

CHAPTER 6

General Discussion

While many of our characteristics and potentialities are genetically determined, innumerable types of experience steer our development and shape our personalities. The development of our brains is especially sensitive to experience during our early formative years, when our abilities to learn and convert experiences into coherent and long-lasting memories are at a maximum. In some cases, this period is crucial for the overall development and fine tuning of specific abilities or skills, and therefore called a “Critical Period”. The duration and the developmental time points for such critical periods vary across different systems. Changes in synaptic properties during the critical period for different sensory networks have been used as models to study experience-mediated synaptic plasticity (Buchwald et al., 1994; Daw et al., 1992; Fox, 1992; McGinn and Henry, 1975). One of the most studied of these is the visual system (Berardi et al., 2000; Hubel et al., 1977; Shatz and Stryker, 1978).

The critical period in the mouse visual cortex starts around P20 and ends around P35 and has its peak at around P28 (Gordon and Stryker, 1996). Visual experience during this period is known to affect the final structuring of the visual cortex circuitry. If, during this critical period, visual input to one eye is more coherent than to the other, the neurons responding to the eye that receives the less coherent input now start responding to the stronger eye (Hubel et al., 1977). This competitive shift in responsiveness is called ocular dominance (OD) plasticity. The inhibitory network is known to regulate the initiation of this critical period (Fagiolini and Hensch, 2000; Hensch et al., 1998). Brain derived neurotrophic factor (BDNF), through its receptor TrkB, modulates the maturation of this inhibitory system and hence, the timing of the critical period (Huang et al., 1999). BDNF is

known to function both antero- and retro-gradely to enhance synaptic connectivity (von Bartheld, 1998). Limiting amounts of BDNF, released from the postsynaptic visual cortical neuron, have been hypothesized to act as the competitive substrate to strengthen the more active thalamic inputs (Cabelli et al., 1995). However, as it is technically a challenge to differentiate between the anterograde and retrograde functions of BDNF, this hypothesis has not been convincingly addressed and opposite views have been put forward (Kaneko et al., 2008).

BDNF expression, which is initially low at eye-opening, reaches maximum levels during the critical period (Castren et al., 1992), at the same time when cortical neuronal connectivity becomes mature. This maturation of the cortical circuitry is reflected by increased stabilization of dendritic spines and axonal boutons, which is stimulated by the development of the mature extracellular matrix (Mataga et al., 2004; Oray et al., 2004; Pizzorusso et al., 2002). Since BDNF and its receptor TrkB are known to effect neuronal structural changes in early development (Alsina et al., 2001; Cohen-Cory and Fraser, 1995; Gorski et al., 2003; Horch et al., 1999; McAllister et al., 1995; Sanchez et al., 2006; Yacoubian and Lo, 2000), we wondered what role TrkB signaling has in the adult visual cortex. Delineating the postsynaptic effects of TrkB signaling in the maintenance of adult visual cortical synapses forms the crux of this thesis.

We needed a system that effectively reduced TrkB signaling after the critical period in either individual or a group of excitatory neurons of the visual cortex, without affecting neuronal development. Since most of the existing transgenic mice had either expression starting earlier on in development (Hanover et al., 1999; Huang et al., 1999) or depended on an

estrogen-dependent inducible system which by itself may influence structural changes (Sakamoto et al., 2003), we had to generate mice with these specific requirements.

Multiple Cre-dependent transgenes can be expressed in isolated neurons of the adult forebrain

In **Chapter 2**, we used the Cre-lox recombination technique (Hamilton and Abremski, 1984) to develop double transgenic mice where the contributing first mouse line expressed the protein of interest in a Cre-dependent manner and the second mouse line expressed the Cre recombinase. In order to compare the effects of expressing the gene of interest and to visualize transgene expression, we decided to generate control transgenic mice that express a standard reporter protein.

So, the first transgenic mouse line expressed a Cre-dependent membrane-associated enhanced green fluorescence protein (EGFP-F) under the Thy1 promoter. The Thy1-promoter controlled the level of EGFP-F expression and also ensured that it was targeted only to excitatory neurons. One of the lines, TLG 498, was used in this initial study. The second transgenic line expressed Cre recombinase under the control of Calcium/Calmodulin dependent kinase II α (CaMKII α) promoter and defined where and when the expression occurred. The Cre line we used in this initial work, Cre-3487, showed Cre recombination only in a small subset of excitatory neurons in the neocortex and hippocampus. Upon crossing the TLG 498 and Cre 3487 mice, we obtained mice that had EGFP-F expression in sparse excitatory neurons in a Golgi-staining-like fashion (Chakravarthy et al., 2008). The

expression started at around 5 to 6 weeks and was found in the neocortex, hippocampus, amygdala, hypothalamus, olfactory bulb, striatum and superior colliculus. The expression was strong enough to label the entire neuron and for performing acute in vivo imaging of dendritic protrusions. However, TLG 1157⁺ Cre 3487⁺ mice had higher levels of EGFP-F expression and were suitable for chronic in vivo imaging of layer II/III pyramidal neurons in the visual cortex.

For this model to be effective, it is essential that the transgene expression not only occurs in the same subset of neurons for comparison across different mouse lines, but also in the same individual neurons. If the mosaicism is brought about by low expression of Cre, then the probability of two or more genes to be expressed in the same neurons is relatively small. However, if the Cre expression itself is mosaic, then we could expect that multiple Cre-dependent genes can be expressed by the same individual neurons. So, we decided to express two different reporter proteins in a triple transgenic approach to check for their expression pattern. First we crossed the EGFP-F expressing mouse line with the R26R reporter line (Soriano, 1999) that had Cre-dependent β -galactosidase expression. The offspring was further crossed with Cre 3487. In the resulting TLG 498⁺ R26R⁺ Cre3487⁺ triple transgenic mice, about 90% of the visual cortical neurons coexpressed EGFP-F and β -galactosidase in the characteristic Golgi-staining-like fashion. This suggested that Cre expression by itself is mosaic and hence a very reliable tool to study the effects of multiple Cre-dependent genes on the structure and function of individual neurons in an otherwise unaltered environment.

It is useful to have a wide array of transgenic mouse lines to study various aspects of development and plasticity in distinct cell types from different parts of the brain. Expression of various spectral variants of GFP in transgenic mice have been used extensively to study in vivo changes during synapse formation and plasticity (Feng et al., 2000). Two recent studies have resulted in exciting, although laborious, transgenic approaches to label small populations of neurons and to study interactions within a network (Livet et al., 2007; Zong et al., 2005). In combination with transgenic mice, viral mediated gene expression is a potent tool that allows spatial and temporal flexibility (**Chapter 3**). We think that our double/triple transgenic approach will be a welcome new tool for characterizing the structural and functional changes in neurons from various parts of the brain. Moreover, the technically less demanding approach and the ability to perform acute and chronic in vivo imaging to dynamically compare the effects of different transgenes on the same subset of neurons in the adult brain makes this a very attractive system.

TrkB-dependent spine maintenance in the visual cortex is regulated by a competitive process.

After the initial validation of the system, we generated transgenic mice that express a Cre-dependent dominant negative TrkB receptor (TrkB.T1) tagged with EGFP (TrkB.T1-EGFP) under the Thy1 promoter. One of the lines, TLT 817, had the strongest TrkB.T1-EGFP expression and when crossed with the Cre 3487 mouse resulted in the expression of TrkB.T1-EGFP in sparse neurons of the neocortex and hippocampus at around 6 weeks. In the visual cortex, expression was found in pyramidal neurons of the

extragranular layers. Granule cells of dentate gyrus and pyramidal neurons of CA1 and CA3 also showed strong expression in the hippocampus. Since transgene expression started in the adult cortex and was confined to a few individual pyramidal neurons, the structural and functional effects of pre- vs post-synaptic interference of TrkB signaling could be studied in a developmentally and structurally unaffected environment.

Expression of TrkB.T1-EGFP for two weeks in layer II/III pyramidal neurons of V1 resulted in a reduction of mature spines (mushroom and stubby) by about 60-85% in comparison with EGFP-F expressing neurons. This was accompanied by a 2-3 times increase in the immature spine forms (long and thin), about 22% increase in filopodia and a 24% reduction in the total density of the protrusions (Chakravarthy et al., 2006). The TrkB.T1-EGFP neurons also showed a reduction in amplitude and frequency of mEPSCs. Moreover, we measured the head size and spine length of mushroom spines from apical dendrites of TrkB.T1-EGFP expressing neurons. There was a 50% reduction in the spine head size and a 30% increase in spine length, further emphasizing their immature morphology (**Chapter 4**). To study whether there was a functional correlate to the morphological changes, we measured mEPSCs amplitude and frequency. We found an excellent correlation between the reduction in head size and that in mEPSC amplitude, and between the mEPSC frequency and spine numbers. TrkB signaling is known to convert silent synapses into mature ones by the insertion of AMPARs in the developing barrel cortex (Itami et al., 2003). So, could the reduction in the large spine be a result of inefficient conversion from immature long and thin spines to a more mature mushroom spine type? Chronic in vivo imaging in the adult visual cortex has shown

that about 70-90% of spines are stable for a few months (Grutzendler et al., 2002; Holtmaat et al., 2005) with mushroom spines being the most stable (Trachtenberg et al., 2002). Since the neurons have been expressing TrkB.T1-EGFP for just about two weeks, it is very unlikely that the natural turnover of spines could account for the profound reduction in the stable spines. In addition, when we measured spine numbers and sizes of spines by making use of DiI labeling at 5 weeks after birth - before the onset of transgene expression - we found that spine morphology was already of a more mature phenotype, indicating that an actual loss of spines and spine size had occurred due to TrkB.T1-EGFP expression. This suggests that intact postsynaptic TrkB signaling is important for the maintenance of stable mushroom spines and synaptic efficacy.

Earlier work had shown that ablating TrkB in either pre- or post-synaptic compartments affected spine formation in the developing CA1 neurons but had no effects on spine density in the adult CA1 (Luikart et al., 2005). This offered us a good opportunity to test our model system as well as understand the underlying mechanisms that may govern synapse formation and maintenance across brain regions. Hippocampal CA1 neurons from TLT 817⁺ Cre 3487⁺ were no different from those of TLG 498⁺ Cre 3487⁺ in total protrusion density or spine types (except for a 35% decrease in stubby spines). More importantly, there was no change in the head size of the mushroom spines although they were much longer. This suggested that postsynaptic TrkB signaling is not essential for spine maintenance in adult CA1, but might be involved in the transition of immature spines to more stable ones. In vivo imaging can give us a better insight into the dynamic

changes that occur during the different stages of spine development and maintenance, reflected by the conversion of one spine type to the other.

So, how is this process regulated? Is there a basal amount of TrkB signaling required for synaptic maintenance in the cortex? Or could competition for TrkB signaling be the underlying mechanism for spine maintenance in the visual cortex? If so, what happens when TrkB signaling is hindered in a majority of neurons in the adult visual cortex? To address this issue, we crossed the TLT 817 mouse to a Cre mouse (Cre G35-3) that mediated transgene expression in all excitatory neurons of the forebrain (Sawtell et al., 2003). This resulted in TrkB.T1-EGFP expression in pyramidal neurons of the visual cortex that started at around 5 weeks, increased with age and reached maximum levels at around 2 months. Interestingly, when compared with TLG 498⁺ Cre G35-3⁺ mice, there was no change in spine density or spine type in layer II/III neurons of V1 at 8 weeks. When we compared the large mushroom spines, the head size of the spine was also the same, suggesting that spine maintenance was not compromised. Similarly, there was no difference in either the amplitude or frequency of miniature excitatory synaptic currents (mEPSCs). This indicated that inhibiting TrkB signaling in most pyramidal neurons of V1 does not alter the number of excitatory synapses (**Chapter 5**).

But what happens to the inhibitory system? We tested for the expression profile of Parvalbumin (Braun et al., 1991), a calcium-binding protein known to be expressed in inhibitory neurons that make perisomatic synapses onto layer II/III pyramidal neurons. TrkB.T1-EGFP expressing neurons had 40% fewer Parvalbumin⁺ puncta around their cell bodies. The size as well as the intensity of these puncta was also reduced by about 10%. The frequency

of miniature inhibitory synaptic currents (mIPSCs) also reduced significantly in these neurons. This implied that perisomatic inhibition is strongly compromised in TrkB.T1-EGFP expressing neurons.

All these data collectively suggest that postsynaptic TrkB signaling regulates synaptic efficiency in a competitive manner that is influenced by the relative, and not the absolute levels of TrkB in comparison to other neurons in the network. Our current hypothesis is that this is brought about by the inhibitory system which acts as a negative feedback, based on the sum total activity in the neuronal network. Reduction of TrkB signaling in an individual cell will reduce excitatory synapses in this particular neuron without affecting the neighboring cells. Thus, the population activity is not modified and hence the inhibitory input to this neuron remains the same. However, when all pyramidal neurons in V1 lack TrkB signaling, the population activity is reduced, thereby triggering a reduction in inhibitory input. This is in line with BDNF's role as a regulator of activity-dependent inhibition of cortical neurons (Rutherford et al., 1997). If such a mechanism is in play, then one would expect that there is a reduced excitatory component involved. Although the spine density and mEPSCs did not show a difference, there is a reduction in the visual responsiveness in these mice (Heimel et al., 2008). We are currently investigating whether this difference in responsiveness is caused by a specific reduction in NMDAR expression. Interestingly, these mice did not show a change in the ocular dominance plasticity. A recent study showed that TrkB signaling in the visual cortex does not affect OD plasticity during the critical period, but is essential for the recovery from OD plasticity (Kaneko et al., 2008).

In this study, by expressing TrkB.T1-EGFP in either sparse or a majority of excitatory neurons, we show that TrkB signaling regulates synaptic morphology in the adult visual cortex in a competitive and bidirectional manner. What is not evident is how this competitive effect of synaptic maintenance correlates to OD plasticity. It could be synergistically brought about by other factors like the extracellular matrix. It is important to develop molecular tools to dissect out the pre- vs post-synaptic effects of BDNF at the thalamo-cortical synapses to study OD plasticity during the critical period. Loss of cortical visual acuity is a hallmark of amblyopic patients and identifying the mechanism(s) by which TrkB signaling affects visual acuity might be of remedial significance. Further studies using *in vivo* imaging will be of great value in understanding some of the fundamental issues about synapse formation and plasticity mediated by BDNF/TrkB signaling.

