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TRKB SIGNALING CONTROLS THE DEVELOPMENTAL EXPRESSION OF NMDAR SUBUNITS IN RAT AND MOUSE VISUAL CORTEX

Thesis submitted for the degree of *Doctor Philosophiae*

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NOTES

The work described in this thesis was carried out at the International School for Advanced Studies, Trieste, Italy, between November 1997 and September 2001. All work reported, with the exceptions listed below, arises solely from my own experiments.

Cloning of immunoadhesins into baculovirus infection system was performed by Dr. Nicola Gargano at MRC, Laboratory of Molecular Biology, Cambridge, UK. I would like to thank him for providing the baculovirus and the anti-camel antibody, and helping me when I started to work with immunoadhesins.

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ABSTRACT

NMDA receptors (NMDARs) are multimeric proteins whose biological and functional characteristics depend on differential subunits assembly during postnatal development. In the present work we deal with the question as to whether the expression of the different NMDAR subunits is influenced by neurotrophins in developing visual cortex. We used a soluble form of TrkB receptor engineered as an immunoadhesin (TrkB-IgG) in order to block TrkB ligands in the rat visual cortex. TrkB-IgG was released through a cannula implanted in the occipital pole and connected to a mini-osmotic pump. TrkB-IgG was continuously released from postnatal day 20-21 (P20-21) to P36-37. These two post-natal ages were chosen for the beginning and the end of the treatment because they represent the peak and the end of the critical period in the visual cortex respectively. In a different group of animals used as controls osmotic pumps were filled with saline. Different antibodies were used to stain neurons expressing NR1, NR2A and NR2B. We counted the number of neurons stained for NR2A and NR2B subunits and expressed it as percentage with respect to the total number of cresyl-violet stained neurons in each cortical layer. In the visual cortex of TrkB-IgG treated rats the percentage of neurons expressing NR2A was significantly increased in all cortical layers. Concerning NR2B subunit, the percentage of stained neurons was not significantly different between TrkB-IgG treated and control rats. The intensity of staining for both NR2A and NR2B was reduced in all cortical layers in TrkB-IgG treated animals with respect to controls. In accordance with these results, the endogenous levels of NR2A and NR2B subunits were reduced in TrkB-IgG treated animals as shown by western blot.

Application of TrkB-IgG presented the disadvantage to block at the same time both TrkB ligands: BDNF and NT-4/5. In order to study separately the effects of deprivation of these neurotrophins we acquired knock-out (ko) mice for BDNF and NT-4/5. We considered 3 post-natal ages (P12-14, before eye-opening; P21-23, peak of critical period; adulthood) and we compared the levels of expression of NMDAR subunits NR1, NR2A and NR2B by immunocyology and western blot, in wild type and in ko animals. The absence of BDNF determines a reduction in the level of NR2A at an early stage of

post-natal development, while NT-4/5 more likely exerts a modulatory action on both subunits.

Thus, TrkB signalling controls the cellular expression of NMDAR subunits in rat and mouse visual cortical neurons during postnatal development and its action is specific for NR2A and NR2B subunits without affecting NR1, whose development is almost over at the time we started our analysis.

1. INTRODUCTION

1.1 NMDA receptors

Most of the brain's excitatory synapses use the amino acid glutamate as a neurotransmitter, released from the presynaptic terminal in packets (Monaghan *et al.*, 1989). The postsynaptic membrane is specialized for the reception of glutamate signals and for the transduction of these signals into the postsynaptic cell. A specialized region of postsynaptic membrane, visible by electron microscopy as a thickening (~30 nm thick), and known as the postsynaptic density (PSD) contains a high concentration of glutamate receptors together with associated cytoskeletal and signaling proteins.

Glutamate receptors (GluR) are classified on the basis of their pharmacological, electrophysiological and biochemical characteristics into ionotropic and metabotropic types. Ionotropic receptors are ion channels permeable to cations and are chemically regulated, while metabotropic ones are coupled to G proteins activating in turn an intracellular second messenger cascade.

Ionotropic receptors are classified (Monaghan *et al.*, 1989) according to their different agonists into: NMDA receptors (NMDARs, whose agonist is N-methyl-D-aspartate); AMPA receptors (whose agonist is α-amino-3-hydroxy-5-methyl-4-isossazolopropionic acid) and KA receptors (whose agonist is kainate). AMPA and KA receptors are commonly grouped together as non-NMDA receptors since they share common characteristics (Moriyoshi *et al.*, 1991).

NMDARs (Fig. 1) are glutamate binding calcium channels activated in the presence of the co-agonist glycine and inhibited, in a voltage-dependent way, by Mg^{2+}. In addition, NMDARs present binding sites for Zn^{2+} (modulator; Westbrook and Meyer, 1987; Hollmann *et al.*, 1993), polyamines (Hollmann *et al.*, 1993) and drugs of the fencyclidin family (current blockers; Nowak *et al.*, 1984; Moriyoshi *et al.*, 1991).

NMDARs are multimeric proteins composed of subunits assembled in 3 different classes: NR1, NR2 and NR3.

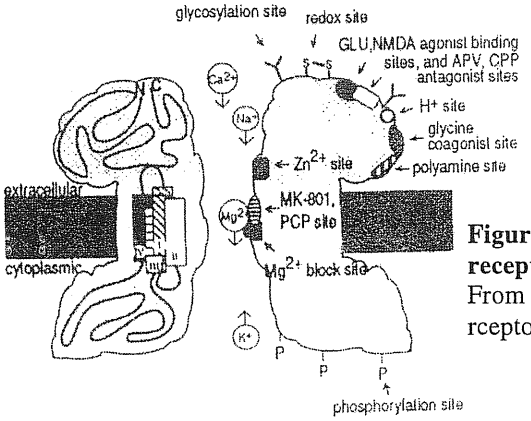


Figure 1. Schematic representation of the NMDA receptor.
From Hollmann and Heinemann. (1994) Cloned glutamate rceptors. *Annu. Rev. Neurosci.*; 17: 31-108

The first subunit (NR1) has been cloned by Moriyoshi *et al.* (1991) by expressing mRNAs obtained from a rat cDNA library in Xenopus oocytes (Masu *et al.*, 1987; Julius *et al.*, 1988). Electrophysiological recordings from oocytes allow a strong response elicited by NMDA, which is peculiar to NMDARs, to be isolated and, consequently, the cDNA clones able to provoke it to be identified.

The NR1 cDNA sequence shows a low but significant homology (22-29% of identity) with genes coding for other glutamate ionotropic receptor subunits. The gene for the NR1 subunit consists of 21 introns and 22 exons: 3 of these exons (5, 21, 22) undergo alternative splicing, giving 8 different splicing forms (Zukin and Bennet, 1995). Seven have in fact been identified in cDNA libraries (Durand *et al.*, 1992; Sugihara *et al.*, 1992); the eighth one has been artificially synthetized (Hollmann *et al.*, 1993). Exon 5 codes a 21 aminoacids (aa) sequence, called N1, at the N-terminal domain. Exons 21 and 22 codify 2 sequences (C1 and C2 respectively) corresponding to the last tract of the C-terminal domain. The 8 splicing forms come from combining 4 different configurations at the C-terminus with 2 different ones at the N-terminus. The corresponding mRNAs are identified with subscripts indicating the presence (1) or the absence (0) of exons 5, 21 and 22 beginning from the 5' end of the mRNA (Durand *et al.*, 1993).

This knowledge has been the starting point to design PCR primers complementary to the most conserved DNA sequences shared by NR1 subunit and other Glu receptors. Such primers allowed the amplification of various rat (Monyer *et al.*, 1992; Ishii *et al.*, 1993) and mouse (Ikeda *et al.*, 1992; Kutsuwada *et al.*, 1992; Meguro *et al.*, 1992) cDNAs. This

step has led to the identification of 4 new subunits with low homology to both other GluRs and NR1 itself (21-27% aa identity), but with high identity in their aminoacid sequences (42-56%). Thus they constitute a new class named, in the rat, NR2. The different subunits are identified as NR2A, B, C and D and are coded by 4 different genes. They associate with the NR1 subunit to form heteromeric receptors. Homomers NR2/NR2 are not functional, while experiments performed in knockout mice lacking the gene for NR1 subunit confirmed NR1 is essential for receptors to be functional (Forrest *et al.*, 1994).

With the same PCR technique, another cDNA has been isolated: it codes for a Glu ionotropic receptor subunit sharing 27% identity at the level of the aminoacid sequence with the other NMDAR subunits, but belonging to a new class; recently it has been named NR3A (Das *et al.*, 1998).

Electrophysiological studies on recombinant NMDARs expressed in *Xenopus laevis* have demonstrated that each of the NR1 subunit splicing forms, except for the truncated one, can give functional homomeric receptors with the properties of the native one (Sugihara *et al.*, 1992). In fact, all binding sites for agonists, antagonists and modulators are present in the homomeric receptor, but the current amplitude is smaller than in the heteromeric ones.

The NR2 subunit, when expressed in oocytes in association with NR1, modulates the activity of the receptor.

Co-immunoprecipitation on rat cortical extracts with specific antibodies of NR1 and NR2 subunits can give some information about receptor composition *in vivo* (Sheng *et al.*, 1994; Luo *et al.*, 1997). The association of NR1 subunit with both NR2A and NR2B is the most frequently represented while heteromers NR1/NR2A or NR1/NR2B are less frequent. Recently it has been proposed that NMDARs are tetrameric proteins (Laube *et al.*, 1998).

Exploiting again the method of expressing the receptors in oocytes, it has been shown that the various splicing forms of the NR1 subunit contribute to define the following physiological and pharmacological properties of the receptor: affinity for agonists, potentiation by polyamines, modulation by Zn^{2+}, regulation by PKC and changes in

current amplitudes (Durand *et al.*, 1992 and 1993; Nakanishi *et al.*, 1992; Zheng *et al.*, 1994; Hollmann *et al.*, 1993; van Hooft *et al.*, 1998).

Co-expression of NR1 and NR2 influences affinity for agonists, potentiation induced by polyamines and Zn^{2+}, inhibition led by Mg^{2+} and channel opening kinetics (Kutsuwada *et al.*, 1992; Stern *et al.*, 1992; Williams *et al.*, 1995; Monyer *et al.*, 1992, 1994; Ishii *et al.*, 1993).

The NR3A subunit expressed in *Xenopus laevis* oocytes weakens the response of NR1/NR2 channels (Ciabarra *et al.*, 1995; Sucher *et al.*, 1995). Other studies were performed on knockout mice unable to produce NR3A protein: these led to the conclusion that this subunit is a regulatory one, involved in synaptic spine development. Knock out mice in fact showed both higher dendritic arborization and abnormalities on spines (Das *et al.*, 1998).

As the composition of NMDARs greatly influences the properties of the channel, the distribution of subunits has been widely studied with respect both to cellular specificity and changes occurring during development.

1.2 mRNA localization and subunit distribution

In situ hybridization and immunohistochemistry studies have demonstrated that the localization of NR2 subunits and NR1 splicing forms is developmentally regulated.

The NR1 subunit is abundant in almost all regions of adult rat brain (neocortex, CA1, CA3, dentate gyrus, thalamic nuclei, striatum, cerebellum); again this evidence suggests that NR1 is an essential component of the receptor (Moriyoshi *et al.*, 1991; Monyer *et al.*, 1992). Even though this subunit is almost ubiquitous in the central nervous system, its splicing forms are heterogeneously distributed, e.g. $NR1_{1XX}$ are present mostly in CA3, in some thalamic nuclei and in the cerebellar granular layer (Standaert *et al.*, 1994). In general, the distribution of NR1 subunits (i.e. proteins) in adult rat brain, defined by immunolocalization, is comparable to the mRNA distribution (Aoki *et al.*, 1994; Petralia *et al.*, 1994a). The NR1 subunit is also widely expressed in the adult mouse brain (Watanabe *et al.*, 1992).

During rat development, NR1 mRNA is slightly detectable at E14; its level then increases gradually until the third postnatal week, before gradually declining again to a stable level at adulthood (Laurie and Seeburg, 1994). Immunoblotting experiments have shown that the amount of protein is also low at birth and increases until the third postnatal week (Luo *et al.*, 1996).

As in other regions of the brain, in the rat visual cortex the laminar distribution of the NR1 subunit develops during the first three post-natal weeks, remaining stable thereafter; it is widely distributed throughout cortical layers and the same pattern is present also in adult animals. In contrast, the level of its mRNA decreases in adult animals (Nowicka and Kaczmarek, 1996).

mRNA localization for the four NR2 subunits shows different distributions in different regions of the rat and mouse brain (Laurie *et al.*, 1997). Like NR1, NR2A mRNA is widely distributed in prosencephalon and cerebellum; NR2B is mainly expressed in the prosencephalon while NR2C is present in the granular layer of cerebellum (Monyer *et al.*, 1994). NR2D is characterized by a limited distribution in the adult brain: in the cortex it is selectively expressed by interneurons, while its mRNA is almost absent in pyramidal neurons (Standaert *et al.*, 1996).

In the adult rat brain, while the NR2A protein is ubiquitous, NR2B is restricted to prosencephalon, NR2C to cerebellum and NR2D to diencephalon and mesencephalon (Petralia *et al.*, 1994b; Wang *et al.*, 1995a; Wenzel *et al.*, 1995).

As for NR1, the expression of NR2 subunits is also developmentally regulated: NR2B and NR2D mRNAs are present before birth, while mRNAs for NR2A and NR2C are detectable only at P0. Their peak of expression is at the third postnatal week, with the exception of NR2D mRNA, which starts to decline at P7 (Monyer *et al.*, 1994).

During the early postnatal period, the NR2B subunit is expressed consistently in hippocampus, while NR2A and NR2C expression is low in all regions of the prosencephalon until P12, when it increases reaching adult levels (Monyer *et al.*, 1994; Watanabe *et al.*, 1992). Taking advantage of immunoprecipitation techniques on rat cortical proteins, Sheng and collaborators (1994) demonstrated that NR2A protein, not detectable at birth, increases during the first three postnatal weeks then and staying stable

until adulthood. NR2B is maximal between P14 and P22 and decreases significantly in adulthood (Cao *et al.*, 2000).

In situ hybridization and northern blot experiments have shown that, in rodents', the NR3A subunit is consistently expressed during the first two postnatal weeks. In adult animals, transcripts decrease to 25% of the quantity detectable at P1. On the other hand the spatial distribution is similar after birth and major levels of expression can be detected in several thalamic nuclei, in CA1 cells and in the cortex (Ciabarra *et al.*, 1995; Sucher *et al.*, 1995).

1.3 Role of NMDARs in neuronal plasticity

Synaptic plasticity is a general term used to describe a great variety of activity-dependent changes in neuronal structure and function. In 1949, the Canadian psychologist Hebb proposed that neuronal plasticity is governed by associative processes between pre- and post-synaptic neurons. During the '70s, the pivotal experiment by Bliss and Lomo (1973) provided the basis for understanding the cellular mechanisms underlying associative neuronal plasticity. They found that an intense pre-synaptic stimulation of the perforant pathway in hippocampus led to a persistent increase of post-synaptic response in the dentate gyrus. They termed this form of synaptic plasticity long-term potentiation (LTP). Subsequently, LTP has been related to neuronal processes like learning and memory (Bliss and Collingridge, 1993; Nicoll and Malenka, 1995). The relationship between associative hebbian plasticity was confirmed in 1986 by experiments showing that low-frequency, low intensity stimuli could produce robust LTP, if repeatedly paired with depolarizing pulses delivered through an intracellular recording electrode (Kelso *et al.* 1986; Sastry *et al.* 1986).

To complete the description of LTP induction, the element needed was a molecular coincidence detector, able to respond to the conjunction of activity in afferent fibres and adequate depolarization in target dendrites.

The first demonstration of the key role of NMDARs in this process involved the blockade of LTP induction by NMDAR antagonists, such as 2-amino-5- phosphovaleric acid (APV) in hippocampal slices (Collingridge *et al.*, 1983). The NMDA receptor satisfies

the requirements for a fundamental role in associative synaptic plasticity. In fact this receptor allows current to flow only when two conditions are simultaneously satisfied: binding of the neurotransmitter released after pre-synaptic activation and depolarization in the post-synaptic cell to release the voltage-dependent block of the Ca^{2+} channel due to Mg^{2+}. Thus, NMDAR is a coincidence detector able to associate afferent electrical activity with the level of neuronal activation, expressed as membrane depolarization, of the post-synaptic cell.

The role of NMDARs in the control of synaptic plasticity has been extensively studied. Because the induction of LTP by tetanic stimulation is prevented by a variety of NMDA antagonists (e.g. AP5, MK-801, Collingridge *et al.*, 1983; Coan *et al.*, 1987), it is clear that the activation of these receptors is an essential trigger for the process. However, the application of NMDA itself is not usually enough to induce LTP (though it readily induces short-term potentiation, STP; Collingridge *et al.*, 1983; Kauer *et al.*, 1988). In an important study, it was found that the induction of LTP could be blocked by intracellular injection of the Ca^{2+} chelator EGTA (Lynch *et al.*, 1983). Because NMDARs are permeable to Ca^{2+}, it is widely assumed that permeation through these channels during tetanic stimulation provides the Ca^{2+} signal necessary for the induction of LTP. Ca^{2+} released from internal stores may also play a role in induction of LTP (Bortolotto and Collingridge, 1993). Since the application of ryanodine or thapsigargin (drugs inhibiting Ca^{2+}-induced Ca^{2+} release and depleting intracellular Ca^{2+} stores respectively), results in substantial reduction of the Ca^{2+} transient associated with synaptic activation of NMDARs (Harvey and Collingridge, 1992), it seems that Ca^{2+} which permeates NMDA channels is increased by Ca^{2+} release from intracellular stores.

Several different Ca^{2+}-sensitive enzymes have been proposed to play a role in converting the entry of Ca^{2+} through NMDARs, the most likely induction signal, into persistent modifications of synaptic strength. One of these enzymes is the Ca^{2+}/calmodulin-dependent protein kinase (CaMKII; Malenka *et al.*, 1989). CaMKII directly interacts with both NR1 and NR2B (Leonard *et al.*, 1999). This interaction places CaMKII in an ideal position for stimulation by Ca^{2+} influx through NMDARs, and brings the kinase in close proximity to AMPA receptors that are phosphorylated and subsequently up-regulated by CaMKII upon NMDAR- mediated Ca^{2+} influx (Lisman *et al.*, 1997; Barria *et al.*, 1997).

A recent study (Gardoni *et al.*, 2001) has demonstrated that NR2A also binds directly to CaMKII, while PSD-95, an abundant post-synaptic density (PSD) protein, competes with the kinase for the same binding domain. Moreover, CaMKII association to NR2A can be affected by activation of the receptor *in vitro* by either pharmacological tools or induction of LTP. Having multiple domains binding to a variety of cytoplasmic proteins, PSD-95 functions as a scaffold for assembling a specific set of signaling proteins around the NMDARs. Thus, an extensive network of protein-protein interactions within the PSD links NMDARs to other classes of post-synaptic glutamate receptors, and couples them to specific intracellular signaling pathways involved in the induction and maintenance of LTP.

Recent experiments suggested the idea that modifications in post-synaptic glutamate receptor levels represent the molecular basis for long-lasting regulation of synaptic strength. Data consistent with this have been obtained from aged rats characterized by a deficit in LTP, learning and memory. Clayton and Browning (2001) demonstrated that these rats have reduced expression of NMDAR subunits. In fact, aged rats have significantly lower levels of NR2B mRNA and protein compared to young animals, whilst no changes are observed in either mRNA or protein level of the NR2A subunit, or in the AMPA GluR2 subunit. Moreover it has been reported (Heynen *et al.*, 2001) that: 1) in the adult brain *in vivo,* synaptic glutamate receptor trafficking is bi-directional and reversibly modified by NMDA receptor-dependent synaptic plasticity; 2) changes in glutamate receptor protein levels accurately predict changes in synaptic strength. These findings indicate that memory can be encoded by the precise experience-dependent assignment of glutamate receptors to synapses, and that alterations in the expression of NMDAR subunits underlies deficits in both LTP and learning/memory.

1.4 NMDARs and neuronal plasticity in developing visual cortex

The mechanism proposed by Hebb in 1949 as the cellular basis for learning was reintroduced by Stent in 1973 in order to explain plasticity in cat visual cortex during the critical period. This period of post-natal mammalian development represents a time window characterized by a high degree of plasticity of thalamo-cortical connections. The

length and duration of the critical period varies between different species. In addition, almost overlapping with the critical period, during early and middle postnatal development, visual cortical connections undergo processes of refinement leading visual cortical network to assume the morphological, biochemical and functional characteristics of adult animals. Afferent electrical activity triggered by vision is the driving force in the active shaping process in the developing visual cortex. It is well-known in mammals, that deprivation of visual input from birth (dark rearing) induces a delay in the maturation of the visual cortex and maintains plastic thalamo-cortical connections after the end of the critical period (Timney *et al.*, 1978; Cynader, 1983; Mower, 1991). The classic paradigm of monocular deprivation was introduced by Wiesel and Hubel (1963) in kitten visual cortex and subsequently applied to other mammalian species (Baker *et al.*, 1974; Drager and Hubel, 1978; Boothe *et al.*, 1985), and used to study visual cortical plasticity during the critical period. The paradigm consists of disrupting vision in one eye by suturing the eyelid. If this manipulation is performed during the critical period, visual cortical neurons cease to respond to the deprived eye, and the territories occupied by thalamic terminals driven by the deprived eye shrink (ocular dominance shrinkage, Shatz and Stryker, 1978; LeVay *et al.*, 1980). In addition, the cell bodies of geniculate neurons receiving inputs from the deprived eye, and sending fibers to the corresponding ocular dominance columns, shrink (Guillery and Stelzner, 1970; Sherman *et al.*, 1974). In subsequent years, the physiological role of afferent neuronal activity in visual cortical plasticity has been studied in detail (for reviews see, Shatz, 1990; Katz and Shatz, 1996; Katz, 1999). The effects of monocular deprivation are activity-dependent and imply competition between thalamic inputs to visual cortical neurons, causing synapses to strengthen or weaken. Indeed, most cells in supragranular and infragranular layers of primary visual cortex receive inputs from both eyes, and therefore are called binocular. The quality and quantity of afferent electrical activity in relationship to post-synaptic neuronal activity determines the fate of synapses (Reiter and Stryker, 1988). In monocular deprivation, the quantity and quality of neuronal activity of thalamic fibers driven by the deprived eye is poor and vague correlated to activity of the postsynaptic target neurons: therefore synapses weaken and finally are lost. This type of synaptic plasticity is considered to be a hebbian process, since it is mediated by the coincidence of pre- and post-synaptic

activity, as explained in the previous chapter. The NMDA receptor satisfies the requirements to play a fundamental role in refining activity-dependent connections in primary visual cortex. Moreover, NMDARs possess other characteristics, which indicate their involvement in post-natal development and plasticity of visual cortex. First, in the developing visual cortex NMDAR function is regulated by visual experience (Fox and Daw, 1993; Fox and Zahs, 1994); in particular the post-natal decrease in the NMDA component of the visual response does not occur in dark reared kittens. Subsequently, it has been demonstrated that infusion of 2-amino-5-phosphovaleric acid (APV), a receptor antagonist, in cortex prevents the functional effects of monocular deprivation during the critical period (Bear *et al.*, 1990). Anatomically, infusion of APV blocks shrinkage of LGN neurons from occurring in monocularly deprived kittens (Bear and Colman, 1990). However, antagonists such as APV decrease geniculo-cortical synaptic transmission (Kasamatsu *et al.*, 1998); hence it is hard to separate effects due to blockade of the receptors on experience-dependent plasticity, from possible alterations of the maturation processes due to a decrease in the cells' ability to respond.

Further evidences comes from studies on NMDAR expression in developing visual cortex. Studies regarding the regulation of NR1 subunit splicing variants and NR2 subunits during development strengthen the hypothesis that the NMDA receptor is involved in refining connections during the critical period (Laurie and Seeburg, 1994; Monyer *et al.*, 1994). In fact, the NR2B subunit is mainly expressed during the early postnatal period, while NR2A increases during the critical period. Together with this change in subunit composition, the response of the channel to its ligand changes in a development-related way; in visual cortex before eye opening, the kinetics of channel inactivation is slower than the kinetics of mature receptor (Carmignoto and Vicini, 1992). This modification is due not only to NR2 regulatory subunits but also to the NR1 splicing variants containing ($NR1_{1XX}$) or not ($NR1_{0XX}$) N1 insert (van Hooft *et al.*, 1998). $NR1_{1XX}$, expressed with NR2 subunit, characterizes receptors with a kinetic of inactivation faster than the second one, thus being an important factor in determining the duration of excitatory post-synaptic currents (EPSCs).

1.5 Neurotrophins and their receptors

Neurotrophins constitute a family of trophic proteins regulating the survival and differentiation of developing neurons, and contributing to the maintenance of their specific functions during adult life (for review see Levi-Montalcini, 1987; Barde, 1990). The neurotrophins characterized to date are nerve growth factor (NGF; Levi-Montalcini, 1951; Cohen, 1960), brain-derived neurotrophic factor (BDNF; Barde *et al.*, 1982; Leibrock *et al.*, 1989; Ernfors *et al.*, 1990a), neurotrophin-3 (NT-3; Ernfors *et al.*, 1990b; Jones and Reichardt, 1990; Kaisho *et al.*, 1990), and neurotrophin-4/5 (NT-4/5; Berkemeier *et al.*, 1991; Hallböök *et al.*, 1991).

The classical actions of neurotrophins include trophic, differentiative and tropic modulation of neurons (Levi-Montalcini, 1987). More recently neurotrophins have been involved in modulation of synaptic transmission (Thoenen, 1995).

The biological action of neurotrophins is mediated by specific receptors present on the plasma membrane of both neurons and other cell types. Various types of neurotrophin receptors have been characterized: a low-affinity receptor (p75) able to bind all neurotrophins and a group of so called high-affinity receptors specific for particular neurotrophins. These high-affinity receptors are tyrosine kinase type receptors (Trk) and they have been identified as: TrkA (Kaplan *et al.*, 1991a,b; Klein *et al.*, 1991), TrkB (Klein *et al.*, 1990a,b; Middlemas *et al.*, 1991) and TrkC (Cordon-Cardo *et al.*, 1991; Lamballe *et al.*, 1991). They respectively bind NGF, BDNF and NT-4/5 (with apparent equal affinity), NT-3; some limited cross-reactivity of NT-3 with TrkA and TrkB has also been reported (Squinto *et al.*, 1991).

p75 was initially described as "the" NGF receptor (Chao *et al.*, 1986; Johnson *et al.*, 1986; Radeke *et al.*, 1987). It is a transmembrane receptor with a large extracellular structure containing four negatively charged cysteine-rich domains and a cytoplasmic domain highly conserved among species and devoid of any homology with protein kinases. The intracellular domain presents a so called "death" domain, involved in signaling pathways modulating apoptosis (for review see Wallach, 1997). There is homology between the p75 receptor and the tumor necrosis factor (TNF) receptor superfamily (Banner *et al.*, 1993; Suda *et al.*, 1993). Interaction of neurotrophins with

p75 may be important for increasing the specific activation of the Trk receptors (Benedetti *et al.*, 1993; Clary and Reichardt, 1994; Chao and Hempstead, 1995). The extracellular portion of Trk receptors possess two cysteine-rich clusters, one of which is located in the amino-terminal region and is separated from the other by three consecutive leucine repeat motifs, and two IgG–related domains in the membrane-proximal region. Previous work has shown that the second IgG-like domain determines the specificity of Trk receptors for neurotrophins (Urfer *et al.*, 1998). The other domains may be required for efficient expression, *in vivo* folding and traslocation across the membrane. Moreover, the first IgG-like domain may contribute to the formation of a stable signal transducing complex by controlling receptor dimerization: this should lead to more efficient autophosphorylation (Urfer *et al.*, 1998). It is conceivable that Trk receptors carry cell adhesion molecule (CAM) binding domains, as CAM are thought to play an important role in the development and maintenance of the nervous system, by promoting morphological plasticity and maintaining stable contacts between cells (for a review see Goodman and Shatz, 1993). A candidate for this function is one of the leucine-rich motifs, since all leucine-rich repeats containing proteins appear to be involved in protein-protein interactions and at least half of them take part in signal transduction pathways (Kobe and Deisenhofer, 1994). Finally, functional studies have reported that neurotrophins bound to Trk receptors are internalized and retrogradely transported to the cell body (DiStefano *et al.*, 1992). Hence an alternative function for the domains not used in binding might involve attachment to transport proteins within the cell (Urfer *et al.*, 1998).

The cytoplasmic region of Trk receptors contains a catalytic tyrosine-kinase domain, a small insert and a short cytoplasmic tail. When bound to their specific neurotrophins, Trk receptors dimerize, increasing their transphosphorylation activity, and trigger a cascade of intracellular events (for review see Barbacid, 1994; Heumann, 1994). TrkB and TrkC also exist in a truncated form, lacking the intracellular domain. These truncated forms might have the function of modulating the local availability of active neurotrophins (Biffo *et al.*, 1995).

Activated Trk receptors induce a rapid increase in the phosphorylation of certain cellular substrates and a subsequent activation of several intracellular pathways. In particular,

these are: 1) activation of phospholipase C leading to activation of protein kinase C and Ca-calmodulin-sensitive kinases; 2) the pathway mediated by p21-ras, leading to the activation of the MEK kinase cascade; 3) the pathway induced by phosphoinositide 3 kinase (PI 3 kinase) (for review see Heumann, 1994; Patapoutian and Reichardt, 2001). In general, the tyrosine phosphorylation events result in downstream regulation of cellular proteins responsible both for cell shaping and survival, and for activating the gene transcriptional machinery that controls growth, migration, morphology and survival of neurons.

Neurotrophins and their receptors are differentially distributed in the CNS (for a review see Davies, 1994; McAllister *et al.*, 1999). In particular, BDNF and TrkB are highly expressed in the cerebellum, the hippocampus and the cerebral cortex. In contrast, NGF is more restricted to areas such as cerebral cortex, hippocampus and striatum. TrkA distribution is limited to systems such as the basal forebrain cholinergic neurons that project to hippocampus and neocortex.

Neurotrophins and their receptors are developmentally regulated (for review see Davies, 1994). Studies in visual cortex have resulted in particularly important insights into the mechanisms regulating the maturation of BDNF and its receptor TrkB. It has been reported that BDNF changes its pattern of expression, and increases its endogenous level, during the postnatal development of visual cortex (Bozzi *et al.*, 1995). Visual experience regulates both the cellular pattern of expression and the endogenous level of BDNF mRNA and protein both during postnatal development and in adulthood. Indeed, dark rearing reduces the level of BDNF and TrkB mRNAs in adult rat visual cortex while in dark-reared pups it retards the increase in BDNF mRNA normally occurring after eye-opening (Castrén *et al.*, 1992). Moreover, dark rearing modifies BDNF mRNA cellular expression (Capsoni *et al.*, 1999a,b). At the level of BDNF protein, dark rearing reduces the number of immunopositive cells, freezing the pattern of expression at the stage before eye opening (Tropea *et al.*, 2001). Also, TrkB receptors increase their expression from an early developmental stage to a middle stage corresponding to the peak of the critical period, remaining stable thereafter (Cabelli *et al.*, 1996).

During development, some neurons change their sensitivity to neurotrophins (reviewed by Davies, 1997). For example, during the earliest stages of target field innervation,

mouse trigeminal neurons display a transitory survival response to BDNF and NT-3. This response is lost as the neurons become NGF-dependent shortly before neuronal death begins in the trigeminal ganglion (Buchman and Davies, 1993).

1.6 Neurotrophins and neuronal plasticity

Neurotrophins appear to control several aspects of synaptic transmission. For example, they influence the neurotransmitter phenotype of neurons in the CNS (Gnahn *et al.*, 1983; Li *et al.*, 1995; Nawa *et al.*, 1994; Jones *et al.*, 1994). In particular, BDNF increases both the expression levels of several synaptic vescicle proteins (Takei *et al.*, 1997) and the phosphorylation of synapsin 1 (Jovanovic *et al.*, 1996).

Neurotrophins are reported to have fast effects on synaptic activity both in the PNS and in the CNS. Acute exposure to BDNF or NT-3 rapidly potentiates spontaneous and evoked synaptic activity of developing neuromuscular synapses in culture (Lohof *et al.*, 1993; Wang *et al.*, 1995b). In cultured hippocampal neurons, BDNF and NT-4/5 enhance synaptic transmission by increasing the frequency of excitatory postsynaptic potentials (EPSPs) (Lessmann *et al.*, 1994); this effect, similar to the action of NT-3 on cortical neurons (Kim *et al.*, 1994), suggests that BDNF acts presynaptically. Further support for this idea comes from Figurov *et al.* (1996): they showed that BDNF enhaces LTP in young hippocampal slices by improving the ability of presynaptic fibers to follow tetanic stimulation. Moreover, in cultured hippocampal neurons, BDNF enhances glutamatergic synaptic transmission via release of Ca^{2+} from IP_3-gated stores (Li *et al.*, 1998). Inhibition of BDNF action using immunoadhesins (TrkB-IgG) or blocking antibodies reduces the magnitude of the late phase of LTP in hippocampal slices (Figurov *et al.*, 1996; Kang *et al.*, 1997).

All these observations indicate an enhancement of transmitter release from pre-synaptic terminals, presumably due to action of neurotrophins via the appropriate Trk receptor. The hypothesis neurotrophins facilitate transmitter release via Trk receptors is further supported by the observation that neurotrophins increase intracellular calcium levels (Berninger *et al.*, 1993; Stoop and Poo, 1996; Canossa *et al.*, 1997). However, this widely accepted hypothesis has been challenged by results showing that BDNF potentiates

synaptic transmission in cultured hippocampal neurons in a rapid and reversible way by increasing postsynaptic responsiveness to excitatory input (Levine *et al.*, 1995, 1996). Further studies have recently shown that neurotrophins cause membrane depolarization within a few milliseconds of application, leading to firing of action potentials (Kafitz *et al.*, 1999; Li *et al.*, 1999; Berninger and Poo, 1999), an effect previously assigned only to classical excitatory neurotransmitters. Thus, neurotrophins may modulate synaptic transmission and efficacy in response to specific neuronal signals by acting at pre- and/or post-synaptic sites.

Structural neuronal changes are the hallmark of plastic re-arrangements occurring in the CNS. For example, both axonal and dendritic structural changes have been described in cortical re-arrangement following monocular deprivation (Hubel and Wiesel, 1970; LeVay *et al.*, 1978; Antonini and Stryker, 1993), eye segregation in LGN layers (Shatz 1983; for a review see Shatz, 1990) and lesion-induced plasticity in the somatosensory (for a review see Merzenich *et al.*, 1990) and visual cortex (for a review see Gilbert, 1998). Neurotrophins are important molecules regulating structural neuronal plasticity both in PNS and in CNS. Initially, NGF was isolated for its ability to stimulate neurite growth (for a review see Levi-Montalcini, 1987). Subsequently, each neurotrophin has been demonstrated to stimulate neurite outgrowth of specific neuronal populations in the PNS, both in vitro and in vivo (for a review see Thoenen, 1991). Neurotrophins exert similar effects also in selected populations of neurons in the CNS. For example, BDNF influences the complexity of retinal axons co-coltured with optic tectum from chicken (Inoue and Sanes, 1997) and BDNF infusion into the optic tectum of Xenopus induces increased branching of retinal axons (Cohen-Cory and Fraser, 1995). In addition to axonal growth neurotrophins control dendritic growth and pattern in pyramidal neurons, as has been shown in organotypic cultures of cortex (McAllister *et al.*, 1995; Baker *et al.*, 1998). A recent experiment in which TrkB receptors were eliminated only in pyramidal neocortical neurons, showed that lack of TrkB produces progressive atrophy of dendritic arborization and finally cell death (Xu *et al.*, 2000).

1.7 Role of neurotrophins in visual cortical plasticity

During the last ten years many studies have been conducted in the visual system with the aim of studying the role of neurotrophins in the control of cortical plasticity and activity-dependent development in mammalian visual cortex. The first experiments were done in the visual cortex of rat: the authors showed that an exogenous supply of Nerve Growth Factor (NGF) is able to prevent the effects of monocular deprivation and strabismus during the critical period (Domenici *et al.*, 1991; Domenici *et al.*, 1992; Maffei *et al.*, 1992; Domenici *et al.*, 1993). In particular, adding NGF prevents the shift of ocular dominance distribution, the reduction of visual acuity in the deprived eye, and the shrinkage of LGN neurons driven by the deprived eye. Subsequently, it has been shown that endogenous NGF is necessary for normal development of the geniculo-cortical system (Berardi *et al.*, 1994) and for the temporal definition of the critical period for monocular deprivation (Domenici *et al.*, 1994). Indeed, NGF blockade by antibodies affects functional properties of visual cortical neurons and morphological characteristics of geniculo-cortical neurons, with the critical period being longer than in normal animals. Interestingly, recent data obtained by Pesavento *et al.* (2000) showed that local supply of NGF inhibits LTP in the visual cortex during the critical period, suggesting that when NGF is increased, synaptic strengthening is inhibited and sensory deprivation is no longer effective.

These data suggestes the hypothesis that LGN fibers compete for NGF released by cortical target neurons. TrkA has not been found in LGN neurons and TrkA mRNA is expressed at low levels in visual cortex (for a review see Cellerino and Maffei, 1996). TrkA has been found in cholinergic neurons of basal forebrain (Li *et al.*, 1995; Cattaneo *et al.*, 1999), which send a well defined projection to several areas, including visual cortex, and are sensitive to NGF withdrawal (Debeir *et al.*, 1999). Recently, it has been shown that intrinsic cortical neurons also express TrkA receptors during postnatal development (Tropea *et al.*, manuscript submitted). Thus, one possible explanation for NGF effects is that NGF regulates developmental cortical plasticity by acting on cholinergic terminals present in the visual cortex and on intrinsic cortical neurons.

More recently, it has been reported that brain derived neurotrophic factor (BDNF) and neurotrophin 4/5 (NT-4/5) are also able to modulate visual cortex development and plasticity. An exogenous supply of BDNF/NT-4/5 prevents the formation of ocular dominance columns (Cabelli *et al.*, 1996). The possible interpretation is that LGN fibers which express TrkB receptors (Cabelli *et al.*, 1996) compete for BDNF instead of NGF. According to this hypothesis, adding an excess of BDNF may reduce the competition between LGN fibers in layer IV, preventing segregation of LGN inputs that normally leads to ocular dominance columns formation and/or maintenance. Similarly, infusion of TrkB fusion proteins, blocking TrkB ligands, also prevents the segregation of LGN inputs (Cabelli *et al.*, 1997), suggesting that adding or reducing BDNF and possibly NT-4/5 prevents ocular dominance maturation. Subsequent studies by Galuske *et al.* (1996; 2000) showed that infusion of BDNF in kitten visual cortex induces a paradoxical shift of ocular dominance distribution, supporting the hypothesis that the endogenous level of BDNF regulates neuronal plasticity in the developing geniculo-cortical cortex.

In agreement with this hypothesis, BDNF both potentiates spontaneous and evoked excitatory transmission (Carmignoto *et al.*, 1997) and enhances LTP (Akaneya *et al.*, 1997) in the visual cortex. Inhibition of BDNF action by immunoadhesins (TrkB-IgG; Shelton *et al.*, 1995) reduces the magnitude of LTP (Sermasi *et al.*, 2000). Also LTD is modulated by BDNF; indeed, induction of LTD is prevented by BDNF (Akaneya *et al.*, 1996, Huber *et al.*, 1998). Another form of synaptic plasticity called bi-directional has been described. This consists of the ability of synapses to revert the sign of a previous long-term change of efficacy; for example passing from LTD to LTP or *vice versa*. This form of synaptic plasticity is expressed during an early period of postnatal development in rat visual cortex and is regulated by BDNF (Sermasi *et al.*, 1999).

Recent experiments showed that focal injections of NT-4/5 in the visual cortex of monocularly deprived ferrets prevents shrinkage of LGN neurons projecting to the deprived cortex (Riddle *et al.*, 1995; 1997). These results suggest that NT-4/5, in addition to BDNF, may also regulate neuronal plasticity in developing visual cortex.

The activity driven re-arrangement occurring in developing visual cortex involves the arrangement of new synapses and withdrawal of other ones. Neurotrophins, as discussed in the previous chapter, seem to be involved in structural changes underlying neuronal

plasticity. In organotypic cultures of the visual cortex, it has been reported that BDNF, as well as NT-3 and to a lesser extent NGF, regulates dendritic growth of pyramidal neurons (McAllister *et al.*, 1997). The effects of the BDNF are layer-specific and indicate that BDNF, and more generally neurotrophins, act to modulate the development of particular dendritic patterns. Interestingly from this point of view, Horch and collaborators (1999) have shown that local modulation of BDNF levels is able to change the site of dendritic spines, in a short time scale suggesting that this neurotrophin contributes to remodel the spatial distribution of synapses along neuronal processes.

These data suggest that neurotrophins operate in neuronal plasticity by regulating synaptic efficacy as well as by influencing morphological changes of synapses and dendrites.

1.8 Transgenic mouse models

Pharmacological studies on the action of neurotrophins have provided interesting results about the functions of neurotrophins in plasticity and activity-dependent development of the nervous system. However, deprivation studies conducted using antibodies or immunoadhesins reported in some cases conflicting results even when applied to the same preparations. This is probably due to lack of availability of specific blocking compounds and methods of chronic delivery in the nervous system. More recently, total deprivation of individual neurotrophins has been achieved by targeted mutations of genes, for which the elective animal model is the mouse. Gene targeting in embryonic stem cells leads to the production of animals containing a deletion or insertion in a gene of interest, thus silencing (knock-out technique) the gene. Knock-out mice have been a powerful tool in neuroscience with which to clarify a series of complex biological problems especially in the field of neuronal plasticity. In one of the first studies, Silva *et al.* (1992) showed that mice deficient in α-calcium-calmodulin kinase type II exhibit abnormal LTP in area CA1 of the hippocampus; defective LTP was associated with alterations of spatial learning. Studies published to date have described knock-outs for individual neurotrophins and their receptors. Knocking out NGF or TrkA resulted in dramatic phenotypes and mice died shortly after birth. These animals are characterized by

depletion of DRG neurons subserving nociception, sympathetic ganglia and trigeminal ganglion in the peripheral and autonomous nervous system and by a mild shrinkage of basal forebrain cholinergic neurons, confirming previous results obtained by using pharmacology techniques (for a review see Snider, 1994). BDNF and TrkB knock-outs are also associated with dramatic phenotype. TrkB(-/-) animals die during the first two days after birth (Klein *et al.*, 1993). BDNF (-/-) animals are characterized by a milder phenotype and they can survive for a few weeks after birth because of abnormal respiration and visceral motility (Jones *et al.*, 1994). Mutant mice are characterized by an abnormal behavior consisting of head bobbing and altered locomotion symptoms that can be re-conducted to the altered vestibular function due to the depletion of vestibular neurons (Ernfors *et al.*, 1994; Jones *et al.*, 1994). Both BDNF and TrkB knock-outs exhibit neuron loss in certain classes of DRG and trigeminal ganglion. Further studies showed that BDNF mutants are characterized by subtle phenotypes in the CNS; for example, in the cortex there is an abnormal expression of neuropeptides, in particular NPY (Jones *et al.*, 1994) and the calcium binding protein parvalbumin in GABAergic interneurons (Baimbridge *et al.*, 1992). More recent results have shown that LTP in the CA1 region of the hippocampus was reduced in both heterozygous and homozygous animals (Korte *et al.*, 1996; Patterson *et al.*, 1996). This deficit can be rescued either by application of exogenous BDNF (Patterson *et al.*, 1996) or by local infection of CA1 cells in hippocampal slices with an adenoviral vector coding for BDNF (Korte *et al.*, 1996). Moreover, a study by Pozzo-Miller *et al.* (1999) showed that BDNF knock-out mice have impairments in pre-synaptic transmitter release in CA1.

Target deletion of NT-4/5 gene resulted in homozygous viable animals without overt phenotype except for partial neuronal depletion in nodose and geniculate ganglia (Liu *et al.*, 1995; Conover *et al.*, 1995). Analysis of NT-4/5 mutants showed that these animals have deficits in hippocampal LTP and long term-memory but no impairment of short-term plasticity and short-term memory (Xie *et al.*, 2000).

The major limitation of gene knock-out technology is that there is no temporal or regional restriction to gene deletion. In addition, as reported above, homozygous mutant mice die prematurely or present dramatic phenotypes that prevent further analysis. To restrict gene targeting to selected cerebral regions and to subgroups of neurons, a new generation of

knock-out mice has been created. The new technology exploits the phage P1-derived CRE/loxP recombination system to delete a certain gene in particular regions of nervous system. In addition, since CRE/loxP mediated recombination does not occur until cells are post-mitotic, this tool allows gene deletion to be temporally restricted. It has been shown that TrkB CRE/lox mutants are viable and develop without dramatic phenotype (Minichiello *et al.*, 1999; Xu *et al.*, 2000a). TrkB CRE/loxP removal in CA1 pyramidal neurons of hippocampus leads to altered expression of LTP associated with selective alterations of spatial learning, but normal brain morphology (Minichiello *et al.*, 1999). Subsequent work of Xu *et al.* (2000b) showed that lack of TrkB in CA1 affects pre-synaptic function in this way reducing the ability of tetanic stimulation to evoke LTP. BDNF CRE/loxP removal in pyramidal neurons of visual cortex induced both a reduction of the number of cortical pyramidal neurons and an alteration of the dendritic processes, which appeared shorter and smaller in diameter with respect to controls. However, these alterations became evident in adult mutant mice, leaving open the question of the effects of BDNF deprivation in cortical development.

Besides the knock-out technique, the "inverse" strategy of over-expressing a gene of interest is also a powerful means of investigation. A recent study by Huang *et al.* (1999) increased our understanding of the role of BDNF in the development and plasticity of visual cortex by exploiting this technology. Huang *et al.* generated a transgenic mouse in which the gene for BDNF was placed under the control of the α-calcium-calmodulin kinase type II promoter. Mutant mice started to overexpress BDNF at an early stage of postnatal development. Interestingly, BDNF overexpression was restricted to cortical pyramidal neurons. They found that the maturation of GABAergic circuitry was altered: inhibition was enhanced and this induced premature ending of the critical period for monocular deprivation. Also, the period of LTP expression was shortened and the maturation of visual function accelerated. Another study (Saarelainen *et al.*, 2000) showed that transgenic mice over-expressing truncated TrkB neurotrophin receptors in neurons have impairments in long-term spatial memory but normal hippocampal LTP.

The results provided by analysis of transgenic animals have clarified the role of neurotrophins and their receptors in the development of different cerebral areas and have contributed to unveil subtle phenotypes important for understanding complex biological

function. Particularly important are studies on neuronal plasticity that relate chronic absence or over-expression of individual neurotrophins/receptors to functional and behavioral changes. Finally, we observe that results obtained in mutant mice are largely complementary to those obtained with pharmacological tools: in mutants, a given molecule is chronically absent or overexpressed, while most pharmacological studies deal with acute/subacute role of the molecule under study.

1.9 Neurotrophins and NMDA receptors

The evidence that both neurotrophins (mainly BDNF and NT-4/5 with respect to the aims of this thesis) and NMDA receptors are involved in the same mechanisms of development and plasticity, have consequently addressed the hypothesis of possible interactions and mutual modulations between these two systems.

It has been shown that in cultured mouse cerebellar granule cells (Muzet and Dupont, 1996), as well as in cortical neurons (Koh *et al.*, 1995), NMDAR maturation is enhanced by BDNF. More recently, it has been reported that BDNF reduces NR2A expression in cultured cerebellar granule cells, suggesting that this neurotrophin controls the synthesis of NR2A, thus changing Ca^{2+} influx and Ca^{2+} dependent processes in neurons (Brandoli *et al.*, 1998).

Another study has shown that neurotrophins can rapidly and reversibly increase NMDA currents in striatal and hippocampal (CA1) isolated neurons (Jarvis *et al.*, 1997) with no involvement of Trk receptors and second messenger cascades. It is likely that BDNF acts directly on NMDA receptors by substituting the coagonist Gly (the same effects were observed applying BDNF and Gly). On the other hand, in whole-cell and single-channel recordings from cultured hippocampal neurons, Levine *et al.* (1998) observed that BDNF augmented glutamate-evoked currents 3-fold and increased the probability of NMDARs being open. For this action of BDNF, activation of TrkB was crucial, as the increase in probability was prevented by the tyrosine kinase inhibitor K-252a. In addition, the NMDAR antagonist MK-801 blocked BDNF enhancement of synaptic transmission, further suggesting that the neurotrophin modulates synaptic efficacy via changes in NMDA receptor function.

BDNF together with NGF induces a rapid increase in the amplitude of EPSCs in rat visual cortex slices recorded by the whole-cell patch-clamp technique (Carmignoto *et al.*, 1997). BDNF also increased the frequency of spontaneous EPSCs and the analysis of the currents revealed that the NMDA receptor-mediated component as well as the AMPA component were potentiated by the neurotrophins.

Neurotransmitter receptors, as well as protein kinases and ion channel proteins, are anchored to the PSD. Recent results showed that NMDARs can be phosphorylated by intrinsic PSD kinases such as PKC (Suen *et al.*, 1998). TrkB receptors have been found in the PSD, and activation of TrkB receptors enhances synaptic transmission not only at the pre-synaptic site (Figurov *et al.*, 1996; Carmignoto *et al.*, 1997) but also at the post-synaptic site (Levine *et al.*, 1995, 1996) where NMDARs are expressed. This led to the hypothesis that the activation of TrkB by its ligands may influence synaptic transmission through phosphorylation of NMDARs. In accordance with this hypothesis, it has been found in postsynaptic fraction of hyppocampal synaptosomes that BDNF enhances synaptic transmission by phosphorylation of NMDAR subunit NR1 (Suen *et al.*, 1997). Moreover, it has been reported (Lin *et al.*, 1998) that incubation of BDNF with cortical or hippocampal post-synaptic densities for 5 min specifically increased NR2B phosphorylation in a dose-dependent manner, while NGF had no effect. The action of BDNF was selective also because it did not alter tyrosine phosphorylation of the NR2A subunit. Together, these results suggest that tyrosine phosphorylation of the NR2B subunits of NMDA receptors may contribute to neurotrophin modulation of the postsynaptic responsiveness and LTP. This is true also in excised patches from hippocampal neurons (Levine and Kolb, 2000).

Taken together, these results indicate that there is an interesting interplay between BDNF and NMDARs, from rapid and acute interactions to control of synthesis transport, insertion and phosphorylation of NMDAR subunits. In the present work, we address the issue related to modulation of NMDARs expression by neurotrophins signalling mediated by TrkB receptors.

1.10 Introducing rat and mouse visual cortex

In rats, the primary visual cortex (area Oc1, area 17) occupies a region in the occipital cortical pole and is flanked by cytoarchitectonic areas 18a and b, considered to be secondary visual areas (Coogan and Burkhalter, 1993). In both rat and mouse, as well as in other mammalian species, primary visual cortex receives visual input from the lateral geniculate nucleus (LGN). LGN is a thalamic nucleus that can be subdivided into two regions, according to segregation of retinal afferents (Reese, 1988). Using cresyl violet staining associated with anterograde tracers injected into one eye, it can be shown that each region receives input from either the controlateral or the ipsilateral retina. In particular, the region receiving input from the controlateral eye is much larger than that receiving input from the ipsilateral eye (Domenici *et al.*, 1993). Both regions project to the primary visual cortex (area 17). In area 17, two sub-areas can be recognized: a medial area (Oc1m) receiving visual input exclusively from the controlateral eye (and the controlateral region of the LGN) and a large lateral area (Oc1b) receiving visual input from both eyes and, consequently innervated by both regions of LGN (Zilles *et al.*, 1984). LGN fibers directed to area 17 terminate predominantly in cortical layer IV. From layer IV, visual information is transferred to layer II-III neurons and, in part, to infragranular layers (Paxinos and Watson, 1986). In the white matter underlying the visual cortex, there are afferents from as well as feedback projections from the visual cortex to LGN, superior colliculus and thalamic nuclei. There are also other afferent fibers coming from basal forebrain nuclei: in particular, from cholinergic neurons (Mesulam *et al.*, 1983; Saper, 1984) and a small group of GABAergic neurons (Gritti *et al.*, 1997). There are also projection from noradrenergic neurons in locus coeruleus (Levitt and Moore, 1979) and from serotoninergic neurons in the raphe nuclei (D'amato *et al.*, 1987). These projections, in particular the cholinergic projections from basal forebrain, are not restricted to layer IV and are distributed throughout the visual cortical layers.

Concerning cellular organization, there are two principal groups of neurons in the visual cortex (Peters and Kara, 1987). Pyramidal cells expressing glutamate and aspartate as neurotransmitters represent 80% of the total neuronal population and are distributed throughout all layers. Non-pyramidal stellate cells are the second neuronal population.

The majority of non-pyramidal cells are GABAergic, with the exception of a small sub-group in layer IV, the so-called spiny stellate cells. In layer IV, geniculo-cortical terminals have synaptic contacts mainly with spines of pyramidal and spiny stellate neurons.

The functional characteristics of visual cortical neurons in the rat and mouse are similar to those described in other mammals. Briefly, visual cortical neurons respond to different parts of the visual field and have well-characterized receptive fields. They respond either to one or other eye (monocular cells) or both eyes (binocular cells, area OC1b) and they are selective for the characteristics of visual stimuli such as orientation and direction of movement (Fagiolini *et al.*, 1994). The key point for the present work is that during post-natal development, responses of visual cortical neurons are sensitive to manipulation of vision. This period is called the critical period and spans three to four post-natal weeks, from eye opening (postnatal day 14-15) to P30-40 (Fagiolini *et al.*, 1994). It is important that, at the time of eye opening: regressive processes such as neuronal death are already over (for review see Zilles, 1985); LGN fibers have already reached the visual cortex, making synapses with the specific targets (Lund and Mustari, 1977); basal forebrain cholinergic neurons are distributed throughout all layers. During the critical period monocular deprivation of the type introduced by Wiesel and Hubel (1965) produces irreversible alterations in the visual cortex, with minor differences between rat and mouse. Visual cortical neurons cease responding to the deprived eye and, as a consequence, the number of binocular units is reduced (Maffei *et al.*, 1992; Fagiolini *et al.*, 1994; Hensch *et al.*, 1998; Hanover *et al.*, 1999). Geniculate neurons receiving input from the deprived eye and sending projections to primary visual cortex shrink (Domenici *et al.*, 1993). These effects are much less pronounced if monocular deprivation is performed after the end of the critical period (Fagiolini *et al.*, 1994). However, neuronal plasticity is not limited to the critical period. Indeed, there are other forms of neuronal plasticity associated with visual learning and changes of the topographic sensory map induced by a peripheral lesion (for example in the retina, Gilbert and Wiesel, 1992; Obata *et al.*, 1999). Deprivation of visual input (dark rearing) results in two types of effects: 1) it increases the length of the critical period for monocular deprivation; 2) it delays the maturation of the functional properties of visual cortical neuron. All these results indicate

that rat visual cortex has well-defined cortical circuitry and is a good model for studing activity-dependent neuronal plasticity and maturation.

1.11 Immunoadhesins

Immunoadhesins are fusion proteins combining the hinge and Fc regions of an immunoglobulin (Ig) with domains of a cell-surface receptor that recognizes a specific ligand. The term immunoadhesin comes from this combination of "immune" and "adhesion" functions (Ashkenazi *et al.*, 1993; for a review see Chamow and Ashkenazi, 1996). These antibody-like molecules were first studied to circumvent the difficulty of obtaining human monoclonal antibodies (mAbs) for use as diagnostic tools and therapeutic agents. In fact, humans are generally tolerant to their own antigens and ethical considerations place restrictions on the active immunization of humans for the purpose of generating mAbs.

The majority of immunoadhesins combine the hinge and Fc regions of an IgG_1 heavy chain with the extracellular domain (ECD) of a type I transmembrane protein, usually a receptor or adhesion molecule. The prototypic immunoadhesin is a disulfide-linked homodimer resembling an IgG_1 molecule which lacks C_H1 domains and light chains. The dimeric structure of the expressed and purified molecule can be confirmed by SDS-PAGE in the presence of a reducing agent to separate the polypeptide chains. In the absence of light chains, deletion of the C_H1 domain improves assembly and secretion of immunoadhesins by the host cell. Placing the fusion junction at the flexible hinge facilitates proper folding of domains and helps to preserve the functions of both parts of the molecule.

Immunoadhesins can be expressed efficiently in a variety of host cells, including myeloma cell lines, chinese hamster ovary cells, monkey COS cells, human embryonic kidney 293 cells and baculovirus infected insect cells. In these systems, the immunoadhesin polypeptides are assembled and secreted into the cell culture medium. The molecule can then be substantially purified by protein A or protein G affinity chromatography, in much the same manner as an immunoglobulin. Indeed, efficient

mammalian cell expression and the ease of this type of purification are significant advantages of expressing proteins of biological interest as immunoadhesins.
In this thesis, the host cells used to express immunoadhesins have been insect cells infected by baculovirus. The specific advantage for using this system is the absence of post-translational modifications, which cannot be excluded in mammalian cells.
Another peculiar feature of our immunoadhesins is that the Fc portion comes from camel IgG_2: this immunoglobulin normally lacks C_H1 domain, avoiding the need for artificially deleting it, as normally done with conventional immunoadhesins (Hamers-Casterman *et al.*, 1993).

1.12 Aim of the thesis

The importance of NMDARs in the plasticity and maturation of the visual cortex has been widely described. These receptors are multimeric proteins and their subunit composition changes during development, together with the functional properties of the receptor. However, the factors and mechanisms regulating the expression of NMDARs and their subunit composition during post-natal development are not yet known. The present work addresses this issue aiming, more specifically, to study the role played by neurotrophins, in particular TrkB ligands, in NMDAR expression.
We used to approaces. First, we blocked both TrkB ligands, i.e. BDNF and NT-4/5, by applying immunoadhesins to the rat visual cortex. Second, we studied the separate effects of BDNF and NT-4/5 on development of NMDAR subunits. For this purpose, we used either BDNF or NT-4/5 knock-out mice.

2. MATERIALS AND METHODS

2.1 Animal treatment and pump implantation

Rats and mice used for this study come from the animal house of the University of Trieste.

Wistar rats were housed in groups of 2-4 per cage, ad libitum access to food and drinks and a 12 hour light/dark cycle. A total of 11 rats were used in this study: N=5 (4 used for immunohistochemistry, 1 for western blot) were implanted with minipumps filled with immunoadhesin; N=5 (4 + 1 as above) were implanted with minipumps filled with physiological solution (control rats). One completely untreated rat was sacrificed as further control in western blot experiment.

Mice (P21-23, adults) were housed in groups of 4-8 per cage, ad libitum access to food and drinks and a 12 hour light/dark cycle. Pups were kept with their mothers. A total of 130 mice were used in this study: N=72 were wild type mice; N=22 were homozygous mutants for BDNF; N=36 were homozygous mutants for NT-4/5 (see section 2.7).

Handling of animals was done in accordance with the Italian law DL 27/1/92 n. 116 based on UE rule n. 86/609.

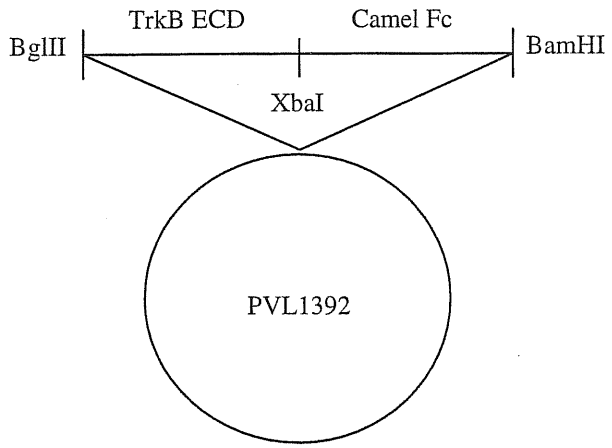
P20 wistar rats were treated either with TrkB-IgG (0.5 mg/ml, 4 animals) or saline (0.9% NaCl, 4 animals) released by mini-osmotic pumps (model 10007D, Alzet, Palo Alto, USA) situated on the rats back. Each pump was implanted unilaterally beneath the skin of the neck and connected with a polyethylene tube to a 30 gauge stainless steel cannula. Pumps were filled with TrkB-IgG containing solution, or saline as control. A small hole was made in the skull in the region corresponding to the right occipital cortex (1 mm from λ scissure, avoiding insertion of the needle of the pump into the ventricle). The needle tip was inserted into the cortex and secured to the skull with dental cement. After the dental acrylic had hardened, the skin was sutured. The mean fill volume of the pumps was 243 µl and the pumping rate 0.5 µl/hr (0.25 µg/hr) of TrkB-IgG. Release was provided for 16 days (i.e., a total volume of about 210 µl) after which P36 rats were sacrificed for immunohistochemistry experiments. Rats were anaesthetized (20%

urethane, 100 µl/100 g body weight) and perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS). Brains were removed and post-fixed in PFA for 1 hour at 4°C, then cryoprotected in 30% sucrose/PBS at 4°C until cutting. Coronal sections (40 µm) containing the primary visual cortex (OC1b; Paxinos & Watson, 1986) were cut with a freezing microtome obtaining about 70 sections that were collected separately for immunohistochemistry.

Mice were perfused and brains collected and treated as described above, but coronal sections cut for immunohistochemistry were 35 µm thick.

2.2 TrkB-IgG production and specificity

Soluble TrkB receptors were engineered as immunoadhesins (TrkB-IgG, Shelton *et al.*, 1995), and were produced by linking the extracellular domain of the human TrkB receptor to the Fc portion of camel IgG_2, constituted of a 35 aminoacid long hinge followed by C_H2 and C_H3 domains (Fig. 2). Briefly, the human TrkB extracellular domain (aminoacids 1-435) was amplified by PCR, cut with the restriction enzymes XbaI/BglII and cloned in pVL1392 where camel Fc was already inserted (XbaI/BamHI).



Actually, the TrkB leader sequence (aminoacids 1-40) was substituted by the gp67 secretion signal sequence that allows secretion in insect cells. Serge Muyldermans (Vrije Universitet Brussels) kindly provided clone CH_2-CH_3 camel IgG_2.

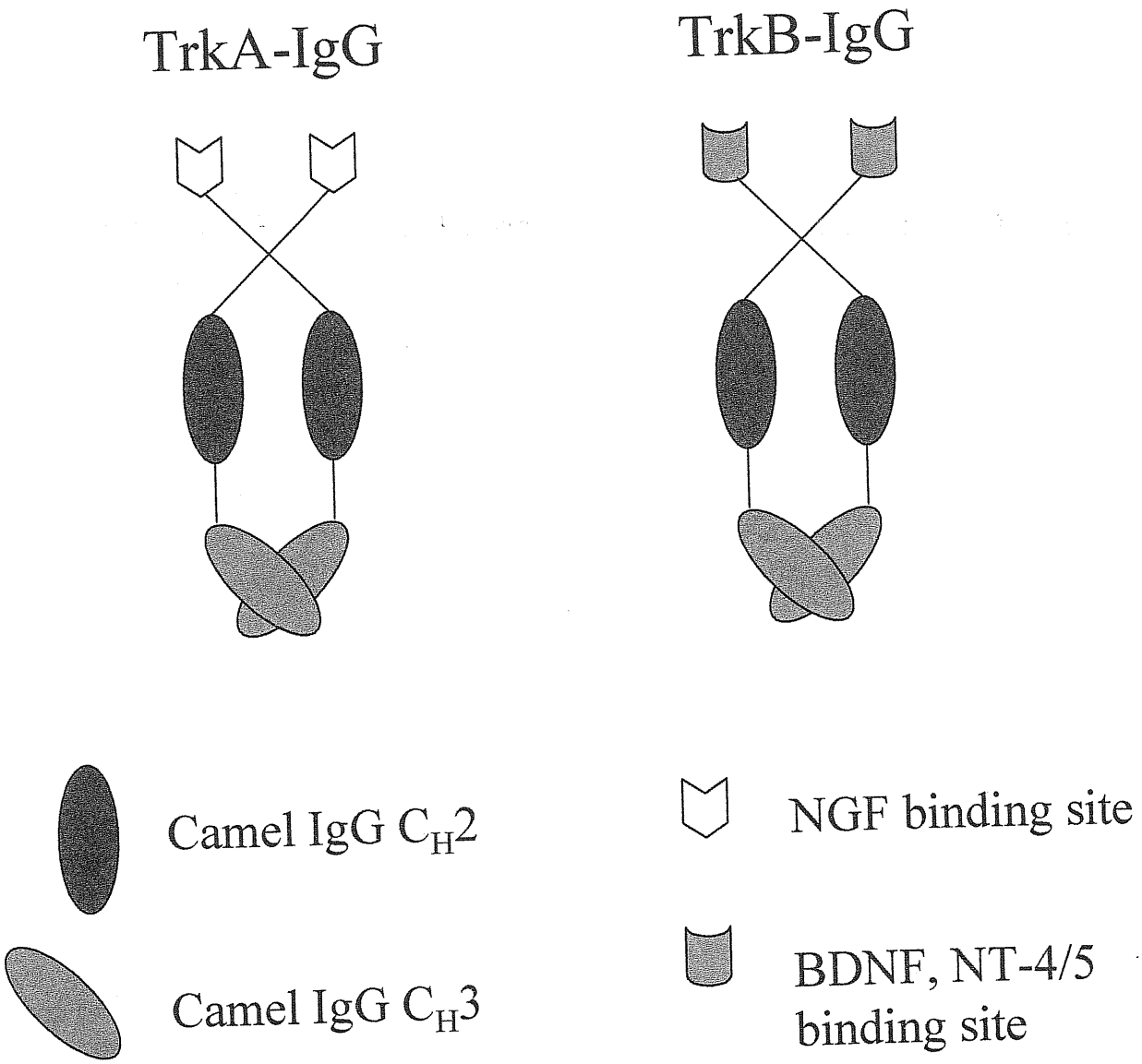


Figure 2. Schematic representation of TrkA and TrkB immunoadhesins (TrkA-IgG and TrkB-IgG respectively).
For the aims of this thesis TrkB-IgG was mainly used. TrkA-IgG shares with TrkB-IgG the same camel Fc region while the ligand-binding region corresponds to the extracellular domain of neurotrophin receptor TrkA. TrkA-IgG was used together with TrkB-IgG in some assays of specificity towards neurotrophins.

DNA sequences were cloned into baculovirus [Autographa californica nuclear polyhedrosis virus (AcNPV)] transfer vectors for expression in Sf9 insect cells. These cells were grown at 27°C, in TNM-FH, 10% fetal calf serum. 72 hours after the first infection, cell supernatant was collected and used for virus amplification, again in Sf9 cells for 72 hours. Immunoadhesins were finally purified by Protein A-Sepharose chromatography from serum-free culture medium of H5 insect cells. Specificity of TrkB-IgG for BDNF was assessed by ELISA. Briefly, different concentrations of BDNF and NGF (10, 100, 500 ng/ml, Alomone Labs, Jerusalem, Israel) were applied on solid phase coupled immunoadhesins (TrkB-IgG and TrkA-IgG as well, where the extracellular domain is that of human TrkA receptor). Incubation with chicken polyclonal anti-BDNF (2.5 μg/ml, Promega Life Science) and rat monoclonal anti-NGF (αD11, 1 μg/ml, Cattaneo *et al.*, 1988) was followed by washes and incubation with biotinilated anti-chicken (3 μg/ml, Promega Life Science) and anti-rat (1:500, Dako A/S, Denmark). After washing, avidin-biotin-peroxidase complex (1:1000, Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) was added. Finally, the reaction was developed with 3,3',5,5'-tetramethyl benzidine, stopped with 0.5 M H_2SO_4 and optical density (O.D.) was read at 450 nm wavelength. TrkB-IgG specificity of binding was tested also for NT-4/5 (Alomone Labs) and NT-3 (PeproTech Inc. Rocky Hill, NJ, USA), applying increasing concentrations of the neurotrophins (0.01, 0.1, 0.2, 0.5, 1 μg/ml) to solid phase coupled immunoadhesin (2 μg/ml). Incubation with chicken polyclonal anti-NT-4/5 and anti-NT-3 (2.5 μg/ml, Promega Life Science) was followed by incubation with biotinilated anti-chicken (3 μg/ml, Promega Life Science); the subsequent steps were as described above.

2.3 Blockade of TrkB ligands and electrophysiological recordings in slices

P17 rat visual cortex slices were incubated in artificial cerebro-spinal fluid (aCSF) containing 1 or 3 μg/ml of TrkB-IgG for 4-7 hours. The aCSF was gassed with 95% O_2 and 5% CO_2, and had the following composition: 126 mM NaCl; 3.5 mM KCl; 1.2 mM

NaH_2PO_4; 1.3 $MgCl_2$; 2 mM $CaCl_2$; 25 mM $NaHCO_3$; 11 mM glucose (Sigma, St Louis, MO, USA). Extracellular field potentials in the inferior half of cortical layer II-III were recorded with an electrode filled with 2 M NaCl and evoked by stimulation of the white matter containing the geniculo-cortical fibres, with a bipolar concentric stimulating electrode. The protocol for inducing LTP consisted of either 3 trains of high-frequency stimulation (HFS, 100 Hz, 1 s, at 10-s intervals) or 3 trains of theta burst stimulation (TBS, 10 bursts at 5 Hz, each burst consisting of 4 pulses at 100 Hz, at 10-s intervals). Control experiments were conducted using slices incubated in both aCSF alone and in aCSF containing 9E10, an antibody recognizing an intracellular epitope (c-myc).

2.4 Immunohistochemistry

2.4.1 Rats

For each treatment, 4 rats were used for immunohistochemistry. For each animal, about 70 coronal sections (40 μm) containing the primary visual cortex (Oc1b; Paxinos & Watson, 1986) were cut with a freezing microtome (total width of Oc1b along the rostro-caudal axis is about 3 mm) and collected separately for immunohistochemistry. One every 8 sections was chosen from rostral to caudal, choosing the first section using a table of random numbers. In this way, 8 series of 8 (or 9) sections each were sampled random uniformly. Six series out of 8 were chosen randomly for immunostaining and 6 different antibodies were used separately so that each series was processed with one antibody. Sections were treated with 0.05% Triton X-100, 10% FCS, 5% bovine serum albumin (BSA) in 0.1 M Tris-Cl pH 7.4, 0.15 M NaCl for 1 hour at RT to block non-specific binding sites. Then, 5 out of 6 series of sections were incubated o/n at 4°C with rabbit polyclonal antibodies raised against NMDAR subunits NR2A, NR2B, NR1 (1:1000, 0.4 μg/ml, 0.5 μg/ml respectively, Chemicon International Inc., Temecula, CA, USA), mouse monoclonal anti-MAP2 (5 μg/ml, Boehringer Mannheim Corporation, Indianapolis, IN, USA) and anti-GFAP (0.1 μg/ml, Amersham International plc.), using just one of these antibodies in each series. The sixth series was incubated with rabbit polyclonal anti-camel (1:5000, previously absorbed on other rat brain slices), to assess diffusion of TrkB-IgG. The next day, sections were incubated with biotinylated anti-rabbit or anti-mouse

IgG (7.5 μg/ml, Vector) for 2 hours at room temperature and then in avidin-biotin-peroxidase complex (1:100, Vector) for 45 minutes. The labelling was revealed by 3,3'-diaminobenzidine HCl (DAB, 10 mg in 25 ml Tris/HCl, 0.1 M, pH 7.5, Sigma), enhanced with Nikel ammonium sulfate (Carlo Erba Chemicals, Italy) for MAP2 and immunoadhesin detection. For cell counting, some sections labeled for NMDA receptor subunits were counterstained with cresyl-violet acetate (0.05%, Sigma).
Control staining was performed by incubation of some slices with the secondary antibody alone.

2.4.2 Mice

Sections from mouse brain containing primary visual cortex were collected separately. One every 6 sections was chosen from rostral to caudal, choosing the first section using a table of random numbers. In this way, 6 series of 9 sections each were sampled uniformly and random. Immunohistochemistry was performed using the same antibodies against NMDAR subunits and according to the same protocol described for rats (these antibodies are expected to work also on mice sections), but the concentrations used were 1:500 (anti-NR2A), 2 μg/ml (anti-NR2B), 0.5 μg/ml (anti-NR1) and the reaction was always developed with DAB, enhanced with Nikel ammonium sulfate. For each different strains and each post-natal ages examined, at least 3 mice were sacrificed for immunohistochemistry.

2.5 Cell counts

Cell counts were performed for NMDAR subunits NR2A and NR2B in rats treated with TrkB-IgG and in control rats treated with physiological solution. Following the methods described above, one series of sections was processed for NR2A and one series for NR2B. Only sections with an evident site of release (TrkB-IgG or saline) were selected for counting. In this way, at least 2 sections, both for NR2A and NR2B, were analyzed for each animal.
After processing for immunohistochemistry and counterstaining with cresyl-violet as described above, OC1b region was selected using a 10X objective (Axiophot, Zeiss).

OC1b was delimited following criteria proposed by Zilles *et al.*, (1984) in cresyl-violet stained sections and validated using electrophysiological recordings (Maffei *et al.*, 1992). A grid was superimposed on OC1b to delimit the area of sampling. The grid was 300 μm wide and spanned the entire visual cortex from the pial surface to the underlying white matter. The grid was composed of rectangular frames of 30 μm, covering 10% of the entire surface of the grid. At the centre of each frame, a reference point (+) was placed to assign the frame to each layer. Cells within each frame were manually counted using the "optical dissector" method (Gundersen, 1986; West, 1993); cells were counted in different focal planes excluding those within the uppermost plane. A 40X objective was generally used. However, overlapping cells were sampled with a 100X objective. Neurons were included in cell counts only if clearly stained in the cytoplasm surrounding the nucleus. Glia was identified as smaller cells without evident nucleoli and with nuclei heavily stained with the Nissl counterstaining. These were discarded (Ramón Y Cajal, 1899; Hedlich, 1988).

In no sections analyzed we observed a cellular labelling restricted to the most superficial focal planes.

The number of NR2A and NR2B subunits-immunopositive neurons per layer was expressed as a percentage of the average number of cresyl-violet stained neurons and was calculated for each cortical layer taking into account all neurons present in OC1b area in each slice considered. Statistical analysis was performed using the Kruskal-Wallis test (SigmaStat, Microsoft Inc., USA). Differences between different groups were considered significant for $P<0.05$.

Immunohistochemistry experiments were conducted blind and sections taken from different groups of animals were processed at the same time.

2.6 Western Blot

2.6.1 Rats

Two rats were treated as for immunohistochemistry experiments, either with TrkB-IgG or with 0.9% NaCl. At P36, portions of visual cortex from both hemispheres were collected separately and homogenized in triple-detergent lysis buffer (Sambrook, Fritsch and

Maniatis, Molecular cloning manual) as described by Molnar *et al.* (1998). Visual cortex was also sampled from a P36 rat that was not treated at all. Sampling of visual cortex was done in the same region investigated by immunohistochemistry. Equal amounts of homogenates were resolved on three 8% polyacrylamide gels and transferred to three nitrocellulose membranes. The membranes were then incubated in PBST (PBS, 0.05 % tween 20) + 5% milk for 1 hour at RT (to block non-specific binidng sites); two membranes out of three were probed with either anti-NR2A or anti-NR2B (the same rabbit polyclonal antibodies used for immunohistochemistry and tested by Chemicon also for working in immunoblotting) respectively diluted 1:500 and 0.8 μg/ml in PBST + 5% milk, o/n at 4°C. After several washes in PBS, membranes were incubated with anti-rabbit secondary antibody alkaline phosphatase conjugated (2 μg/ml, Vector) for 2 hours at RT. After washing with PBS, proteins were visualized using p-nitro blue tetrazolium chloride (NBT; 0.5 mg/ml, Sigma) and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP; 0.25 mg/ml, Sigma) in developing buffer (0.1 M Tris-HCl pH 9.5, 0.05 M $MgCl_2$, 0.1 M NaCl, 1 mM levamisolhydrochloride; Sigma). The same amounts of homogenates were probed, on a third membrane, with anti-tubulin (mAb YOL-1, 1:250, kindly provided by Cesar Milstein, MRC Laboratory of Molecular biology, Cambridge, UK) to compare the results with the corresponding amounts of proteins loaded in the lanes.

As a control, one membrane blotted as those used for the experiment was incubated with the secondary antibody alone.

2.6.2 Mice

Samples of mouse visual cortex were collected and homogenized according to the protocols described for rats. For each different strains of mice, and each post-natal ages considered, cortice were taken from 8-10 brains to perform the experiment 4 times (samples from 2 or eventually 3 mice had to be pooled together in order to get enough material). Protein concentration was assessed by Lowry colorimetric assay (Lowry *et al.*, 1951) so that known amounts of protein were loaded in each lane of 8% polyacrilamide gels. Western blot was performed for NMDAR subunits NR2A and NR2B according to the same protocol and using the same antibodies as already described. Bands analysis was

performed with NIH-Scion Image software (Scion Corporation, Frederick, MD, USA), and results were reported as mean ± sd. Differences were considered significant for $P < 0.001$ (statistical analysis was performed using the t test, SigmaStat, Microsoft Inc., USA).

2.7 Molecular biology

From Jackson Laboratories we acquired 2 different strains of knock out (ko) mice: (1) $Bdnf^{tm1Jae}$ where a part of exon 5 of the Bdnf gene is deleted and replaced by a neomycin cassette, leaving 49 and 30 aminoacids in the N and C termini respectively (Ernfors *et al.*, 1994); (2) SvJae-$Ntf5^{tm1Jae}$ where the entire Ntf5 gene (coding the neurotrophin NT-4/5) is replaced with a PGK-neomycin cassette (Liu *et al.*, 1995). "tm1Jae" indicates "targeted mutation 1, Rudolf Jaenisch", as the $Bdnf^{tm1Jae}$ mutant strain was developed in the laboratory of Dr. Rudolf Jaenisch at the Whitehead Institute for Biomedical Research at the Massachusetts Institute of Technology.

Heterozygous Bdnf mice were kept mating and DNA extraction from tails followed by PCR was performed for genotype screening of newborn pups.

DNA extraction: small portions of mouse tails were incubated in TB buffer (50 mM Tris-Cl, pH 8; 100 mM EDTA; 100 mM NaCl; 1% SDS; 0.6 mg/ml proteinase K; all chemicals from Sigma except for proteinase K from Roche) o/n at 55°C with continuous mixing. An equal volume of 1:1 (v/v) equilibrated phenol/chloroform (Sigma) was added; the tubes were then gently shaken on a rocker for 30 min and centrifuged for 10 min at 11000 rpm. Superior fractions were collected and 2 volumes of ethanol were added to precipitate DNA. DNA was finally resuspended in distilled water.

PCR: primers (Roche) were designed and the protocol set up according to the information provided by Jackson Laboratories:

oIMR0132; 5' – ggg AAC TTC CTg ACT Agg gg – 3'.

oIMR0133; 5' – ATg AAA gAA gTA AAC gTC CAC –3'; with oIMR132 this primer amplifies a ~250 bp DNA fragment from the wild type allele. With oIMR0134, it amplifies a ~340 bp DNA fragment from the disrupted allele.

oIMR0134; 5' – CCA gCA gAA AgA gTA gAg gAg – 3'.

5-20 ng DNA were used per reaction; the reaction mix contained also: 1x PCR buffer (10 mM Tris-HCl, 1.5 mM Mg^{2+}, 50 mM KCl, pH 8.3; Roche), 0.2 mM dNTP mix (Roche), 0.5 μM primers, 0.5 U Taq polymerase (Roche), distilled water to a final volume of 20 μl.

PCR products were separated by 2% agarose gel electrophoresis and results were: a single band of about 250 bp for Bdnf +/+ mice; a single band of about 340 bp for Bdnf -/- mice; both bands for heterozygous mice.

3. RESULTS

3.1 Immunoadhesins: production and selectivity

In order to sequester endogenous TrkB ligands in the visual cortex we made use of a soluble form of TrkB receptor engineered as an immunoadhesin (Chamow and Ashkenazi, 1996). We call this particular immunoadhesin TrkB-IgG (Shelton *et al.*, 1995), to identify a chimaeric molecule where the extracellular domain of the TrkB receptor is fused to the Fc portion of camel immunoglobulin G_2 (IgG_2). The reason for selecting this unusual mammal is that its IgG_2 naturally lacks the C_H1 domain of the Fc portion (Hamers-Casterman *et al.*, 1993), avoiding the need to artificially delete it, as with "conventional" immunoadhesins. Moreover, IgG2 has a 35 aminoacid long hinge connecting the C_H2 domain directly to the variable region. This hinge is rather rigid, being rich in proline residues, and therefore should make the TrkB receptor more accessible to its ligands. BDNF binds TrkB-IgG with a binding affinity (Kd) of 10^{-10} M, as determined with Biacore by surface plasmon resonance (unpublished data). This affinity is similar to that of BDNF with the cell bound receptor (Dechant *et al.*, 1994).

We purified immunoadhesins from the culture medium of insect cells infected by baculovirus (bv). One of the main problems with immunoadhesin production concerns possible post-translational modifications: therefore, choosing the proper infection system is vital for successful experiments. The bv infection system is particularly suitable because, in insect cells, there is no risk of post-translational modification of engineered proteins. The procedure for purification is simple and efficient: after just one step on a ProteinA-sepharose column we could obtain pure immunoadhesins, as verified each time with SDS-PAGE (Fig. 3A,B). With one purification (from culture medium of 150 million insect cells), we could get up to 1-1.5 mg of immunoadhesin a quantity that allows us to perform release of immunoadhesins in animals *in vivo*.

The specificity of TrkB-IgG for BDNF was tested by an enzyme-linked immunosorbent assay (ELISA). This was performed by applying three different concentrations of BDNF (0.01, 0.1 and 0.5 μg/ml) to solid phase coupled TrkB-IgG (1 μg/ml), followed by BDNF antibodies. ELISA was also performed using the same concentrations of NGF, a

neurotrophin whose structure is related to BDNF. TrkB-IgG showed high specificity for BDNF but not NGF (Fig. 4A,B). Following a similar procedure, as described in the Materials and methods section, the ability of TrkB-IgG to bind NT-4/5 was also tested. Increasing concentrations of NT-4/5 were applied to a fixed concentration (2 μg/ml) of solid phase coupled immunoadhesin: we observed saturation of TrkB-IgG binding sites (for that concentration) with 0.2 μg/ml NT-4/5 (Fig. 4C). On the other hand, NT-3 showed only a slight reactivity towards TrkB-IgG at the highest concentration tested (1 μg/ml; Fig. 4D). In the presence of BDNF and NT-4/5, it is very unlikely that NT-3 can compete for binding the immunoadhesin and interfere with the results of our experiments.

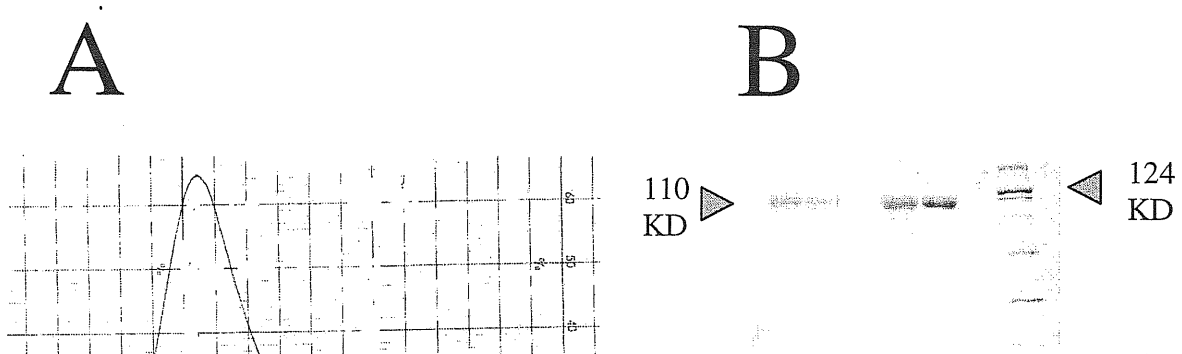


Figure 3. TrkB-IgG purification.
TrkB-IgG is purified from insect cell medium by affinity chromatography on a proteinA-sepharose column. One single peak of elution is obtained (A) and purity of the immunoadhesin is tested by SDS-PAGE loading the eluted fractions on a 10% polyacrylamide gel stained with Coomassie brilliant blue (B). From each fraction one band of expected molecular weight is detected.

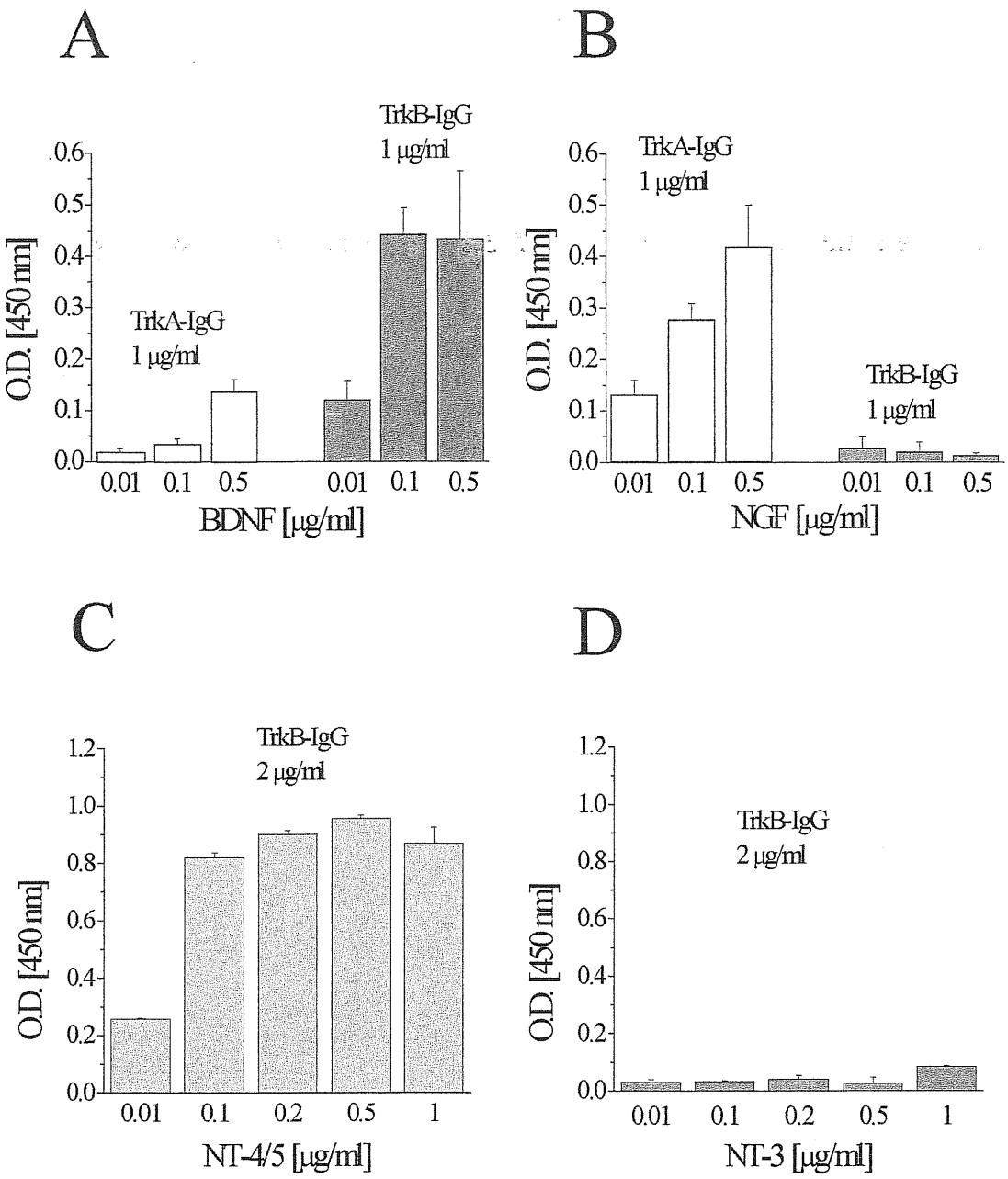
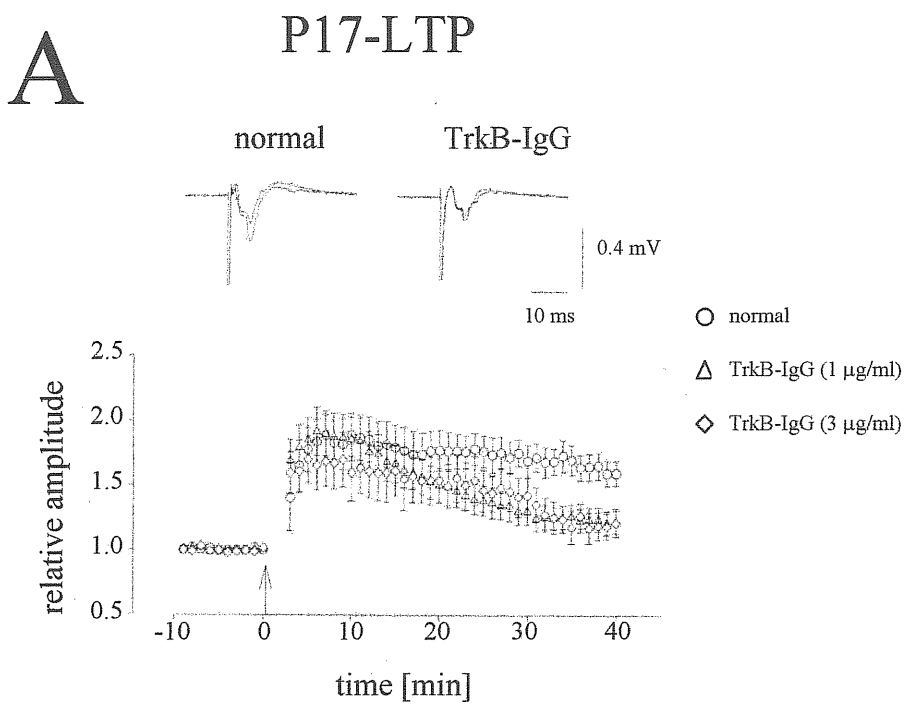


Figure 4. Specificity of immunoadhesins for neurotrophins.
ELISAs were performed to assess the specificity of TrkB-IgG for BDNF and NT-4/5. Increasing concentrations of BDNF (A) and NGF (B) were applied to a fixed concentration (1 µg/ml) of solid phase coupled TrkB and TrkA immunoadhesins. Each immunoadhesin shows high specificity for its corresponding neurotrophin. (C) Increasing concentrations of NT-4/5 were applied to solid phase coupled TrkB-IgG (2 µg/ml). TrkB-IgG binding sites seem to be saturated by 200 ng/ml NT-4/5. (D) Increasing concentrations of NT-3 were applied to solid phase coupled TrkB-IgG (2 µg/ml) resulting in almost undetectable binding.

3.2 Blockade of TrkB ligands affects LTP in the rat primary visual cortex

To assess the effectiveness of TrkB-IgG in blocking TrkB ligands such as BDNF we decided to use a functional test in *in vitro* slices containing the visual cortex. Previous work, has shown that certain forms of long term synaptic plasticity depend on BDNF for their expression (Akaneya *et al.*, 1997); in particular long term potentiation (LTP) is enhanced by BDNF supply. In the rat visual cortex, LTP elicited by stimulation of white matter is expressed from an early stage after eye opening until P30-35, an age almost coincident with the end of the critical period. To sequester endogenous TrkB ligands, slices containing the primary visual cortex were incubated in aCSF containing TrkB-IgG at two different concentrations (1 and 3 µg/ml). Note that 1 µg/ml of TrkB-IgG corresponds to a normal concentration of 66 nM, which is much greater than the Kd of the TrkB ligands filled receptors (in the order of 0.1 nM). Moreover, similar concentrations of TrkB-IgG were shown to be effective in hippocampal slices (Figurov *et al.*, 1996; Kang *et al.*, 1997). As control, we used the same concentrations of 9E10 an antibody recognizing an intracellular epytope (c-myc). Slices were incubated for 4-7 hours and then transferred to the recording chamber. To study the penetration of immunoadhesin into the tissue, slices were incubated with either aCSF containing 1 µg/ml or aCSF alone, and then visualized using a secondary antibody directed against the Fc portion of the immunoadhesin. The general staining in slices incubated with TrkB-IgG is higher than in slices incubated in aCSF alone, demonstrating that TrkB-IgG was penetrated into the tissue (data not shown).

Extracellular recordings were performed in order to study the role of endogenous TrkB ligands in regulating the expression of LTP during the postnatal development of visual cortex. This was studied at P17, LTP being evoked by high frequency stimulation (HFS, 100Hz, 1s) to the white matter, and calculated relative to the averaged baseline amplitude of field potentials. Control LTP at P17 was followed for 40 min and was characterized by a percentage change from control baseline (PCCB) of 166 ± 10 %, (n= 5, Fig. 5A).



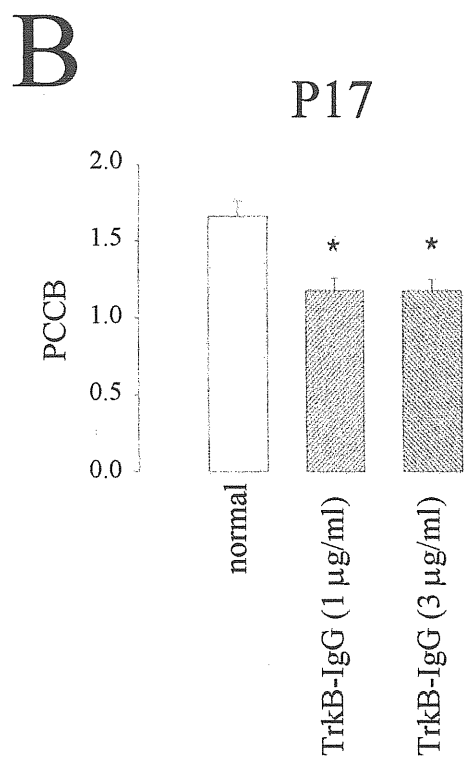


Figure 5. LTP at P17 requires TrkB activation.
(A) Averaged (± SEM) responses representing the amplitude of the maximum negative field potential recorded in layer II-III following stimulation of the white matter. Values were normalized to average baseline. The arrow indicates the conditioning protocol for LTP (three trains of 1 s at 100 Hz). At P17 LTP is equally impaired at the two concentrations of TrkB-IgG used. (B) Summary of the effects of TrkB-IgG at 1and 3 µg/ml on LTP expression at P17. Histograms show the percentage change from control baseline (PCCB) of LTP in slices incubated in the indicated conditions. *P < 0.05. Adapted from Sermasi et *al.* (2000); Eur. J. Neurosci.; 12: 1411-1419; figure 2A and figure 4A.

In experiments using TrkB-IgG incubated slices, tetanic stimulation (100 Hz, 1s) to the white matter resulted in a temporary increment in the amplitude of the recorded responses lasting around 30 min (fig. 5A). PCCB at 30-40 min after tetanus was dramatically reduced with respect to control slices (118 ± 8 % vs. 166 ± 10 %, n=8). Evoked responses did not completely recover to baseline values but the difference in PCCB at 30-40 min after tetanic stimulation between control and TrkB-IgG incubated slices was statistically significant (t test, $p < 0.05$, Fig. 5B).

We then examined whether the residual LTP still present in the TrkB-IgG incubated slices at 1 μg/ml reflected an incomplete effect of the TrkB-IgG. To check this possibility, in a further set of experiments slices were incubated with a three fold higher concentration of TrKB-IgG (3 μg/ml). The resulting impaired LTP (Fig. 5A,B) was statistically indistinguishable from that observed with 1 μg/ml (PCCB at 30-40 min of 118 ± 7 %, n= 5, t test, $p > 0.05$). Incubation with 9E10 was used as a negative control for possible non-specific effect on LTP expression. These results confirm that the observed impairment of LTP is specifically due to the blockade of TrkB ligands. Moreover these results indicate that TrkB-IgG is an effective tool for blocking TrkB ligands in visual cortex.

3.3 NMDARs in rat visual cortex

3.3.1 Pump implantation and compound diffusion

The effects of TrkB signalling blockade on the developmental expression of NMDARs were analysed during the window of postnatal development between P20-21 and P36-37. This particular period was chosen on the basis of results obtained in parallel studies by E. Tongiorgi, F. Ferrero, A. Cattaneo and L. Domenici (paper submitted to Neuroscience). Comparing the pattern of cellular expression of NMDAR subunits at P21 and P37, the authors observed that between P20 and P36 there are no gross changes in the cellular distribution of immunoreactivity for NR2A and NR2B. A small change was described in layer V, where the number of stained cells for NR2A was slightly but significantly lower. In contrast, the staining level for NR2A was highly increased in P21 compared to P37.

The conclusion of this work was that NMDARs undergo developmental regulation during the period between P20 and P36. The second main reason to choose this age period was that we did not want to interfere with developmental regressive phenomena such as cell death, which by P21 are largely complete in the visual cortex.

TrkB-IgG, or physiological solution as control, were infused into the right occipital pole by means of mini-osmotic pumps connected to a cannula inserted at 1 mm lateral to midline (Fig. 6A) following methods previously described (Lodovichi *et al.*, 2000). We used TrkB-IgG at a concentration of 0.5 mg/ml, roughly corresponding to 33 μM, i.e. in vast excess of the concentration used for bathing slices before LTP protocols (as mentioned above), that was however efficient in sequestering TrkB ligands.

In fig. $6B_1$ it appears that the cannula produced a lesion whose diameter was roughly 0.5 mm.

The center of the site lesion was localized as 1.5-2 mm away from primary visual cortex. To test if TrkB-IgG really reached the primary visual cortex, immunohistochemistry for TrkB-IgG was performed in serial slices adjacent to those used for NMDAR expression analysis. To study the diffusion of TrkB-IgG, we used an antibody against camel IgG.

The results clearly showed that immunoreactivity for TrkB-IgG was present at a distance of 2.5-3 mm from the injected site, i.e. TrkB-IgG released by the implanted cannula was able to reach the primary visual cortex (Fig. $6B_2$). Staining was more intense within 1mm on either side of the cannula insertion site (Fig. $6B_1$). In order to exclude possible mechanical damage to the tissue, we checked the layer organization and the distribution of cresyl-violet stained cells. Laminar organization was altered in the region of the lesion site, whilst in the primary visual cortex reached by TrkB-IgG, the cytoarchitecture was completely normal throughout all cortical layers (data not shown).

Before investigating possible changes in NMDAR subunit expression pattern, we also verified if blocking TrkB signalling had any effects on the cell cytoskeleton. To this end we performed immunohistochemistry with antibodies directed against two specific markers: the first one, MAP2, is an essential cytoskeletal protein of neurons and

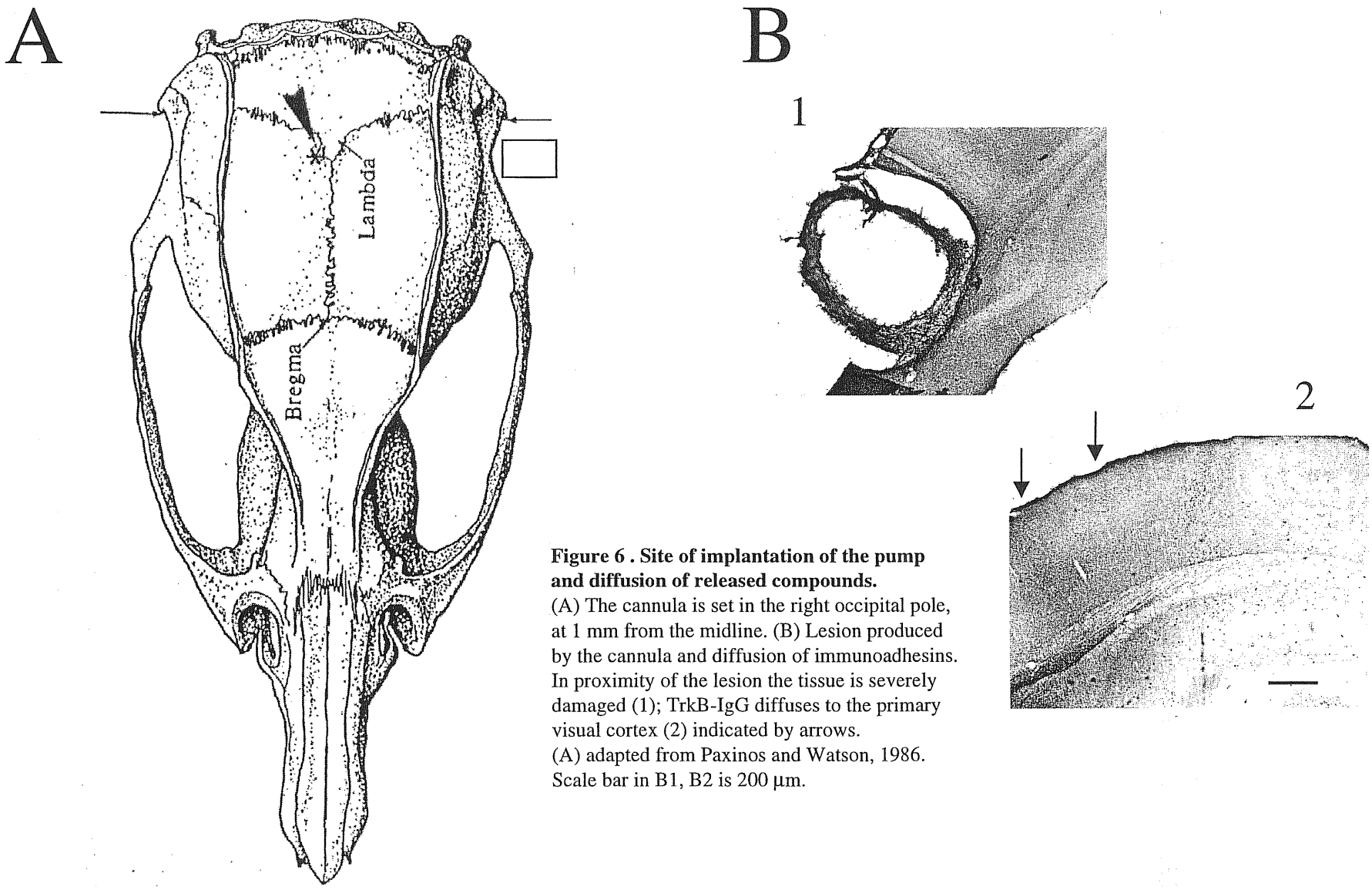


Figure 6 . Site of implantation of the pump and diffusion of released compounds.
(A) The cannula is set in the right occipital pole, at 1 mm from the midline. (B) Lesion produced by the cannula and diffusion of immunoadhesins. In proximity of the lesion the tissue is severely damaged (1); TrkB-IgG diffuses to the primary visual cortex (2) indicated by arrows.
(A) adapted from Paxinos and Watson, 1986.
Scale bar in B1, B2 is 200 µm.

the second one, GFAP, is a cytoskeletal protein specific for astrocytes. We focused our attention on the primary visual cortex, i.e. away from the region containing the lesion induced by the cannula. In fig. 7A, we report staining for MAP2 in cortical regions of control (1) and TrkB-IgG treated (2, 3, 4) rats; in particular, in TrkB-IgG treated cortex, regions in 2 and 3 are at different distances (2 and 2.5 mm respectively) from the site of implant. It appears that staining for MAP2 in TrkB-IgG treated rats was not different from the staining observed in control animals. Also, the staining for GFAP showed no differences between treated and control rats (Fig. 7B).

3.3.2 NMDARs: immunohistochemistry and cell counts

To detect NR1 in visual cortical neurons, we performed immunohistochemistry using a commercial antibody specific for four out of the eight splicing forms of this subunit, namely 1a, 2a, 1b and 2b. These have previously been shown to be expressed in the rat visual cortex (Sugihara *et al.*, 1992). In P36 TrkB-IgG-treated and control rats, most cells throughout all cortical layers expressed NR1. Immunostaining was prevalent in the cell perykaria and in proximal dendrites (Fig. 8A,E). Fig. 8 shows that TrkB-IgG treatment did not produce changes in the distribution of NR1 throughout the different cortical layers; also, the intensity of immunostaining was not different in treated and control animals.

In control animals, staining for the NR2A and NR2B subunits was less intense and occurred in less cells compared to NR1. NR2A and NR2B, like NR1, were abundant in the cell perykaria and were often present also in basal and apical dendrites. In TrkB-IgG treated rats, immunostaining for the NR2A subunit was less intense compared to control rats and the stained neurons were more numerous (Fig. 8B,E). Also, immunostaining for the NR2B subunit differed between the two groups of animals and fig. 8C,F shows that the staining for NR2B was less intense in treated compared to control animals, while the laminar distribution of labeled cells was unchanged.

To obtain a quantitative evaluation of these changes, some serial sections of visual cortex from each animal within the region of immunoadhesin diffusion, were processed for

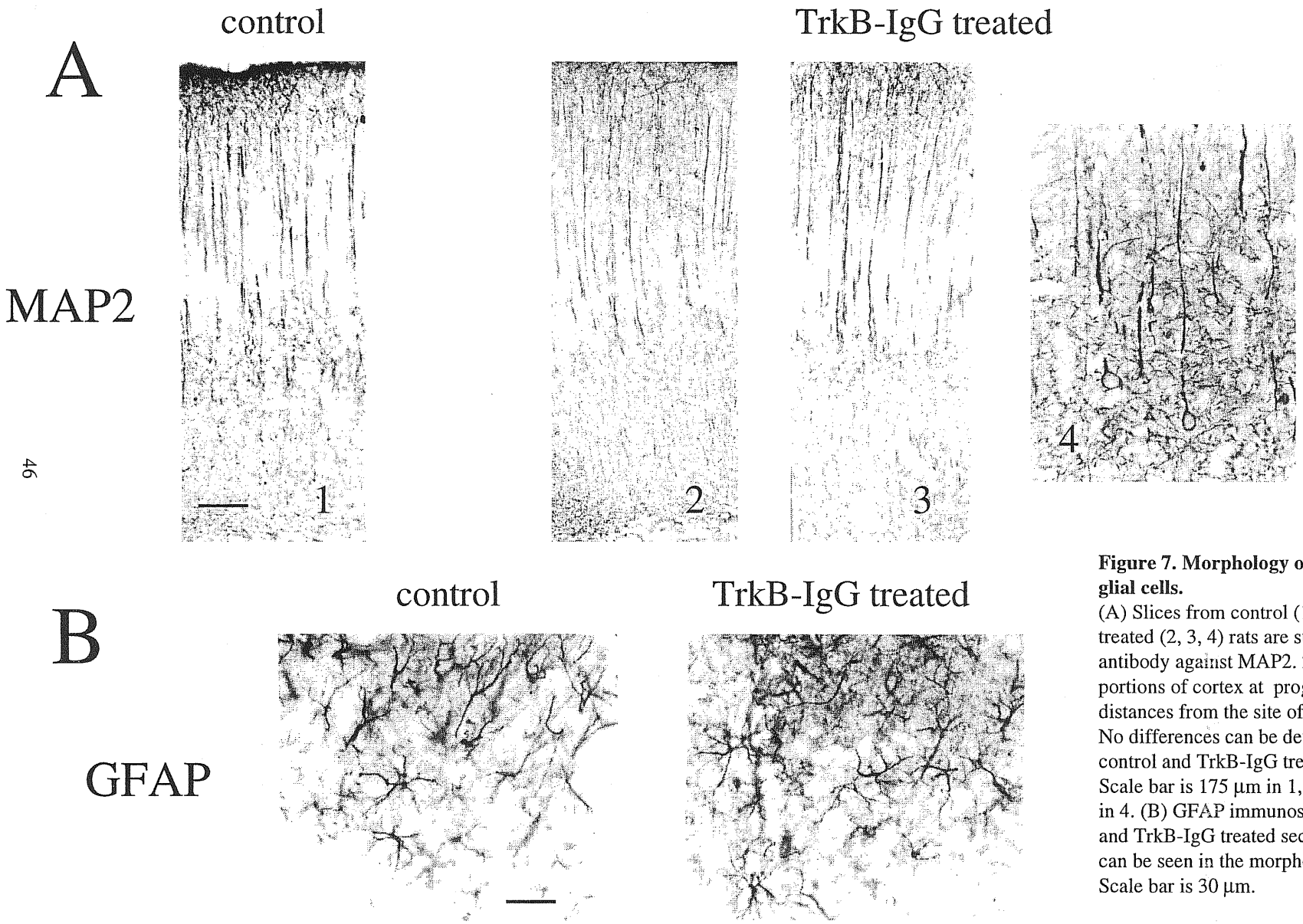


Figure 7. Morphology of neurons and glial cells.
(A) Slices from control (1) and TrkB-IgG treated (2, 3, 4) rats are stained with an antibody against MAP2. 2, 3 represent portions of cortex at progressively higher distances from the site of injection. No differences can be detected between control and TrkB-IgG treated sections. Scale bar is 175 μm in 1, 2, 3 and 60 μm in 4. (B) GFAP immunostaining in control and TrkB-IgG treated sections: no differences can be seen in the morphology of glial cells. Scale bar is 30 μm.

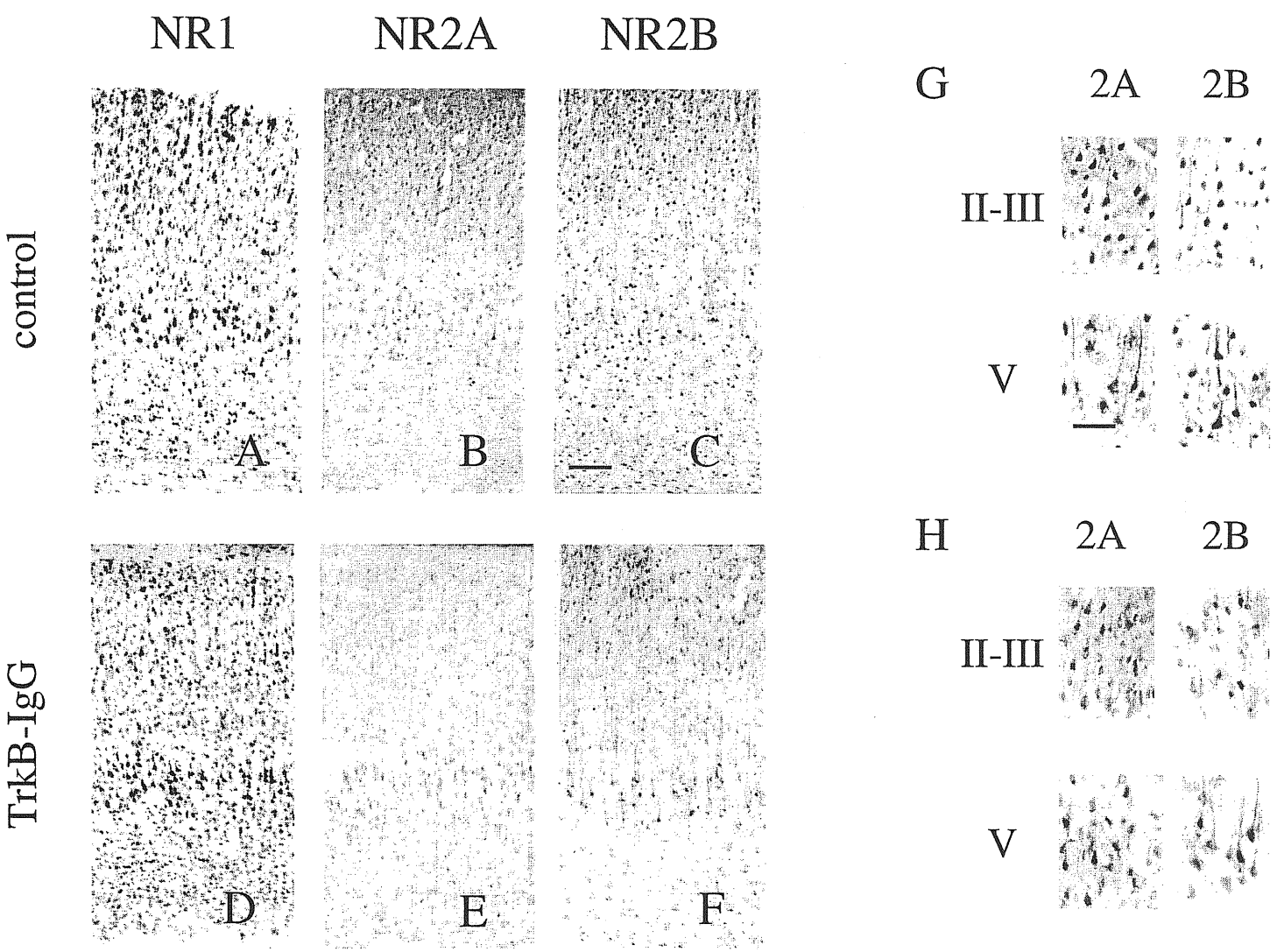


Figure 8. NMDAR subunits immunostaining.
(A-C) Staining for NR1, NR2A, NR2B in primary visual cortex of control rats. Scale bar is 80 μm. (G) Enlargements of layers II-III and V neurons. Scale bar is 55 μm. (D-F) Staining for NR1, NR2A, NR2B in primary visual cortex of TrkB-IgG treated rats. Immunoreactivity for NR1 is not significantly different from control. Intensity of staining for NR2A and NR2B is weaker than in controls and especially as far as NR2A concerns, the number of stained neurons seems to be increased. Scale bar as for (A-C). (H) Enlargements of layers II-III and V neurons. Scale bar as for (G).

immunohistochemistry and counterstained with cresyl-violet (Fig. 9). To avoid bias due to cell shrinkage, the number of immunopositive neurons was expressed as a percentage of the number of cresyl-violet stained cells. To sample cells in different cortical layers, we defined a 300 μm wide grid, spanning all cortical layers. We used this method to evaluate the percentage of neurons expressing NR2A and NR2B subunits of both P36 TrkB-IgG treated and control animals. Fig. 10 shows that the percentage of NR2A immunopositive neurons was significantly higher ($P< 0.05$) in all cortical layers in TrkB-IgG treated animals compared to controls. No significant differences were found in the mean percentage of NR2B expressing neurons, when TrkB-IgG treated animals were compared to controls (Fig. 10).

The quantitative results confirmed the qualitative ones; when TrkB activation is blocked, the NR2A subunit is expressed in a higher percentage of neurons, although at a lower staining level.

3.3.3 Western blot analysis

Since blockade of TrkB signalling reduces cellular staining intensity of both NR2A and NR2B, we predicted that, in the absence of TrkB activation, NR2A and NR2B would be expressed at lower levels in visual cortex. To test this prediction we performed immunoblotting for these subunits on visual cortex homogenates from animals treated with TrkB-IgG and saline, as previously described. Homogenates were obtained from portions of visual cortex precisely corresponding to the region chosen for immunohistochemistry and cell counts.

All antibodies used recognize a single band on the blotted membrane, of molecular weight corresponding to that described in the literature (180 KDa for NMDAR subunits NR2A and NR2B; about 55 KDa for tubulin).

To control for non-specific results, the first lane on each gel was loaded with homogenates from a normal P36 rat (not implanted with an osmotic pump). In this case, we expected to see the same amount of NR2A and NR2B subunits as in the untreated hemispheres of implanted rats. In fig. 11, the bands for the normal rat (lane 1) had the same intensity as those for controlateral (untreated) hemispheres of implanted rats

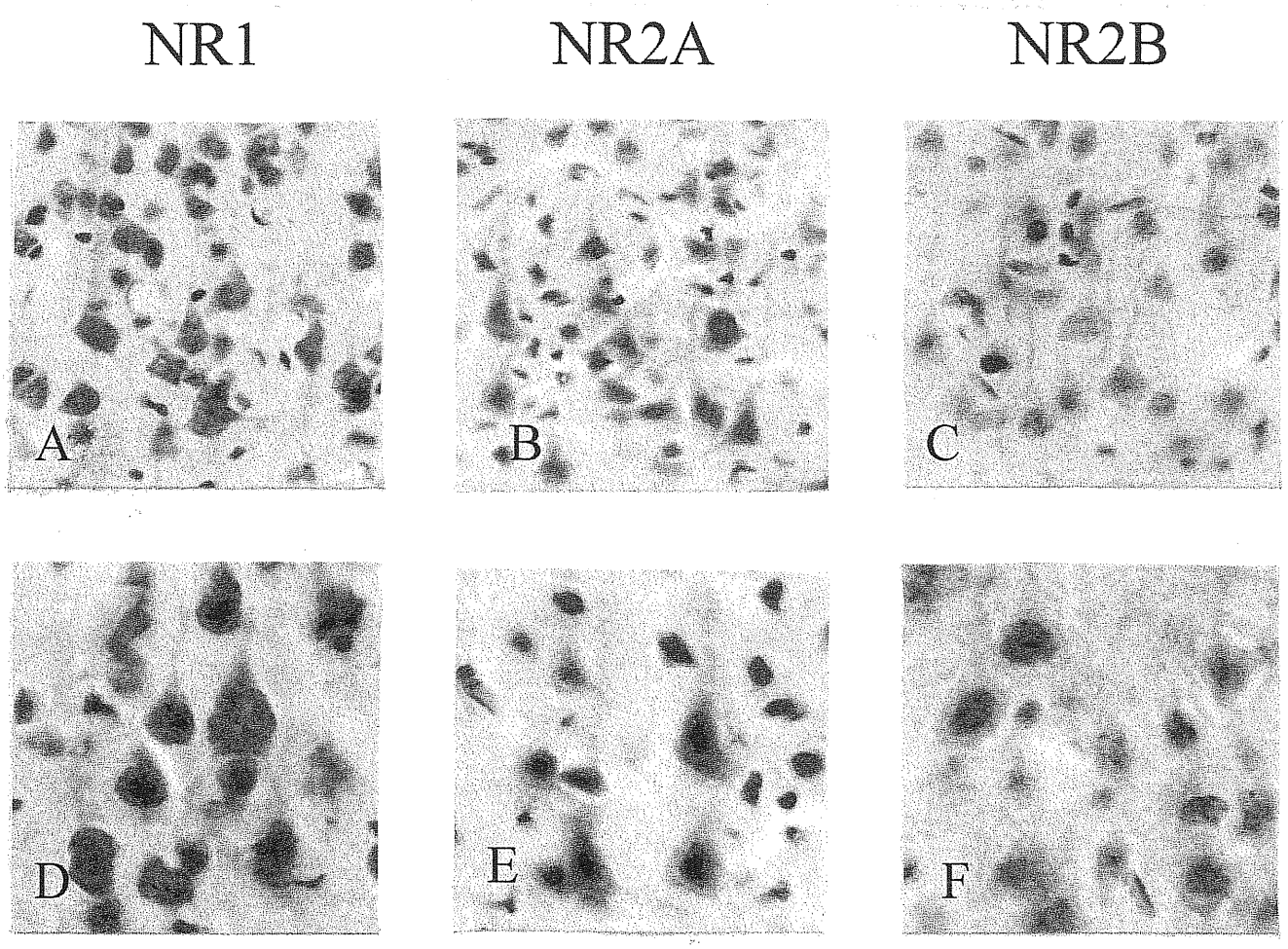


Figure 9. Imunohistochemistry for NMDAR subunits and counterstaining with cresyl violet.
Layer V pyramidal neurons are stained for NMDAR subunits NR1, NR2A and NR2B and counterstained with cresyl-violet. Scale bar is 25 μm in A, B, C and 15 μm in D, E, F.

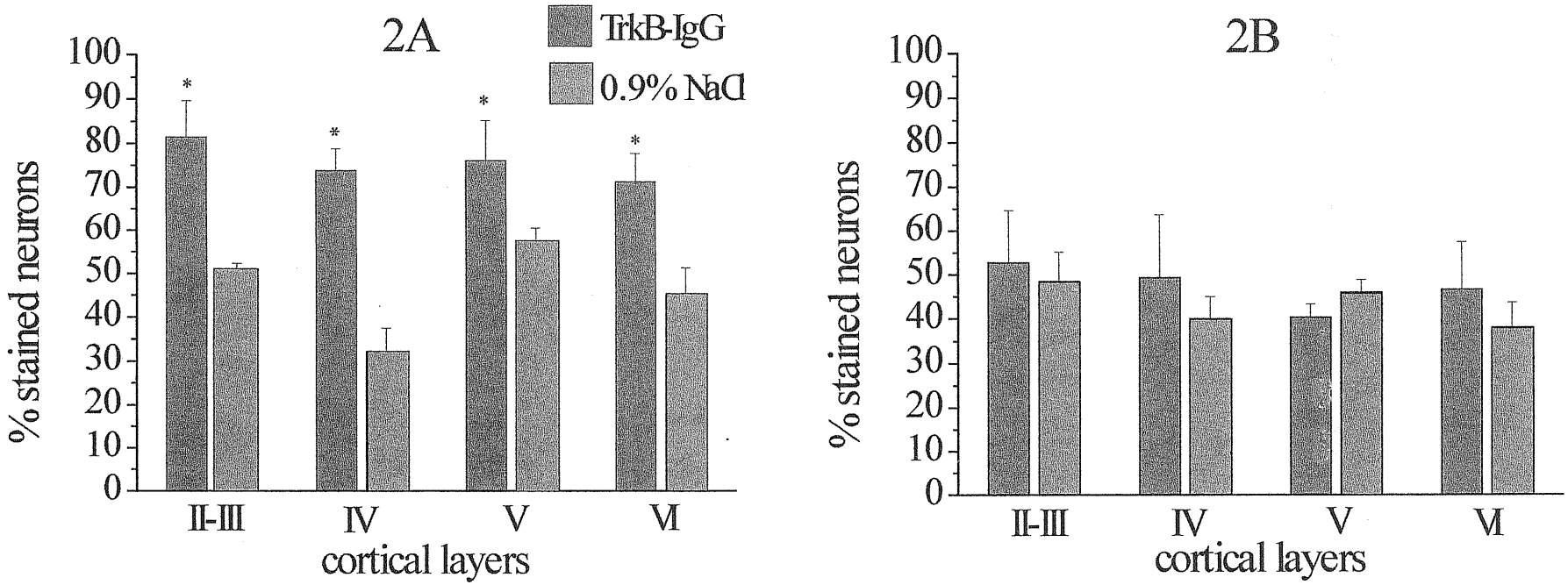


Figure 10. Blockade of TrkB signaling differently regulates NR2A and NR2B subunits in primary visual cortex during post-natal development. Percentages of stained neurons are expressed as means ± SD. In TrkB-IgG treated animals, in all cortical layers, percentage of neurons stained for NR2A is significantly increased (*) with respect to control ($P < 0.05$). Differences in percentages of NR2B stained neurons are not significant.

(lanes 3 and 5) when normalized to tubulin for the total amount of extracted protein. Note that the bands in lanes 3 and 5 show the same intensities; this represents an internal control for our experiment.

The bands corresponding to TrkB-IgG treated cortices (lane 4) were less intensely stained than both untreated cortex (lane 5) and control cortex treated with physiological solution (lane 2). Thus, blockade of TrkB signalling reduces the level of both NR2A and NR2B in visual cortex.

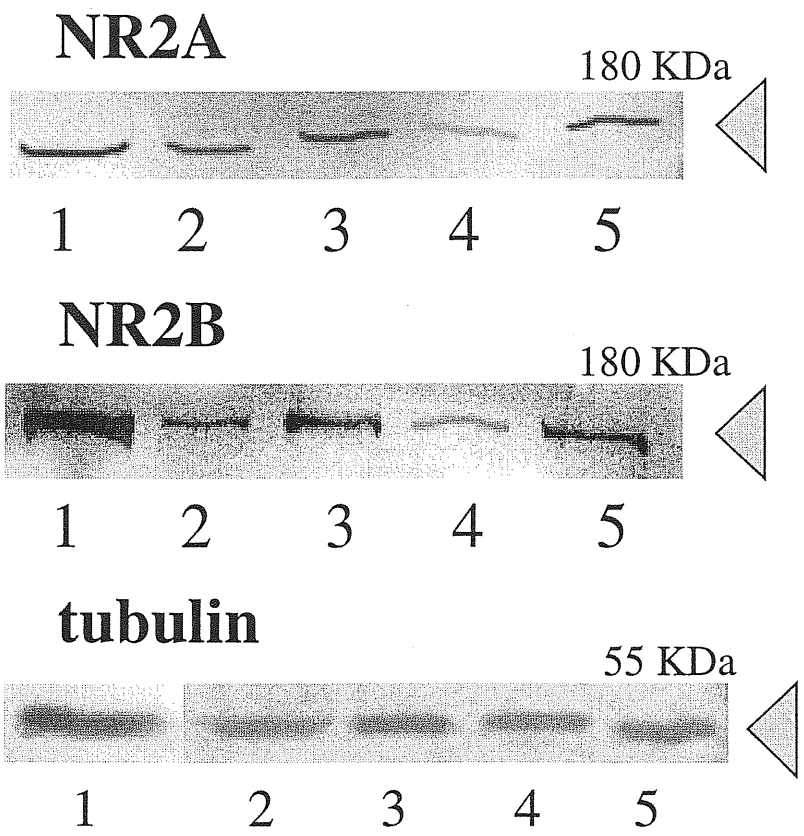


Figure 11. Western blot
Equal amounts of homogenates were loaded on three 8% polyacrylamide gels: lane 1 contains visual cortex homogenate from untreated P36 rat; lanes 2 and 3 contain homogenates from control rat, respectively the hemisphere ipsilateral and controlateral to the site of injection of the pump; lanes 4 and 5 were loaded with homogenates from TrkB-IgG treated rat, respectively the hemisphere ipsilateral and controlateral to the site of injection. Bands in lane 4 are less intensely stained both for NR2A and NR2B, than those in lanes 2 and 5. Blotting for tubulin was performed to have an internal control on the amount of extracted proteins.

3.4 NMDARs in developing visual cortex of knock-out mice

The specificity of TrkB-IgG for both BDNF and NT-4/5 led to the blockade of TrkB activation in the visual cortex of treated rats, probably causing more complex effects than simply blocking these two neurotrophins separately. This aspect of our methods might account, at least in part, for the discrepancies between the results just presented and those published by other authors (Small *et al.*, 1998, see discussion).

In order to study separately the influences of BDNF and NT-4/5 deprivation on the development of NMDARs in the visual cortex, we acquired 2 strains of knock-out (ko) mice: $Bdnf^{tm1Jae}$ and 129S4/SvJae-$Ntf5^{tm1Jae}$, as described in Materials and methods (and abbreviated Bdnf and Ntf5 respectively). Bdnf heterozygous mice (+/-) are phenotypically indistinguishable from homozygous ones (+/+), while homozygous mice (-/-) are smaller than normal siblings and most die within the second postnatal week. With particular care and attention we managed to take a few of them to the third postnatal week). In addition, observing their general locomotion behavior, it appears that these mice have defective coordination of movement and balance. They also exhibit head-bobbing and spinning during periods of hyperactivity. Although the proportion of Bdnf -/- homozygous should be ¼ of the total number of newborns when mating +/- with +/-, we observed that they are in fact less numerous. We suggest that this is due to embryonic death.

In contrast to Bdnf -\- mice, Ntf5 (+/+ and -/-) homozygous mice are phenotypically indistinguishable, viable and fertile both during post-natal development and in adulthood.

3.4.1 DNA extraction and PCR

We extracted total DNA from pieces of tail cut from each newborn mouse in Bdnf +/- x +/- matings. Polymerase chain reaction (PCR) was performed with 5-20 ng DNA and with primers amplifying both the Bdnf wild type allele and the disrupted one. After PCR, the products of amplification were separated on a 2% agarose gel (Fig. 12). Sham reaction did not present any bands, ensuring absence of contamination. One single band of about 250 bp indicates +/+ homozygous mice (same genotype as wild type); one single

band of about 340 bp indicates -/- homozygous mice; the simultaneous presence of both bands indicates +/- heterozygous mice, presenting one copy of the wild type allele and one copy of the disrupted one.

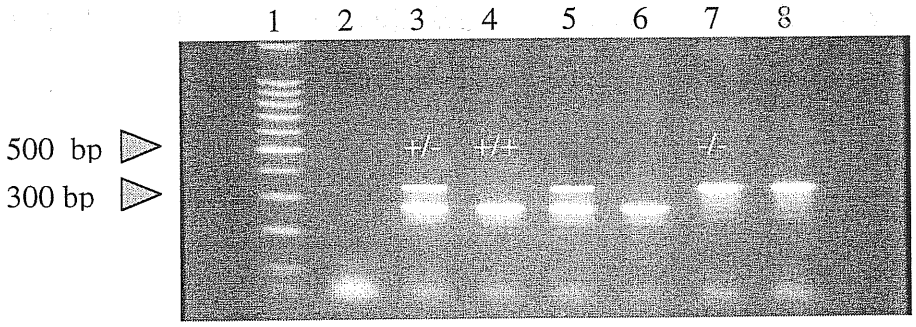


Figure 12. PCR products of DNA extracted from Bdnf^{tm1Jae} mice tails.
After DNA extraction from mice tails, PCR is performed to amplify Bdnf gene. To reveal genotypes, samples are loaded on a 2% agarose gel. Lane 1: marker; lane 2: sham reaction; lanes 3, 5: +/- mice; lanes 4, 6: +/+ mice; lanes 7,8: -/- mice.

3.4.2 Immunocytology

To evaluate the cellular expression of NMDAR subunits in the visual cortex during post-natal development, we analyzed their cellular distribution and level of cellular staining using the same antibodies and following the same procedures described above for rats. Our investigation concerned homozygous animals (both +/+ and -/-) of Bdnf and Ntf5 mice. We considered three post-natal ages: P12-14 (before eye opening); P21-23 (peak of the critical period); P50-60 (adulthood). In case of Bdnf -/- mice, as mentioned above, it was not possible to investigate post-natal ages older than P20-21 because of death of these animals.

Interestingly, eye opening is delayed in these knock-out animals. Indeed, at P20-21 when they were sacrificed, their eyes were still closed, whilst wild type mice opened their eyes around P14.

3.4.2.1 Bdnf^{tm1Jae} mice

Staining for the NR1 subunit showed a cellular pattern that did not change at different ages and between the different genotypes (Fig. 13): in fact cells were uniformly and intensively stained throughout all cortical layers. This suggests that development of the NR1 subunit is almost complete by the time when started our analysis (P12-14) and is not influenced by BDNF.

Concerning the NR2A subunit (Fig. 14), in wild type mice stained cells were generally more numerous and intensively labeled in both layers II-III (and mainly in the uppermost portion) and layer V at all post-natal ages. Layers IV and VI presented sparse cells. The general cellular distribution was similar at all ages considered. The intensity of staining was slightly weaker at P21-23 than at P12-14, and stronger in adulthood.

P12-14 knock-out animals exhibited sparse cells, whose staining was quite uniform and low in intensity. Indeed, the intensity of staining was lower at both post-natal ages in -/- mice compared to age-matched wild type. At P21-23 however, the intensity of staining was stronger than at P12-14. At this stage of development the general distribution of stained cells was similar in -/- and +/+ mice, even though stained cells appeared more numerous in layers II-III in knock-out animals with respect to wild type. Thus, cellular expression of NR2A appears to be reduced in knock-out mice at P12-14, while at a later age (P21-23) this reduction tends to disappear.

Concerning the NR2B subunit, in wild type mice immunopositive cells were generally more intensely stained in layer V than in the other layers, and the distribution of stained cells was uniform throughout the whole cortex (Fig. 15). The intensity of staining was slightly reduced at P21-23 and slightly increased at adulthood.

In P12-14 -/- mice, cells were strongly stained in layers II-III and V, and the overall level of staining was highly similar to that found in age-matched wild type mice (Fig. 15). At P21-23, stained cells were more regularly distributed in all cortical layers with the same intensity of staining between layers, but weaker than at P12-14 (Fig. 15). Thus, cellular expression of NR2B is not grossly altered in knock-out mice.

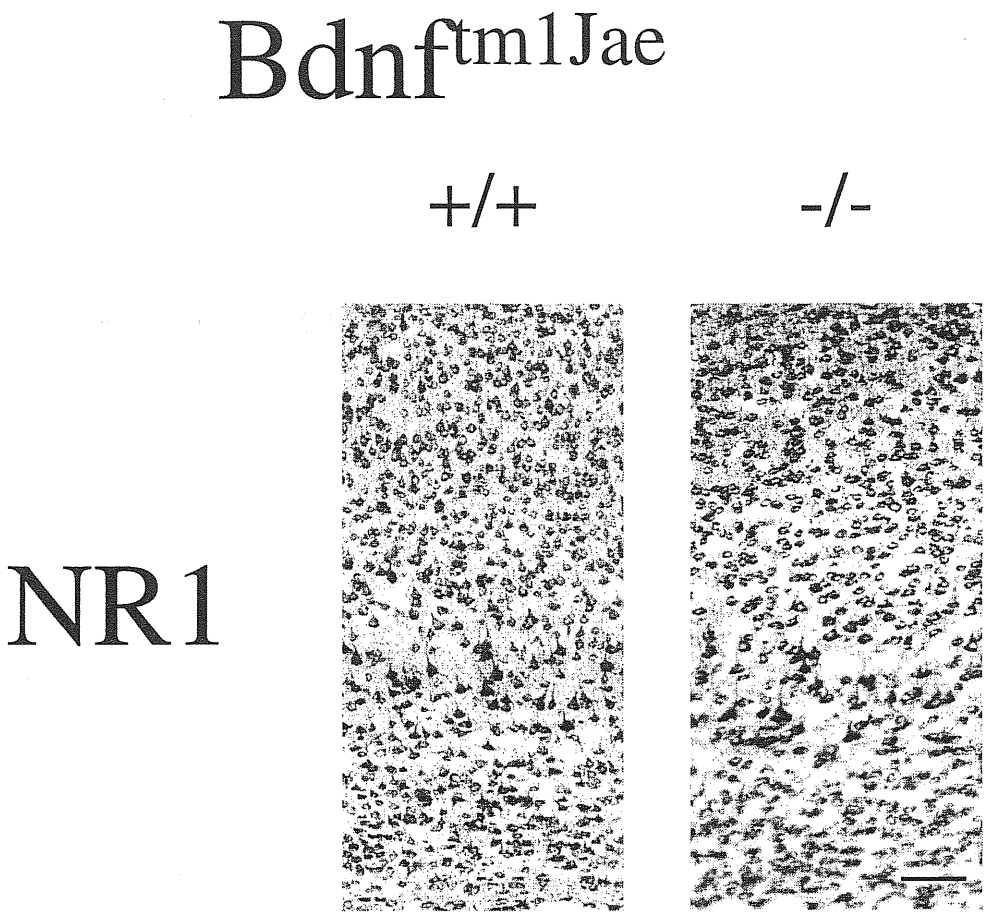


Figure 13. Immunohisotchemistry for NR1 subunit in Bdnf^{tm1Jae} mice visual cortex.
Primary visual cortex of wild type (+/+) and Bdnf ko mice (-/-) was stained for NR1 subunit of NMDARs. The sections are taken at different post-natal ages to show that the development of this subunit is over at the time we start our study and does not depend neither on genotype nor on age. Scale bar is 40 μm.

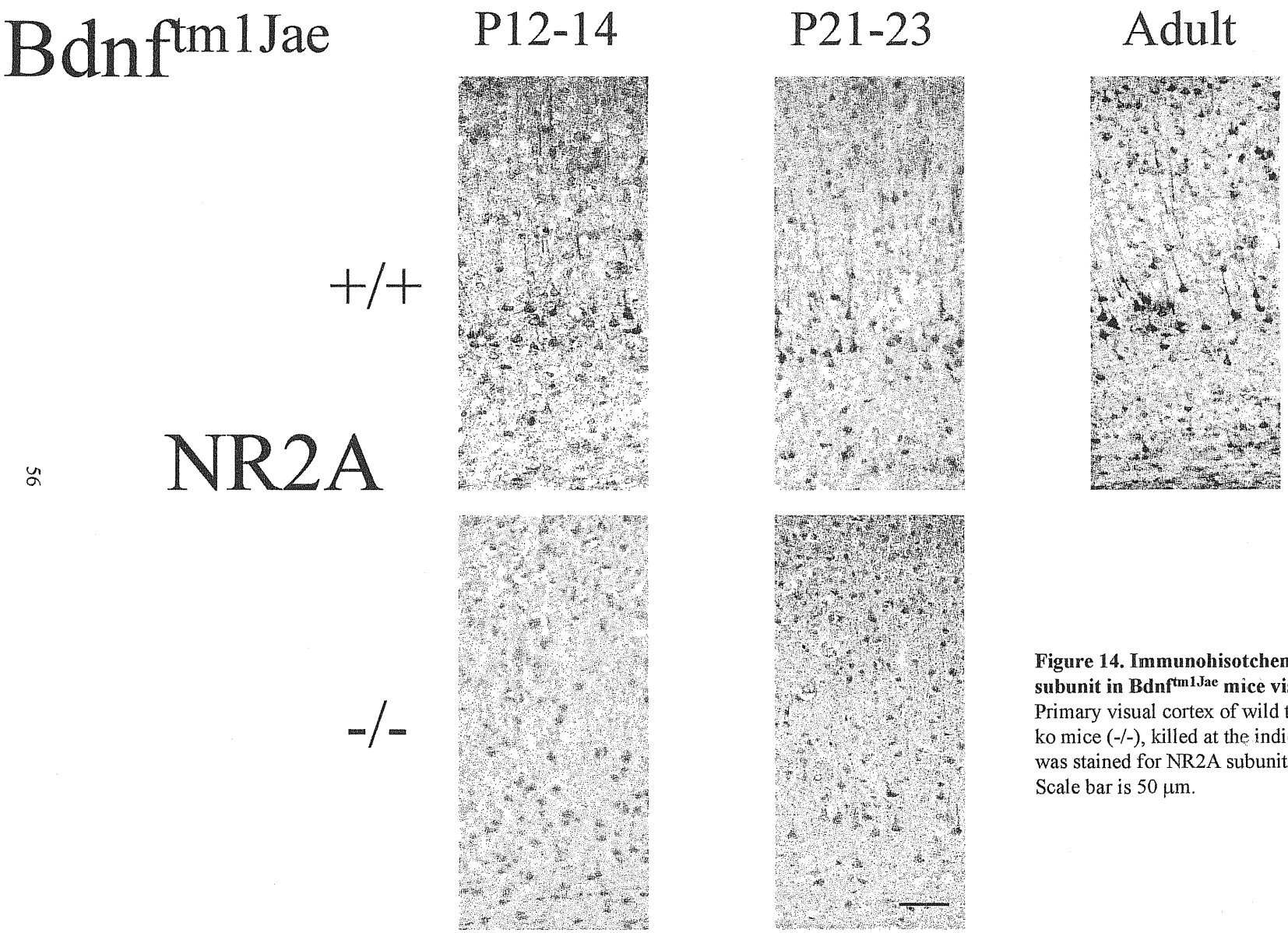


Figure 14. Immunohisotchemistry for NR2A subunit in Bdnf^{tm1Jae} mice visual cortex.
Primary visual cortex of wild type (+/+) and Bdnf ko mice (-/-), killed at the indicated post-natal ages, was stained for NR2A subunit of NMDARs. Scale bar is 50 μm.

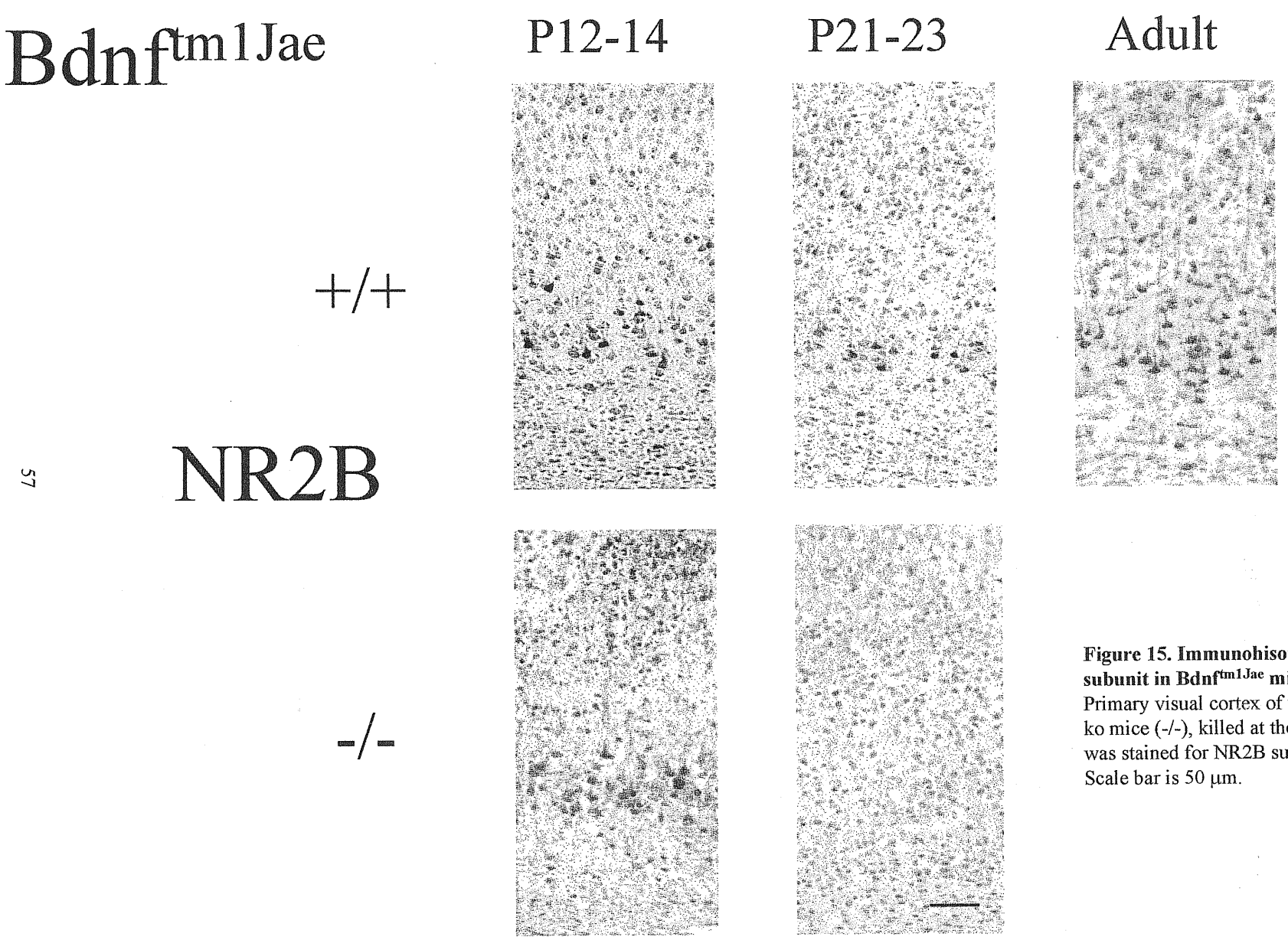


Figure 15. Immunohisotchemistry for NR2B subunit in Bdnf^{tm1Jae} mice visual cortex.
Primary visual cortex of wild type (+/+) and Bdnf ko mice (-/-), killed at the indicated post-natal ages, was stained for NR2B subunit of NMDARs. Scale bar is 50 μm.

3.4.2.2 SvJae-Ntf5^{tm1Jae} mice

As for Bdnf mice, development of the NR1 subunit appears to be complete by the age at which we started our analysis (P12-14), and no differences were evident between the different genotypes (Fig. 16).

Concerning the NR2A subunit (Fig. 17), in P12-14 +/+ mice, cells in layers II-III and V were more strongly labeled than those in layers IV and VI, where only few cells were evident. In P21-23 animals, stained cells were less concentrated in layer II-III and V, appearing uniformly distributed throughout all cortical layers. Adult animals presented a pattern of cellular distribution similar to that found at P21-23, although layers II-III and V were slightly more strongly labeled than IV and VI (where many cells were stained).

No striking differences were observed in the overall distribution of stained cells between mutant mice and age-matched wild types. The only exception was at the P12-14 stage: indeed, -/- animals exhibited cells uniformly labeled for NR2A and distributed throughout all cortical layers. Thus, in knock-out mice no differences are evident at the different ages. Alterations in NR2A induced by absence of NT-4/5 appear to be subtler and attenuated with respect to those shown in Bdnf mutant mice.

With respect to NR2B subunit (Fig. 18), wild type mice exhibited, at P12-14, stained cells uniformly distributed throughout all cortical layers. Moreover, the intensity of staining was stronger in layers V and II-III (the uppermost portion). At P21-23 and in adulthood, layers II-III and V were more strongly stained and cells were less numerous than at P12-14.

In mutant mice at P12-14, the distribution of NR2B was not grossly different compared to wild type age-matched mice. However, the stained cells appeared to be more numerous in layer II-III, although the level of staining was lower. At P21-23 and in adulthood, the general pattern of cellular distribution was the same: stained cells were uniformly distributed throughout all cortical layers, more numerous than in age-matched wild type animals, less numerous than in P12-14 mutant mice. In adult animals the general intensity of staining seemed more similar to P12-14 -/- mice, while at P21-23 cells were more strongly labeled. Thus, the cellular distribution of immunostained cells is not grossly altered in the absence of NT-4/5, with the exception that at an early post-natal stage stained cells appear more numerous in layer II-III.

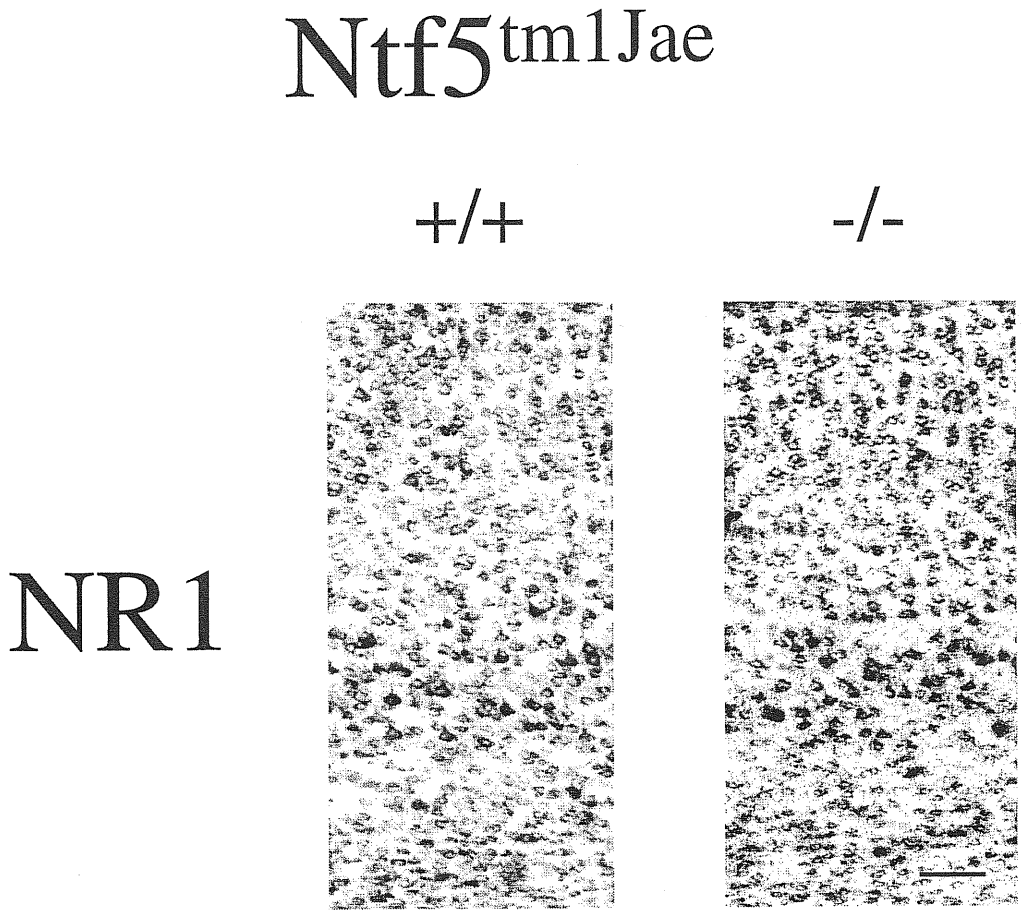


Figure 16. Immunohisotchemistry for NR1 subunit in Ntf5^{tm1Jae} mice visual cortex.
Primary visual cortex of wild type (+/+) and NT-4/5 ko mice (-/-) was stained for NR1 subunit of NMDARs. The sections are taken from different post-natal ages to show that the development of this subunit is over at the time we start our study and does not depend neither on genotype nor on age. Scale bar is 40 μm.

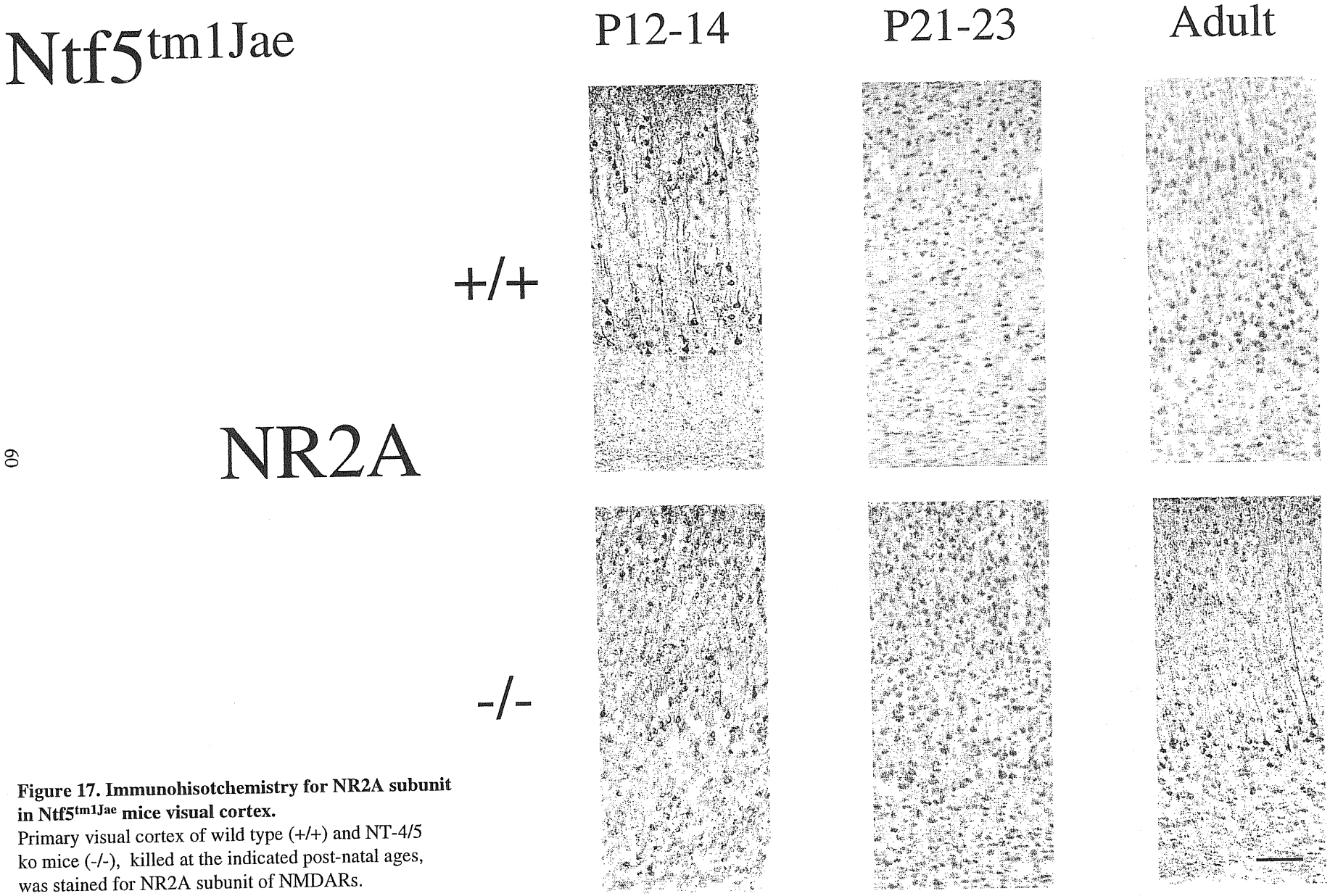


Figure 17. Immunohisotchemistry for NR2A subunit in Ntf5^{tm1Jae} mice visual cortex.
Primary visual cortex of wild type (+/+) and NT-4/5 ko mice (-/-), killed at the indicated post-natal ages, was stained for NR2A subunit of NMDARs.
Scale bar is 50 mm.

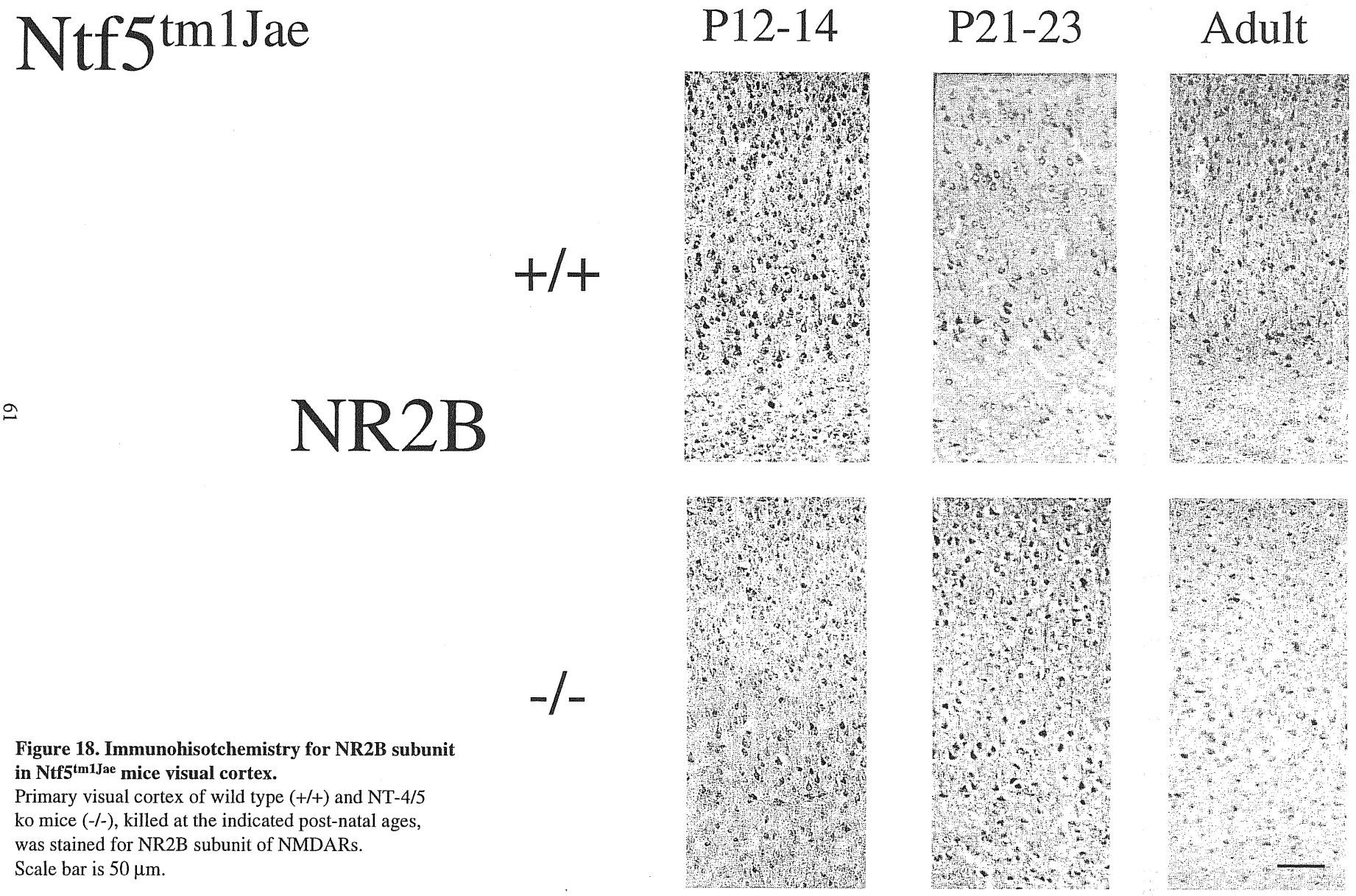


Figure 18. Immunohisotchemistry for NR2B subunit in Ntf5^{tm1Jae} mice visual cortex.
Primary visual cortex of wild type (+/+) and NT-4/5 ko mice (-/-), killed at the indicated post-natal ages, was stained for NR2B subunit of NMDARs. Scale bar is 50 μm.

3.4.3 Western blot

To evaluate the levels of NMDAR subunit expression in visual cortex during development, we performed immunoblotting with the same antibodies and the same procedures used for rats. For all samples homogenized, protein concentration was determined by using a colorimetric assay (Lowry *et al.*, 1951) in order to load the same amount of proteins in each lane of the gels. However, note that the efficiency of protein extraction was quite variable from case to case; thus we maximized the amount of homogenates to load on the basis of the worst extraction efficiency. Thus, in the case of $Bdnf^{tm1Jae}$ mice, 13±2 μg of proteins were loaded on each lane while in case of $Ntf5^{tm1Jae}$ mice, 29±2 μg of proteins were loaded. Analysis was limited to subunits NR2A and NR2B and did not include NR1 since, as shown in the previous chapter, NR1 cellular expression was not altered in mutant mice.

Measurements of band intensity were taken (and plotted) as correspondent to NMDAR subunits content. Due to the nature of the output of the software used for analysis, we have indicated in all graphs in figures 19 and 20 that NMDAR subunit content is given in arbitrary units.

3.4.3.1 $Bdnf^{tm1Jae}$ mice

In Bdnf +/+ mice, the level of NR2A subunit did not change from P12-14 to adulthood (Fig. 19A, lanes 1, 2, 3). In -/- mice, the level of NR2A subunit was reduced at P12-14 (lane 4) recovering to a normal level at P21-23 (lane 5; Fig. 19, table 1). Thus, in quite good agreement with immunocytology, absence of BDNF causes a reduction of NR2A level at an early stage of post-natal development (P12-14), while later this reduction disappears.

Concerning the NR2B subunit (Fig. 19B), +/+ mice exhibited a high level at P12-14 (lane 1). At P21-23 (lane 2), NR2B was significantly reduced compared to P12-14, while no differences were found between P21-23 and adult animals (lanes 2, 3). Bdnf -/- animals were characterized by abundant level of NR2B at P12-14 (lane 4). As with the results obtained in wild type mice, the level of this subunit at P12-14 was higher compared to P21-23 mice of same genotype (lane 5). No significant differences were found at either

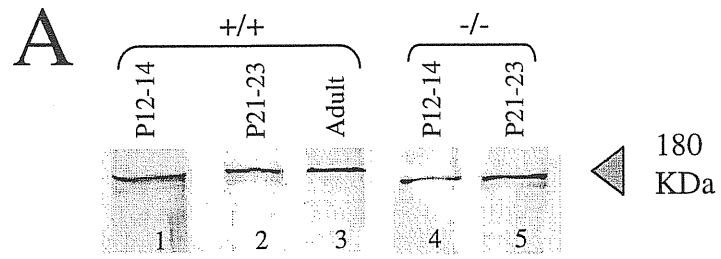
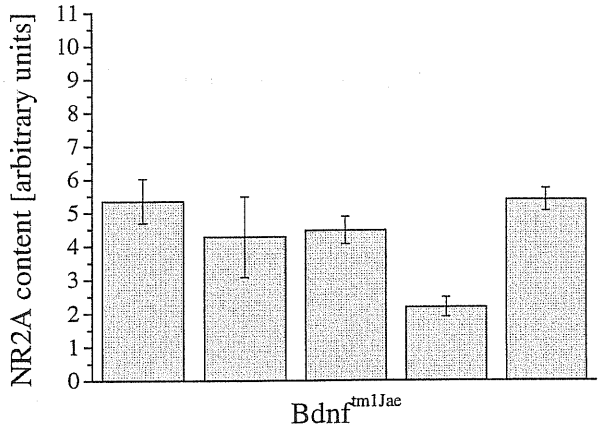


Table 1

lanes	1	5
4	*	*



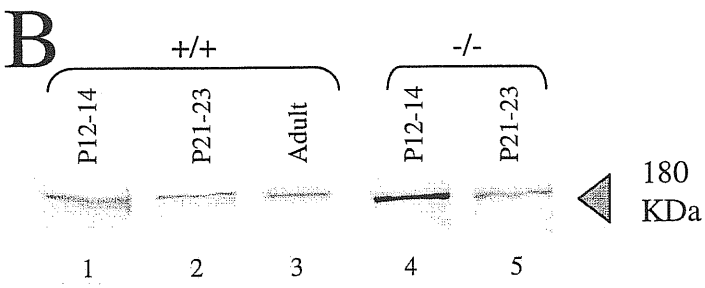


Table 2

lanes	1	5
2	*	
3	*	
4		*

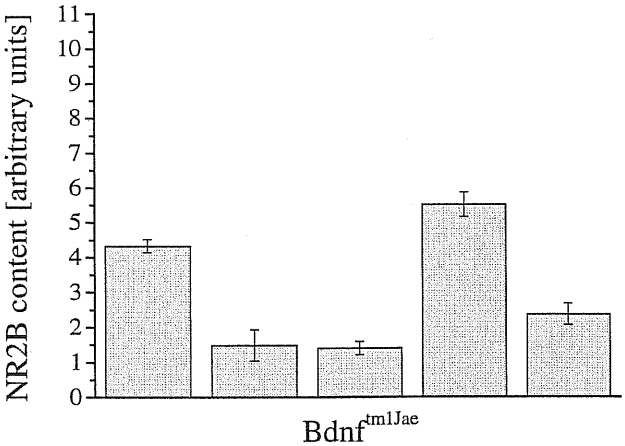


Figure 19. Western blot on Bdnf^{tm1Jae} mice homogenates.
The same amount of proteins was loaded in the different lanes (13±2 μg). Immunoblotting was performed for the NMDAR subunits NR2A and NR2B. For the different post-natal ages and genotypes indicated 8-12 mice were sacrificed; experiments were repeated 4 times. Results have been expressed as mean ± sd. Differences were considered significant for $P<0.001$ (*), as summarized in tables 1, 2.

postnatal age between mutant and wild type mice (Fig. 19, table 2). Therefore, the level of NR2B develops normally in BDNF knock out mice.

3.4.3.2 SvJae-Ntf5^{tm1Jae} mice

In wild type mice, the level of NR2A was not significantly different at P12-14 and P21-23 (Fig. 20A, lanes 1, 2), while it was significantly reduced in adulthood (lane 3). In P12-14 mutant mice, NR2A was slightly, but not significantly, reduced when compared with age-matched wild type mice (lanes 4, 1). It was slightly, but again not significantly, increased compared to P21-23 and adult mutant mice (lanes 5, 6). P21-23 mutant mice exhibited reduced levels of this subunit compared to age-matched wild type mice, while in adulthood there were no differences (Fig. 20, table 1).

In Ntf5 +/+ mice, NR2B subunit decreases from P12-14 to P21-23 (Fig. 20B, lanes 1, 2), remaining stable thereafter (lanes 2, 3). In Ntf5 -/- animals, the level of this subunit was high at P12-14 (lane 4) and decreased in P21-23 and adult (lanes 5, 6) mutant mice, as found for wild type mice. However, at P12-14 the level of NR2B in Ntf5 mice was significantly higher than in wild type mice (lanes 4, 1; Fig. 20 table 2).

Together, these observations suggest that NT-4/5 plays a modulatory action on NR2A and NR2B, and its absence affects differentially the developmental expression of these subunits.

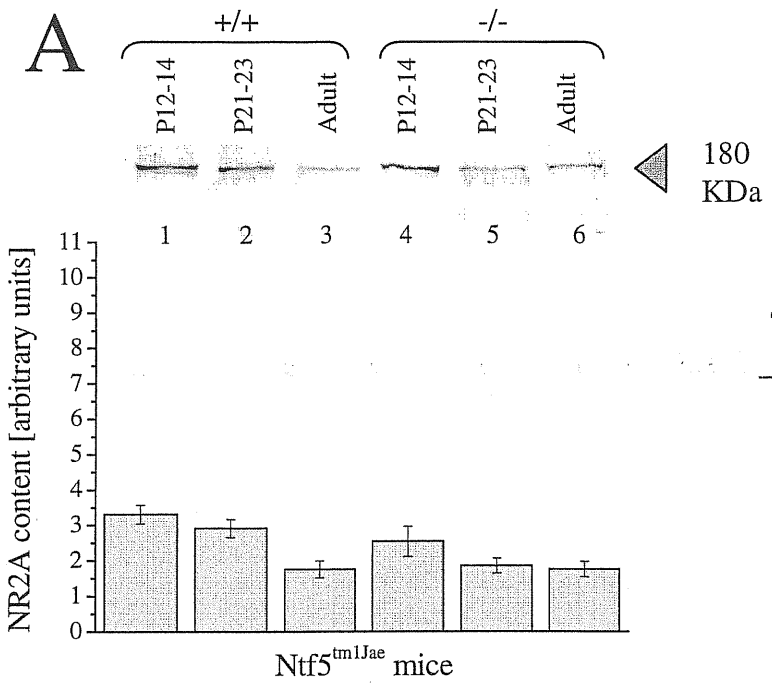


Table 1

lanes	3	5
2	*	*

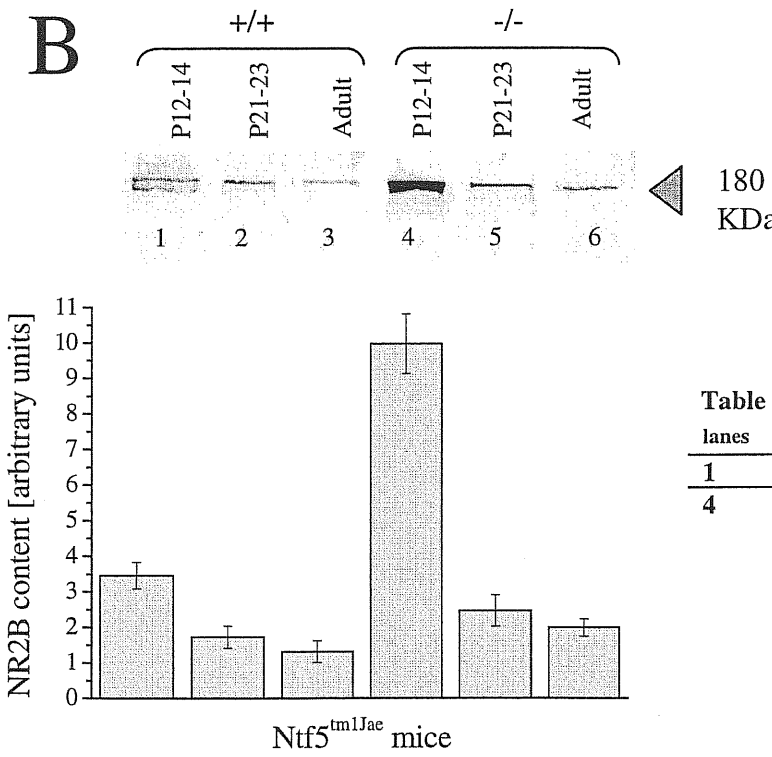


Table 2

lanes	2	3	4	5	6
1	*	*	*		
4				*	*

Figure 20. Western blot on Ntf5^{tm1Jae} mice homogenates.
The same amount of proteins was loaded in the different lanes (29±2 µg). Immunoblotting was performed for the NMDAR subunits NR2A and NR2B. For the different post-natal ages and genotypes 8-12 mice were sacrificed; experiments were repeated 4 times. Results are expressed as mean ± sd. Differences were considered significant for $P<0.001$ (*), as summarized in tables 1, 2.

4. DISCUSSION

4.1 A general overview

During the last decade several investigators started to be interested on the expression of NMDARs in different areas of the brain and in particular in the visual cortex. The visual cortex is a structure widely used to study factors and mechanisms involved in activity-dependent plasticity and maturation of neurons. As pointed out in the introduction the primary visual cortex of mammals is highly plastic and sensitive to visual manipulation during a restricted time window of postnatal development called critical period. For example a monocular deprivation performed during the critical period induces functional and morphological alterations such as lost of binocular cells, shift of ocular dominance distribution towards the undeprived eye and shrinkage of neurons in LGN receiving the input from the deprived eye. During an almost coincident postnatal period also the visual cortical connections undergo processes of refinement leading to the acquisition of an adult phenotype. Visual deprivation during this period (dark rearing) alters the functional properties of visual cortical neurons (Fagiolini *et al.*, 1994) and increases the length of the critical period for monocular deprivation.

The interest on NMDARs in developing visual cortex moves from three fundamental studies. The first study done in middle eighty showed that the pharmacological blockade of NMDARs by local supply of APV in the primary visual cortex was able to prevent the functional (Kleinschmidt *et al.*, 1987) and morphological (Gu *et al.*, 1989; Bear *et al.*, 1990) effects of monocular deprivation. Although the enthusiasm risen by these results was successively damped by the demonstration that blockade of NMDARs interferes with synaptic transmission, thus inducing effects similar to visual deprivation, the idea emerged that NMDAR is essential to couple neuronal activity triggered by vision with synapses strength. This idea was reinforced by the demonstration that NMDARs are necessary to induce LTP in different areas of the brain (Harris *et al.*, 1984; Kanter and Haberly, 1990), including the visual cortex (Artola and Singer, 1987).

More recently, an elegant study by Carmignoto and Vicini (1992) brought the important evidence that the length of NMDA dependent EPSP changes at different stages of

postnatal development. In visual cortical neurons of layer IV NMDA current shortens as postnatal development proceeds. From the functional point of view this result suggests that NMDARs are involved in the maturation of visual cortical neurons by influencing the entry of Ca^{++} into neurons and increasing the length of EPSP in this way allowing to recruit different inputs and consequently to reach the neuronal firing threshold. Another prominent hypothesis was that the developmental changes of the biophysics properties of NMDARs may regulate the duration of the critical period and its relation with the period of expression of LTP in developing visual cortex (Kirkwood *et al.*, 1995). This last hypothesis is not yet confirmed by the experimental results and at least in another area of the brain, the somatosensory cortex, has not been proven (Barth and Malenka, 2001).

The third group of studies moves from the discovery that NMDARs are heteromers composed by different subunits. Molecular cloning has identified three classes of NMDAR subunits designated as NR1, NR2 and NR3. Although the NMDAR subunits stoichiometry in the CNS remains unclear, an elegant study exploiting co-immunoprecipitation with specific antibodies for NR1, NR2A and NR2B showed that NR1 is associated with NR2A and/or NR2B to form different heteromeric composition of NMDARs in the developing cortex (Sheng *et al.*, 1994).

In the last years evidence has been provided that NMDAR channel kinetics depend on subunit composition (Moriyoshi *et al.*, 1991; for a review see Cull-Candy *et al.*, 2001). For example in recombinant NR1-NR2 channels the biophysics properties of NMDARs are determined by the NR2 subunit, with the NR1-NR2A receptor showing the fastest kinetic (Monyer *et al.*, 1994). The different composition of NMDARs is responsible for the functional heterogeneity observed in the visual cortex at different developmental stages. In particular, changes in the functional properties of NMDARs during postnatal development mirror the modifications in their subunit composition (Monyer *et al.*, 1994; Flint *et al.*, 1997). Recently, it has been shown that the developmental increase of NR2A corresponds to shortening of NMDA dependent EPSP, thus suggesting that shortening of EPSP corresponds to a developmental switch from NR2B to NR2A (Nase *et al.*, 1999).

Taken together, these data suggest that NMDARs are developmentally regulated in their expression and that changes in subunit composition may account for functional changes of NMDARs occurring in developing visual cortex.

In the visual cortex NMDAR subunits are expressed in different types of neurons including pyramidal and stellate cells (Rumpel *et al.*, 2000; Huntley *et al.*, 1994). Several studies conducted in cat, ferret and rat agree that the different NMDAR subunits undergo developmental changes in their pattern of expression (Chen *et al.*, 2000; Roberts and Ramoa, 1999; Wenzel *et al.*, 1995). NR1 is expressed early during postnatal life with a progressive increase during the first postnatal week followed by a progressive decrease of staining, especially in layer IV and VI, at later ages. In the adult rat visual cortex NR1 is expressed in all cortical layers with a minimum in layer IV (Petralia *et al.*, 1994a; Tongiorgi *et al.*, 1999).

The developmental expression of NR2A and NR2B subunits has been less intensively analyzed. It has been reported that NR2A increases in cortical layer IV during the critical period (Nase *et al.*, 1999) while NR2B decreases (Nase *et al.*, 1999; Cao *et al.*, 2000). In a previous study (Tongiorgi *et al.*, 1999) it has been shown that before eye opening NR1, NR2A and NR2B are largely expressed in all cortical layers; in addition, in most neurons the pattern of NR2A and NR2B expression almost overlaps that of NR1. These results suggest that NR2A and NR2B are regulated during postnatal development and, in particular, during the critical period in the visual cortex.

More recently, Tongiorgi *et al.* exploited the possibility that NR2A and NR2B change their expression during the critical period. To this aim they considered two postnatal ages, namely P21 and P37, corresponding to the peak and the end of the critical period in the rat visual cortex, respectively. By using a stereological methods to count neurons in different cortical layers they found that the pattern of cellular expression throughout cortical layers and the staining intensity of NR2B does not change during the period P21-P37. Concerning NR2A, cellular expression is almost identical between P21 and P37 while the staining intensity is higher at P37 with respect to P21, suggesting that visual cortical neurons are rich of NR2A at the end of the critical period when NMDAR EPSPs are shorter.

Recent experiments in rat visual cortex pointed out the importance of NR2A/NR2B ratio in visual cortical neurons. This ratio measured in synaptosomes from visual cortex increases during postnatal development, reaches the maturity around the fourth postnatal week and correlates with the duration of NMDA current (Quinlan *et al.*, 1999b). The

hypothesis has been raised that an increase of NR2A expression would be responsible for change of NR2A/NR2B ratio and this would account for change of NMDAR functional properties (Flint *et al.*, 1997, Nase *et al.*, 1999, Quinlan *et al.*, 1999a,b). The results obtained by Tongiorgi *et al.* support this model since the expression of NR2A at the end of the critical period is higher than at an earlier stage of postnatal development.

4.2 Factors regulating NMDARs in developing visual cortex

Factors influencing NMDAR subunit expression in different areas of the CNS include calcium and protein kinase C (Umemiya *et al.*, 2001; Logan *et al.*, 1999), dopaminergic system (Yang, 2000) and visual experience (Binns *et al.*, 1999). A great part of the data concerning NMDAR expression was inferred from changes in NMDAR-mediated currents in certain peculiar conditions.

It is well known that visual experience represents the driving force for the maturation of mammalian visual cortex. This prompted several authors to raise the question of whether also NMDAR circuitry develops under the control of visual input. To answer this question dark rearing has been widely used to exploit the effects induced by absence of vision on the maturation patterns, including NMDARs, in the visual cortex. Visual deprivation does not affect maturation of NR1; only when retinal spiking activity was totally blocked by binocular injections of tetrodotoxin (TTX) development of NR1 was altered (Catalano *et al.*, 1997). Concerning NR2A, it has been reported that developmental increase of NR2A mRNA is altered in the layer IV of visual cortex of dark reared rats (Flint *et al.*, 1997, Nase et. al, 1999). Examining the cellular expression of the protein, Tongiorgi *et al.* showed that deprivation of visual experience between P21 and P37 alters the expression of NR2A in all cortical layers. Indeed both the proportion of immunopositive neurons and the intensity of cellular staining are decreased in dark reared rats when compared with normally reared rats. These data are in accordance with recent results obtained by using western blot showing that dark rearing induces a reduction of NR2A in synaptoneurosomes of visual cortex (Quinlan *et al.*, 1999a,b). In contrast, regulation of NR2B is not under the control of visual experience because in dark reared animals the cellular expression of NR2B was not affected although slightly

decreased expression level was observed (Tongiorgi *et al.*, submitted). Taken together, these data suggest that the regulation of the expression pattern of NR2A and NR2B subunits in the visual cortex is likely to follow different genetic and epigenetic programs
The results obtained by Quinlan *et al.* are also relevant because they showed that the regulation of NR2A by light in synaptoneurosomes of visual cortex is extremely rapid and dynamic, suggesting that synthesis and possibly assembling of NMDAR subunits might occur locally, at the level of synaptic terminals.
One crucial question is whether in absence of visual input the developmental expression of NR2A is altered or only shifted in time. Nase *et al.* (1999) showed that visual deprivation delays the maturation of NR2A mRNA. Quinlan *et al.* (1999b) showed that the developmental increase of NR2A, normally occuring in synaptoneurosomes prepared from visual cortex of normally reared animals, is attenuated in dark reared animals. These data couple with previous work where it is apparent that dark rearing induces change of EPSP kinetics; NMDAr responses are larger that in normally reared animals (Carmignoto and Vicini, 1992).

4.3 Factors regulating the expression of neurotrophins and their receptors

In the last decade studies on the role exerted by the neurotrophic factors of NGF family attracted the attention of investigators due to the fact that neurotrophins exert fundamental actions on synaptic plasticity and visual cortex maturation (for a review see Cellerino and Maffei, 1996) as well as previous reports showed for NMDARs.
The cellular expression of neurotrophins, and in particular of BDNF, has been widely investigated in the visual cortex. BDNF mRNA expression changes during postnatal development. Before eye opening, the total level of BDNF mRNA is low (Bozzi *et al.*, 1995) and neurons expressing BDNF mRNA are evenly distributed throughout the cortical layers (Capsoni *et al.*, 1999a). After eye opening, BDNF mRNA level increases (Bozzi *et al.*, 1995) while the number of neurons expressing BDNF mRNA in layer IV and V decreases (Capsoni *et al.*, 1999a). Alteration of normal visual experience influences the endogenous level of BDNF mRNA in the visual cortex. (Castrén *et al.*,

1992; Bozzi *et al.*, 1995; Schoups *et al.*, 1995; Capsoni *et al.*, 1999a,b). The demonstration that visual experience influences the endogenous level of BDNF mRNA in the visual cortex (Castrén *et al.*, 1992) was fundamental to support the hypothesis that neurotrophins are involved in activity-dependent neuronal plasticity and development. Castrén and collaborators (1992) investigated how light regulates expression of BDNF mRNA in rat visual cortex. Keeping adult rats in the dark or preventing normal activity of retinal ganglion cells by injection of tetrodotoxin significantly decreased the levels of BDNF mRNA in the visual cortex but not in other cortical areas. Exposure to light after a period of darkness rapidly restored the mRNA to control levels. Interestingly these alterations in visual input had no effect on NGF mRNA. Also TrkB mRNA was decreased in darkness although less than BDNF mRNA. Moreover, the increase in BDNF mRNA normally occurring in the visual cortex of newborn rats after eye-opening is retarded, although not completely abolished, by rearing the pups in the dark. Dark rearing induces changes in the cellular expression of BDNF mRNA throughout visual cortical layers: in neurons of layers IV and V, BDNF mRNA is reduced but distributed in the great majority of neurons (Capsoni *et al.*, 1999a).

Also BDNF protein decreases in rats deprived of visual experience (Tropea *et al.*, 2001). The cellular pattern of expression changes, and both mRNA and protein disappear from dendrites where they are normally expressed. Thus, visual input controls the synthesis of BDNF and possibly its targeting to dendrites (Tongiorgi *et al.*, 1997; Righi *et al.*, 2000; Tropea *et al.*, 2001)

These studies indicate that BDNF cortical expression undergoes developmental changes and is regulated by visual experience.

4.4 Interactions between NMDARs and neurotrophins

Evidences that NMDARs influence BDNF synthesis are quite extensively described. It has been shown that activation of glutamate receptors enhances the synthesis of BDNF in hippocampal neurons both *in vitro* and *in vivo* (Zafra *et al.*, 1991). The same authors showed also that blockade of NMDARs by MK-801 decreases BDNF mRNA. Following these cues, Castrén and collaborators (1992) proved that the injection of MK-801

blocking NMDARs did not prevent the light-induced increase in BDNF mRNA in dark reared rats.

Recent studies showed that BDNF increases the phosphorylation of different NMDAr subunits in this way enhancing synaptic transmission (Suen *et al.*, 1997; Lin *et al.*, 1998; Levine and Kolb, 2000). These results together with the evidence that EPSCs is influenced by neurotrophins (Muzet and Dupont, 1996; Carmignoto *et al.*, 1997) and that kinetic of the receptors depends on their subunit composition (Carmignoto and Vicini, 1992; Monyer *et al.*, 1994; Flint *et al.*, 1997; Nase *et al.*, 1999), induced us to propose that the NMDAR subunits composition itself might be regulated by BDNF.

In 1999 Tongiorgi *et al.* showed that at an early stage of postnatal development, before eye opening, TrkB, the receptors for BDNF and NT-4 and NMDAR subunits NR1, NR2A and NR2B are largely coexpressed within visual cortical neurons.

The results on the coexpression of NMDAR subunits NR1-C1, NR2A, NR2B with full length TrkB (TrkB-fl) mRNA in the primary visual cortex of rat indicate the presence of precise postnatal developmental patterns. At an early phase of postnatal development (P10) and at the peak of the rat critical period (P22) both NMDAR subunits and TrkB-fl mRNA were expressed throughout all cortical layers but layer I and coexpressed in large cell population. These data suggest that at least during early postnatal development neurons expressing NMDAR subunits are a potential target of BDNF. BDNF is present in the visual cortex of mammals both during postnatal development and in the adulthood. Recent papers on the rat visual cortex showed that BDNF is low at the time of birth, increasing during the second and third postnatal week, reaching a plateau around postnatal day 20 (Castrén *et al.*, 1992; Bozzi *et al.*, 1995; Schoups *et al.*, 1995). Thus, the period of increase in BDNF synthesis does correspond to the maximal coexpression between NMDAR and trkB in visual cortical neurons. Poor information is presently available on the possible interactions between NMDAR and TrkB ligands during development. Recently it has been reported that BDNF accelerates the maturation of NMDAR in cortical neurons (Koh *et al.*, 1995) and in cerebellar isolated granular cells (Muzet and Dupont, 1996). The hypothesis has been raised that endogenous TrkB ligands, such as BDNF, might directly regulate the heteromeric composition of NMDAR. In the paper by Tongiorgi *et al.* (1999) it has been reported that TrkB is expressed in a

large population of cells distributed throughout cortical layers at P10 and P22 while it appeared reduced in cortical layer IV of adult rats. Interestingly, also the coexpression of NMDARs with TrkB-fl is reduced in layer IV and to lesser degree in layer V of adult rats with respect to an early stage of postnatal development. Although reduced, the splicing variant C1 of the subunit NR1 and TrkB-fl continues to be coexpressed in all labelled cells even in adulthood while several cells in layer IV and V expresse NR2A or NR2B but not TrkB-fl and, consequently, not NR1-C1. Thus, in the rat visual cortex, cells that loose TrkB during postnatal development are progressively characterized by a different expression of NMDAR subunits in the adulthood. It is therefore tempting to speculate that cells becoming progressively unresponsive to BDNF and possibly to NT-4/5, the other TrkB ligand, change the composition of their NMDARs.

Moving from these results, here we have tried to clarify the role played by neurotrophins of the TrkB signalling in the regulation of cellular expression of NMDARs. The strategy we followed was to block TrkB ligands, altogether first, by applying TrkB-IgG to rat visual cortex, then separately, taking advantage of 2 strains of knock-out mice, for BDNF and NT-4/5 respectively.

4.5 Blockade of TrkB ligands alters the post-natal development of NMDARs

Our results show that blockade of TrkB activation by TrkB immunoadhesin alters the postnatal development of NMDAR subunits in the rat visual cortex. In particular, blocking TrkB ligands: 1) reduces the expression of NR2A and NR2B subunits as measured by western blot; 2) increases the relative number of neurons expressing NR2A in all cortical layers. In contrast, NR1 subunit appears completely unaffected by TrkB signalling blockade.

Quantitative cell counts data might be potentially sensitive to three kinds of artifacts: 1) a bias in the counting method; 2) an uneven penetration of the antibodies; 3) possible changes in the cell population during post-natal development due to cell death.

Concerning the first type of artifact, two systematic errors can occur. The first one can be termed sampling error: in the present work we used a random sampling method and avoid

this possibility. The second type of error is related to changes of the criteria used to select cells; in rats treated with TrkB-IgG the number of cells stained for NR2A increases although the overall intensity of staining is lower than in control rats. This change in the intensity of staining could induce a tendency to include cells which otherwise would be discarded. In principle we cannot exclude this possibility. However, a systematic error of this kind should affect to the same extent both NR2A and NR2B positive neurons, while in case of NR2B subunit the reduction in the intensity of staining was not coupled to the increase in the number of stained cells. This can be considered as internal control for the validity of the selected method of cell-counting.

Artifacts related to uneven penetration of antibodies can be easily worked out by looking at the intensity of staining in different focal planes: staining restricted to the most superficial planes strongly suggests such kind of artifact. Again we can discard this possibility since the observed developmental changes are selective for NR2A and NR2B subunits; indeed NR1 is not sensitive to blockade of TrkB signalling. In addition, as reported in the "Materials and Methods" section, at the examined postnatal ages we did not observe a penetration of our antibodies restricted to the most superficial focal planes.

Finally, we must consider the possibility that cell death could be still active during post-natal development, and potentially interfere with developmental changes of NMDAR subunits. To avoid this possibility we have selected a post-natal period when the process of cell death is over. Indeed, in a previous study, by application of a stereological method, Tongiorgi *et al.*, showed that the number of cortical neurons in rat visual cortex does not change after P21 (submitted to Neuroscience).

4.5.1 Validity of the methodological approach

We have blocked TrkB activation using a soluble form of TrkB receptor engineered as an immunoadhesin (TrkB-IgG). We have shown that this immunoadhesin recognizes BDNF but not a closely related molecule as NGF (Pesavento *et al.*, 2000). Moreover, we have reported that TrkB-IgG is able to block endogenous TrkB ligands, altering the expression of synaptic plasticity forms dependent on BDNF in the visual cortex (see also Sermasi *et*

al., 2000). These results indicate that this immunoadhesin is particularly suitable to block in a specific way TrkB ligands in animals *in vivo*.

TrkB-IgG was continuously released through a cannula inserted into the medial border of occipital pole, following methods previously described (Lodovichi *et al.*, 2000). Implantation of the cannula and release of substances induce a lesion around the site of insertion as shown in the present paper, both in control animals treated with physiological solution and in TrkB-IgG treated animals. Analysis of NMDAR expression was performed in the primary visual cortex, i.e. outside the release site. By using specific marker for TrkB-IgG we demonstrated that the immunoadhesin was able to diffuse to regions adjacent to the release site, reaching the primary visual cortex.

To check for the absence of alterations in the primary visual cortex due to mechanical tissue damage we assessed the pattern of expression of markers specific for neurons and astrocytes. We used antibodies recognizing MAP2 a microtubule associated protein expressed in dendrites and cell perykaria and which is also a good marker for cell morpholgy and extension of dendritic arborizations. The aim was to demonstrate that neuronal processes such as dendrites and cell bodies were not structurally altered by diffusion of TrkB-IgG. Our results clearly show that blockade of TrkB ligands, *in vivo*, did not result in gross morphological alterations of visual cortical neurons cytoscheleton. Also astrocytes resident in the visual cortex are not affected by TrkB-IgG treatment, as shown by using GFAP as marker, thus excluding the possibility that mechanical damage due to vehicle diffusion or cannula implantation could induce gross morphological alterations.

These data indicate that both neurons and astrocytes have maintained their morphological features in spite of the presence of TrkB-IgG. In addition, in a previous paper, the same delivery method has been successfully applied to study the effects of different neurotrophins on the functional characteristics of neurons in the developing rat visual cortex excluding unspecific alterations (Lodovichi *et al.*, 2000).

Thus, the method used to deliver TrkB-IgG is particularly suitable for studying the effects of neurotrophins and/or their blockers on different aspects of visual cortex maturation.

4.5.2 Changes in NMDARs during postnatal development. Role of TrkB ligands

All previous reports agree that NMDARs are developmentally and activity regulated, however, none of these previous studies have investigated so far the molecular factors controlling the cellular expression of different NMDAR subunits during postnatal development.

Following a previous study demonstrating the co-expression of NMDAR subunits and TrkB mRNA (Tongiorgi *et al.*, 1999) we have now investigated whether the expression of NMDAR subunits is influenced by neurotrophins involved in TrkB signalling.

The greatest effect of TrkB-IgG treatment is on NR2A subunit. We provided evidence that the intensity of staining and endogenous level of NR2A is reduced in TrkB-IgG treated animals, similarly to what shown for NR2B. In addition, also the relative number of cells expressing NR2A is different between TrkB-IgG treated and control animals while the cellular pattern of NR2B is unchanged. Indeed, the percentage of NR2A but not NR2B stained cells increased in all cortical layers of TrkB-IgG treated animals. Thus, blockade of TrkB ligands induces two opposite effects on NR2A expression: (1) reduction of the cellular content of NR2A and more in general of the endogenous level of this subunit in the whole visual cortex, (2) increase of the number of neurons expressing NR2A. This represents an important result suggesting that endogenous TrkB ligands might regulate the cellular level of NR2A and contribute to sculpt the circuit expressing NR2A.

Recently, it has been reported that in organotypic slices dendritic growth of pyramidal neurons is modulated by neurotrophins, including BDNF and NT-4/5, in different layers of developing visual cortex (McAllister *et al.*, 1995); in particular, TrkB-IgG treatment altered the dendritic growth of pyramidal cells (McAllister *et al.*, 1997). This observation raises the possibility that changes of NMDARs could be secondary to abnormal development of visual cortical neurons and in particular of dendritic processes. However, two considerations induced us to discard this hypothesis. First, TrkB-IgG treatment does not affect equally all NMDAR subunits considered in the present study. Indeed, NR1 subunit is unaffected by blockade of TrkB activation. Second, TrkB-IgG treatment started

at P20, i.e. at a relatively late stage when the gross process of dendritic growth is almost over, although changes of dendritic length can occur until adulthood (Juraska, 1982; Miller, 1986).

Our data partially disagree with the results of Small *et al.* (1998). These authors showed that BDNF treatment causes an increase of NR2A and a decrease of NR2B in cultured cortical neurons, thus predicting that blockade of BDNF signalling would lead, at least in the case of NR2B, to opposite results with respect to our data. Possible discrepancies might be due to results obtained in *in vivo* animals and chronic treatment versus cell cultures and acute treatment. In addition, in our case we blocked not only BDNF but also NT-4/5 and therefore the absence of TrkB signalling may cause more complex phenomena with respect to those induced by the exposure of cortical neurons to BDNF. Beside these discrepancies, our paper and that by Small *et al.* (1998) point to the importance of TrkB ligands as possible modulators of NMDAR subunits expression.

The data reported in the present paper suggest that TrkB ligands might modulate the cellular expression of distinct subunits of NMDARs during postnatal development and support the specificity of the effects induced by blockade of TrkB ligands on various subunits. The result that after TrkB-IgG prolonged treatment the percentage of visual cortical neurons expressing NR2A increases suggests that a group of neurons retain the capability to synthesize NMDARs although at low level, that otherwise would be lost during postnatal development. We advance the hypothesis that at an early stage of postnatal development NR2A is expressed in a large number of neurons. As development proceeds some neurons reduces their expression of NR2A, which becomes undetectable while in other neurons the expression of NR2A increases reaching an adult level. This hypothesis is consistent with the data showing that before eye opening (Tongiorgi *et al.*, 1999) most neurons express NMDAR subunits, including NR2A. At later postnatal ages the cellular expression of NR2A and NR2B becomes more discrete and appears restricted to different subgroups of neurons suggesting that several neurons loose the possibility to synthetize NR2A and/or NR2B.

BDNF and NMDAR subunits are developmentally regulated in the primary visual cortex. Although only speculative at the present time, the possibility exists that, by modulating the expression of NMDAR subunits, TrkB ligands might contribute to NMDAR

variation. It has been reported that in the visual cortex the NR1 subunit co-immunoprecipitates preferentially with NR2A and/or NR2B thus suggesting the presence of heterodimers, containing NR2A or NR2B or heterotrimers of the type NR1/NR2A/NR2B. TrkB ligands by changing the expression of NMDAR subunits might change the subunit composition of heteromeric NMDAR. Changing subunit composition of NMDARs influences NMDA current, Ca^{2+} influx and neuronal plasticity. In particular, the gating properties of NMDARs are determined by the NR2A subunit with the complex NR1-NR2A showing the fastest decay of NMDA current. Thus, following the idea that TrkB ligands control the maturation of NMDAR subunits we may establish a molecular link between visual experience and biophysics/molecular changes of NMDARs.

What are the evidences favoring the idea that visual input modulates the expression of NMDAR subunits through TrkB ligands? It is well known that the maturation of visual cortical circuitry depends on visual experience. A bulk of studies showed that neurotrophins (Castrén *et al.*, 1992; Schoups *et al.*, 1995; Capsoni *et al.*, 1999a; Tropea *et al.*, 2001) and NMDARs (Flint *et al.*, 1997; Nase *et al.*, 1999; Quinlan *et al.*, 1999a,b; Tongiorgi *et al.*, submitted) are under the control of visual input. For example, visual deprivation reduces the level of BDNF mRNA and increases the number of cells expressing it, resuming a pattern of cellular expression present before eye opening. These data have been interpreted by assuming that visual experience exerts a fundamental role in shaping the cortical circuitry expressing BDNF. In absence of light expression of BDNF mRNA in the visual cortex re-assume the characteristic of an early stage of postnatal development, before eye opening, when it is expressed at a low level in almost all cortical neurons.

Concerning NMDARs, visual deprivation reduces the expression of NR2A while leaving unaffected NR2B (Tongiorgi *et al.*, submitted). In addition, results obtained by using western blot (Quinlan *et al.* 1999a,b) showed that dark rearing induces a reduction of NR2A level in synaptosomes. By modulating endogenous TrkB ligands, the visual input might regulate the expression of NMDAR subunits in developing visual cortex. One possibility is that visual deprivation by reducing the endogenous level of BDNF mRNA and accordingly its cellular expression of BDNF protein (Tropea *et al.*, 2001) may cause a reduction of the cellular expression of NMDAR subunits, such as NR2A, and an

increase of the cell population expressing them, thus resuming a pattern present at an early stage of postnatal development, before eye opening. In accordance with this idea, in the present work we have provided the evidence that blocking TrkB signalling affects the postnatal maturation of NR2A by inducing a reduction of the staining level and an increase of the number of stained cells. However, the effects on NMDARs induced by the blockade of TrkB ligands are not completely identical to those caused by dark rearing. Indeed, in rats treated with TrkB-IgG also the expression of the subunit NR2B is altered with a reduction of staining intensity with respect to controls while in dark reared rats the level of NR2B is not changed. In addition, in dark reared rats the level of NR2A and the number of cells expressing it are both reduced while in TrkB-IgG treated rats the population of immunopositive cells is enlarged. Possible discrepancies may arise from the fact that TrkB-IgG blocks not only BDNF but also NT-4/5 and the data on the effects of dark rearing on NT-4/5 are still lacking. Thus, the idea that TrkB ligands may represent the molecular link between visual input and the cellular expression of NMDARs subunits is intriguing but still far from being proven.

4.6 The pattern of expression of NMDAR subunits is altered in the visual cortex of knock-out mice

To investigate separately the effects induced by absence of BDNF and NT-4/5 on NMDAR subunit expression during post-natal development, we acquired BDNF and NT-4/5 knock-out mice. The BDNF (-/-) animals are characterized by a dramatic phenotype and they can survive for a few weeks after birth in accordance (Jones *et al.*, 1994). In our animal house BDNF (-/-) animals survived until P21-23. These mice were characterized by an abnormal behavior consisting of head bobbing and altered locomotion symptoms that can be re-conducted to the altered vestibular function due to the depletion of vestibular neurons as shown by Ernfors *et al.* (1994). BDNF heterozygous mice were viable and without phenotypic differences when compared with wild type animals this rendering feasible analysis at different postnatal ages.

Target deletion of NT-4/5 gene resulted in homozygous viable animals without overt phenotype thus allowing an extensive analysis at different postnatal ages.

Up to now very little is known about NMDAR subunit composition during development in mouse brain, and possible factors influencing their expression. A study investigating the developing cerebellum (Takahashi *et al.*, 1996) has shown that the expression of NR2C subunit late in development is responsible for a reduction in the sensitivity of NMDA-EPSCs to block by extracellular Mg^{2+} and that receptors containing the NR2A subunit determine the fast kinetic of NMDA-EPSCs (as seen also in rats). Another work (Follesa and Ticku, 1996) showed that chronic ethanol treatment of mouse cortical neurons in culture produced an increase in NR1 and NR2B subunits (NR2A subunit was not expressed in these neurons). The same effect was observed also after chronic treatment with NMDAR antagonist CPP, and using ethanol and CPP in combination.

Being our work mainly focused on cellular expression of NMDAR subunits and since the expression of NMDAR subunits in the mouse visual cortex has not been described it, was mandatory to describe the developmental expression of NMDAR subunits in the normal visual cortex. With respect to the staining of NMDAR subunits described in the rat visual cortex we observed similarities and differences. NR1 patterns of immunostaining are almost identical in rat and mouse. Immunostained cells are uniformly and intensively stained throughout all cortical layers and this pattern is already present at an early stage of post-natal development (P12-14) indicating that maturaion of NR1 subunit is almost completed by this stage. Concerning NR2A and NR2B subunits, the expression in the mouse visual cortex appears more discrete and mainly concentrated in layers II-III and V with respect to the rat visual cortex. In general, the maturation of NMDAR subunits is faster in the mouse visual cortex, at least for subunit NR1 and NR2A. Indeed, these two subunits present an adult-like pattern of cellular expression already at an early stage of post-natal development, before eye-opening. The level of NR2B is still altered at an early stage of postnatal development (12-14), before eye opening. At P21-23, roughly corresponding to the peak of the critical period (Huang *et al.*, 1999) the level of NR2B is reduced with respect to P12-14, remaining stable thereafter. Thus, the development of NR2B is similar to that described for rat visual cortex (Nase *et al.*, 1999; Cao *et al.*, 2000).

The two strains of mice were not derived from the same wild type line, probably due to the difficulty in knocking-out Bdnf gene and to the fact that Bdnf -/- mice do not reach

adulthood, while Ntf5 -/- can be mated together. Thus, internal controls were represented by +/+ animals of the two colonies (sometimes however referred to as wild type) and this might be the reason for a few discrepancies found between the two groups. For example, in Bdnf +/+ mice the endogenous level of NR2A evaluated by western blot is stable during development while in Ntf5 +/+ animals this subunit is reduced in adulthood.
The data coming from immunocytology and western blot are generally in quite good agreement with the exception of those relative to NR2B subunit in Ntf5 mice (both wild type and mutant). The fact that we observe a discrepancy in this case could depend on more subtle variability between animals that could not be detected simply by qualitative analysis of cellular expression.

4.6.1 Bdnf knock-out mice

We showed that in absence of BDNF, the cellular pattern of NR1 subunit is normal, while NR2A and NR2B are altered. BDNF deficient mice are characterized by a reduction of NR2A level, as observed by western blot, at an early stage of postnatal development (P12-14) while later this reduction disappears. Also at the level of cellular expression NR2A appeared reduced in Bdnf -/- at P12-14, in accordance with western blot data. In contrast with NR2A, expression of NR2B in BDNF looks normal in BDNF (-/-) mice at both postnatal ages examined in the present study.
Thus, absence of BDNF resulted in the retarded maturation of NR2A but not NR1 and NR2B.
As already mentioned Bdnf -/- animals still present their eyes closed at P20 while, normally, mice open their eyes around P14. Thus, due to strong regulation of NMDAR subunits by visual input shown in rat visual cortex, we must consider the possibility that alterations of NR2A and NR2B in BDNF knock-out mice could be the direct consequence of visual deprivation. Literature is quite poor of studies on the consequences of dark rearing in the mouse visual cortex. In general, visual deprivation induces morphological alterations consisting of a retarded development of dendritic spines (Freire, 1978). Since NMDARs are mostly localized in dendritic spines (Takumi *et al.*, 1999) it is possible that retarded maturation of spines might cause an altered expression of NMDAR subunits. However, in our opinion this hypothesis is unlike since reduction

of NR2A is not accompanied by a concomitant alteration of NR1 and NR2B subunits, as it would be expected in case of retarded development of the visual cortex. However, we can not completely exclude the possibility that a retarded maturation of the brain could differently affect the different subunits of NMDARs.

4.6.2 Ntf5 knock-out mice

Ntf5 -/- mice are characterized by milder and subtler alterations of NMDAR expression with respect to BDNF knock-out mice. As in BDNF mutant NR1 looks normal in Ntf5 -/- mice, this indicating that the effects induced bu lack of NT-4/5 are specific. Results obtained by western blot suggest that absence of this neurotrophin leads to a slight reduction of NR2A subunit at an early stage of postnatal development; in fact, NR2A levels is decreased at P12-14 with respect to wild type, whilst at a middle stage and in adulthood NR2A looks normal.

Western blot results suggest an abnormal maturation of NR2B. In fact, at P12-14 NR2B is expressed at an higher level in knock-out mice with respect to age-matched wild types; as the development proceeds NR2B reduces reaching a normal level at P21-23. Immunocytology does not support completely western blot results since the distribution pattern of stained cells is not grossly altered in P12-14 knock-out mice with the only exception that immunopositive cells in layers II-III appear more numerous and less stained compared to wild type mice. However, it will be desirable to apply methods of quantitative analysis in order to get a definitive conclusion on the effects induced by absence of NT-4/5 on the cellular expression of NR2B.

Although it could be surprising that western blot and immunocytology showed not completely overlapping results this observation may depend on methodological differences; indeed, by using immunocitology we evaluate the staining of neuronal cell perykaria while western blot takes into account NMDARs expressed in cell bodies and neuropil.

We want to notice that both NR2A and NR2B resulted altered in NT-4/5 knock-out mice at an early stage of postnatal development recovering a normal phenotype at later ages. What is the reason for these transient alterations? One possibility implies that several

factors may control the development of NMDAR subunits; in absence of NT-4/5 other factors can prime the development of NMDARs allowing a rescue from alterations.
Together, the results obtained in Bdnf and Ntf5 ko mice support the conclusion that TrkB ligands represent factors modulating the maturation of NMDAR subunits in developing mouse visual cortex.

4.7 Concluding remarks and future strategies

The uncontroversial conclusion of the present work is that blockade or absence of TrkB ligands, namely BDNF and NT-4/5, alters the maturation of NMDAR subunits. The different observations obtained in rats, by blocking both TrkB ligands at the same time, and knock-out mice resulting in the absence of BDNF or NT-4/5 can form a unitary scheme of action of these neurotrophins on NMDAR subunit NR2A in developing rat and mouse visual cortex. Indeed, absence of BDNF seems to affect especially NR2A delaying its development, or in other words, blocking its expression at early stages of post-natal development. NT-4/5 more likely exerts a role similar to that of BDNF: indeed, in Ntf5 ko mice NR2A appears reduced at an early stage of post-natal development. Thus it is not surprising that blocking both TrkB ligands results in reduction of NR2A level, as we have reported in the visual cortex of the rats treated with TrkB-IgG.
More complex is the interpretation of results obtained on NR2B. Blockade of TrkB ligands induces a reduction of NR2B level without changing its cellular distribution pattern. Absence of BDNF leaves the level of expression of NR2B completely normal while absence of NT-4/5 induces a transient increase of NR2B level at an early stage of postnatal development. Possible discrepancies between rats treated with TrkB-IgG and NT-4/5 ko mice may depend on: 1) blockade of both BDNF and NT-4/5 in TrkB-IgG treated rats versus absence of the 2 neurotrophins, separately, in ko mice; 2) blockade of neurotrophins during a middle stage of postnatal development in TrkB-IgG treated rats versus absence of neurotrophins from an early stage of development in ko mice; 3) differences linked to the two different animal species, namely rat and mouse, used in the present study.

We decided to study knock-out mice models because treating rats with immunoadhesins did not allow separate the distinct effects of BDNF and NT-4/5. However, classical knock-out mice do not represent the elective model to study the effects induced by lack of a molecule on defined periods of post-natal development and in defined areas of the brain. Indeed, knock-out mice used in the present work lack neurotrophins early in development and in the whole brain. This could trigger a series of undesirable processes such as changes in the expression of other molecules inducing indirect effects on the function under study and priming of the same developmental function by different molecules. Thus, the ideal condition would be to block or stop the synthesis of such molecule during defined periods of time and in precise areas of the brain. For further improving the models we could consider for example the possibility to acquire conditional knock-out mice for TrkB ligands.

A second strategy consists in exploring the possibility to implant, in defined areas of the brain, engineered cells able to differentiate into neurons and to release molecules under investigation, in our case neurotrophins or their blockers. The ideal model would be represented by neuronal progenitors injected in the visual cortex and able to integrate into the nervous circuitry without causing tumors or any other kind of mechanical problems. Cattaneo *et al.* (1994) described a cell line derived from rat embryonic striatal primordia (ST14A) and conditionally immortalized (temperature sensitive) with T antigen that could be suitable for our purpose. After ensuring that ST14A are viable in rat visual cortex for longtime (and rats of course are viable as well) we could transfect them in order to induce the synthesis and release of BDNF or TrkB-IgG or NT-4/5. Of course this improvement in the model would bring many more opportunities of studying different aspects of cortical development.

During the last years several observations suggested that NMDAR channel kinetics depend on subunit composition. For example, in recombinant NR1/NR2 channels the biophysical properties of NMDARs are determined by the NR2 subunit, with the NR1/NR2A heteromer showing the fastest kinetic (Monyer *et al.*, 1994). Recently, it has been shown that the developmental increase of NR2A corresponds to shortening of NMDA dependent EPSP, thus suggesting that shortening of EPSP corresponds to a developmental switch from NR2B to NR2A (Flint *et al.*, 1997; Nase *et al.*, 1999).

According to these observations we can expect that the molecular changes we have observed are reflected as well in different physiological properties of the receptors. For example in case of blockade of TrkB ligands in rats and absence of BDNF in ko mice we found that expression of NR2A is reduced during post-natal development. We predict that reduction of NR2A would result in changes of NMDA current. In vitro electrophysiology in visual cortex slices coming from the different types of animals analyzed in the course of our study should be performed in order to verify our prediction.
An interesting unsolved problem concerns the site of synthesis of NMDAR subunits with respect to their localization in neurons. Both TrkB receptor and NMDARs are localized in dendrites, besides cell bodies. Thus, certainly the presence/absence of BDNF and NT-4/5 exerts local influences; synthesizing NMDAR subunits directly in dendrites rather than in the cell body (to be then transported to processes) would allow more rapid responses to neurotrophin action. To test this hypothesis, a first set of experiments could be performed on cells cultured in presence/absence of neurotrophins. Cycloheximide (an inhibitor of protein synthesis) could be injected directly in dendrites. Immunocytochemistry should then be performed to compare the pattern of distribution of NMDAR subunits in processes treated with cycloheximide with respect to controls that did not undergo protein synthesis inhibition.

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