylor et al., 2016). Blocking OTX2 transfer in the adult can reopen a window of plasticity in the cortex: examining changes in gene expression in reactivated adult plasticity might provide new strategies to reopen plasticity in the adult cortex. By studying the regions implicated in hypo-anxious phenotypes in *Otx2*<sup>+GFP</sup> mice, we could draw a parallel between the role of OTX2 during postnatal development and psychiatric disorders.

Identifying the non-cell autonomous roles of OTX2 in specific brain regions could be achieved by injection of Cre-expressing viruses in target regions of the scFv-OTX2 mouse. Alternatively, conditional scFv-OTX2 viruses could be injected in target regions of PV::Cre or TH::Cre mice. Such strategies will permit the expression and secretion of the antibodies in a particular cortical region of

interest and help identify the sources and target regions of the transfer of OTX2 in the hypo-anxious phenotype.

## GENERAL CONCLUSION

The aim of my project was to understand some mechanisms by which OTX2 regulates CPs of plasticity through transcriptional mechanisms and epigenetic modifications. Thanks to my studies we have a better idea of these mechanisms (Figure 24). In my first study, we demonstrated that OTX2, once internalized, is able to control the transcription of some plasticity genes via the action of GADD45 $\beta$ , which in turn participates in the demethylation of plasticity gene promoters. In my second study, I have brought to light that during the peak of plasticity there is a remodeling of chromatin and an increase in DSBs. Importantly, we show that OTX2 is able to bind the *Line-1* promoters. It is known that LINEs can cause DSBs (Thomas et al., 2012). This could facilitate the access of RNA-PolIII and induce the transcription of plasticity genes (Madabhushi et al., 2015). Finally the two models proposed are not exclusive as illustrated in the conclusion figure.

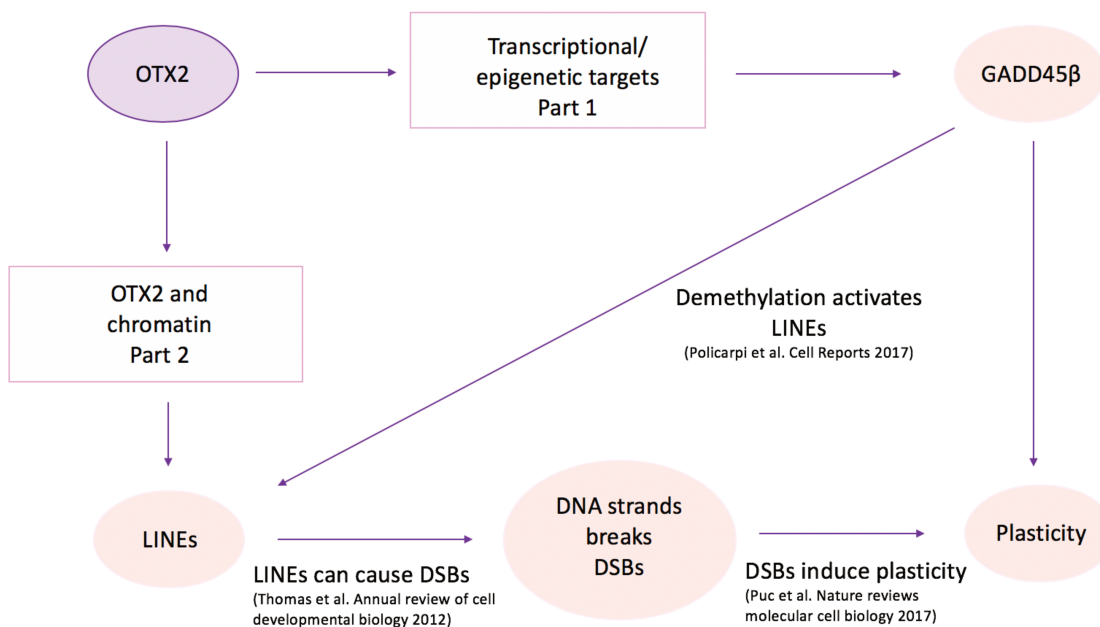


Figure 24. Mechanisms by which OTX2 may control plasticity

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During postnatal development, the cerebral cortex has limited temporal periods of high plasticity, called critical periods (CPs). These windows, which allow neural circuitry to be shaped by external stimuli, are found in many cortical regions and are regulated by several factors that influence the balance between excitation and inhibition. The most studied CP is that of ocular dominance in the visual cortex, and the OTX2 homeoprotein transcription factor plays an essential role. OTX2 is transferred from extra-cortical sources into a specific class of interneurons, the parvalbumin (PV) cells, and this transfer controls CP onset and closure in several cortical regions. The aim of my thesis was to reveal the mechanisms underlying OTX2 non-cell autonomous activity implicated in CP regulation.

My first objective was to identify the targets of OTX2 in PV cells for the control of the ocular dominance CP within V1. We sought direct transcription targets of OTX2 through *in vivo* and biochemical approaches, and we discovered that OTX2 binds to the *Gadd45 $\beta$ / $\gamma$*  promoter. Cortical OTX2 infusion in juvenile mice induced *Gadd45 $\beta$ / $\gamma$*  expression through direct regulation of transcription. Intriguingly, a reverse effect was found in the adult, where reducing cortical OTX2 resulted in *Gadd45 $\beta$ / $\gamma$*  upregulation. Viral expression of *Gadd45 $\beta$*  in adult visual cortex activated plasticity gene expression and directly induced ocular dominance plasticity. One function of GADD45 $\beta$  is to participate in activity-induced DNA demethylation. Indeed, this *Gadd45 $\beta$*  overexpression in adult resulted in concomitant changes in MeCP2 foci within PV interneurons and in methylation states of several plasticity gene promoters, suggesting epigenetic regulation.

My second objective was to explore whether OTX2 could control the maturation of PV cells through epigenetic modifications. I found different epigenetic markers could be modified by OTX2 during juvenile development and in the re-opening of adult plasticity. I identified markers associated with mobile element expression, suggesting CPs involve genomic instability. I found that LINE-1 retrotransposon expression in V1 is heightened during juvenile development and adult plasticity, and characterized an interaction between LINE-1 promoter and OTX2.

Altogether my work extends our current understanding of cortical plasticity and the role of OTX2 during CPs. I have shown that non-cell autonomous OTX2 regulates cortical postnatal plasticity through direct transcriptional mechanisms and epigenetic modifications.