Injury-induced and hormonal state dependent plasticity in the rat central nervous system

Ph.D. Thesis

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Szüleimnek...

A természet egyre mélyülő megértése során minden nagy siker forrása
a természet megfigyelésének öröme és a rajta való szüntelen gondolkodás.

Lenard Fülöp

I. ABBREVIATIONS

AD	Alzheimer's disease
ANOVA	analysis of variances
AOB	accessory olfactory bulb
ARC	arcuate nucleus
Ba-DupLac	bioengineered PRV-Ba virus strain for neural tract tracing studies
Bcl-2	B cell lymphoma related protein 2
BclXL	B cell lymphoma related protein X, long product
BNIP2	Bcl-2 interacting protein 2
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
DMEM	Dulbecco's modified minimum essential medium
E2	17-β estradiol
EP	evoked potential
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
GABA	gamma–amino–butyric acid
GLUT	glutamate
HSPG	heparan sulphate proteoglycan
HSV	herpes simplex virus
ION	infraorbital nerve
LTP	long-term potentiation
MEG	magnetoencephalography
MEP	motor-evoked potential
M1	primary motor cortex
M1 I	ipsilateral primary motor cortex
M1 C	contralateral primary motor cortex
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
N7x	facial nerve transection
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
OVX	ovariectomized rat
OVX + E	estrogen treated ovariectomized rat
PBS	phosphate buffer solution
PET	positron emission tomography
PND	postnatal day
PRV	pseudorabies virus
PRV-Ba	attenuated PRV strain Bartha
pt	photothrombotic lesion
SD	standard deviation
SEM	standard error of the mean
SFO	subfornical organ
S1	primary somatosensory cortex
TMS	transcranial magnetic stimulation

II. AIMS AND SCOPE OF THIS WORK

The term neuronal plasticity is rich and difficult to explain; its most general definition is the capability the nervous system to undergo adaptive changes. This capacity of the nervous system is very diverse, ranging from the reflex loops through the responses to various types of injuries up to complicated learning and cognitive processes.

In one part of the investigations, we (my colleges and me) examined the physiological consequences of peripheral nerve transection in the central nervous system (CNS). Contrary to the classical view of a predetermined wiring pattern, there is considerable evidence that cortical representation of body parts is continuously modulated in response to activity, behavior and skill acquisition. Both animal and human studies showed that following injury of the peripheral nervous system such as nerve injury or amputation, the somatosensory cortex (S1) that responded to the deafferented body parts become responsive to neighboring body parts. Similarly, there is expansion of the motor representation of the stump area following amputation.

However, it is more than probable that the changes in the cortical representational maps are consequences of molecular biological and biochemical changes in the neurons and the glial cells and in their connections. The peripheral nerve injury-induced changes in the function of neuronal connections (the plasticity itself) and its modifiability in the cortex and the hormonal state dependent plastic changes in hypothalamic structures were studied with immunohistochemical, electrophysiological and behavioral methods. To study the neuronal plasticity, a relatively new method was introduced in our department: a pseudorabies virus (PRV) was utilized as a transneuronal tract-tracing tool.

Of course the work presented here is not done by one person, but is a real team-work. I have been the mean investigator in the below listed experiments where my name is indicated. I have been only co-investigator for statistical analysis, animal preparation, etc. and discussion in the other works. But for better understanding of the enigmatic biological phenomenons neuronal plasticity and estrogen effect in neural tissue - I include also their work with their permission.

The aims and scopes of my work included in the present thesis are as follows:

1. **that facial nerve transection (N7x) (the model of human facial palsy) induces changes in the neuronal connections of the primary motor cortices (M1s) in both hemispheres, which influence the transcallosal PRV labeling pattern (main investigator *Szatmár Horváth*).**
2. **test the hypothesis that even a minimal conflict with NMDA receptors in the early critical age of life, which results in mild if any detectable change in daily behavior, induces hidden but life-long dysfunctions, which can be detected in different parts of the CNS with appropriate methods (main investigator *Hajnalka Németh*).**
3. **both denervation and ischemia-induced cortical changes indicate that widespread remote decreases in inhibition are a common feature of these peripheral and central injuries. We have examined whether the two manipulations interact or not in causing disinhibition (main investigator *dr Tamás Farkas*).**
4. **to investigate whether the 17-β estradiol (E2)-induced decrease in inhibitory synaptic inputs on the arcuate neurons results in a change in their electrophysiological activity, and to study the possible effects of activation of the olfactory and somatosensory systems on the activity of the neurons in the arcuate nucleus (ARC) (main investigator *dr Zsolt Kis*).**
5. **to test whether estrogen really does influence the susceptibility of CNS structures to PRV infection, we examined the estrogen-dependent spread of PRV between the neurons of the ARC and the subfornical organ (SFO), the neurons of both of which possess estrogen receptors (Ers) (Shughrue et al., 1997) (main investigator *Szatmár Horváth*).**
6. **to test the function of estrogen in one of the most plastic part of the rat CNS, in the barrel field of the somatosensory cortex (main investigator *dr Zsolt Kis*).**

III. OVERVIEW

III.1. NERVOUS SYSTEM REORGANIZATION FOLLOWING INJURY

It is a common clinical observation that functional recovery (reorganization of cortical representation) frequently occurs following a peripheral injury such as amputation or a brain injury such as stroke. These changes in plasticity may account for recovery of function after injury. There are many reasons for the different degrees of recovery, including age of the patient, location and extent of the lesion as well as individual variations in anatomical and functional connections. There is now considerable evidence that cortical representation of body parts is also continuously modulated in response to activity, behavior and skill acquisition (Kaas, 1991; Sanes and Donoghue, 2000).

III.2. REORGANIZATION FOLLOWING PERIPHERAL NERVE INJURY

III.2.1. Amputation and peripheral nerve lesions

III.2.1.1. *Animal studies*

Plasticity of the somatosensory system has been studied extensively, and dramatic changes in the organization map of the S1 occur after removal of afferent input.

Lesions in the rat trigeminal system have become an important model for the study of CNS pattern generation and plasticity (Kossut, 1992; Hoeflinger et al., 1995; Jacquin et al., 1995). Our previous results demonstrated that temporary infraorbital nerve (ION) damage (crush) not only resulted in a transient disappearance of the contralateral vibrissa-evoked responses from the barrel cortex, but also produced an expansion of the neighboring representation area of the digits of the contralateral forepaw (Kis et al., 1999b) *(see **Figure 1**, next page)*.

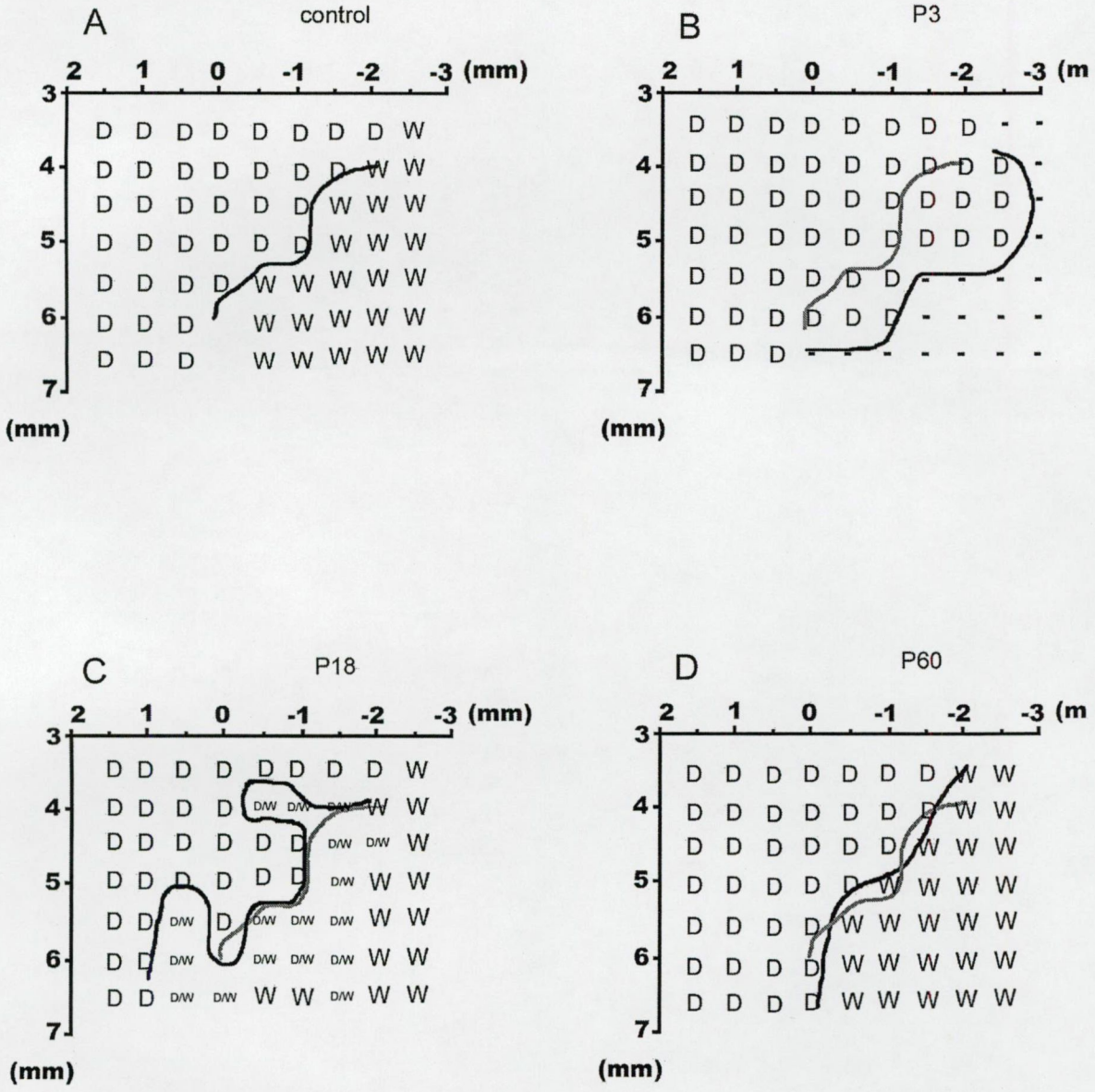


Figure 1 Reorganization in cortical maps of digit and whisker representations induced by injury and subsequent regeneration of the contralateral infraorbital nerve (ION). **A** The posterolateral border of the physiological representation of digits 2, 3, and 4 in a control. **B** Contralateral ION crush resulted in a large unresponsive area to whisker deflection in the posteromedial barrel subfield, but stimulation of the digits evoked responses in the enlarged representation. The border of the digital representation was shifted posteriorly. *Grey line:* The original border between the digits and whisker regions, ***black line*** the posterior border of the enlarged digital representation. The shift in digital representation could be observed within minutes/hours after the ION crush. **C** The expanded digital representation and the reappeared whisker responses resulted in an overlapping zone between the pure digital and the vibrissal cortical areas (*D/W*). *Grey line* The original border between D and W areas, ***black line*** posterior border of the pure digital area. **D** 60 days after ION crush, the representational maps of the digits and vibrissae in the primary somatosensory cortex were found to be quite similar to those of the controls. *D:* Digit representation, *W:* whisker representation. –: no evoked response. Zero on the horizontal scale represents the bregma

In adult monkeys, following peripheral nerve lesion or digit amputation, parts of the S1 that previously responded to the deafferented body parts became responsive to inputs from neighboring body parts (Kelahan et al., 1981; Merzenich et al., 1983). These changes can be reversed after nerve regeneration (Wall et al., 1983). Although initial studies suggested that the upper limit of cortical expansion is 1-2 mm (Merzenich et al., 1983), corresponding to the projection zone of single thalamocortical axons, it is now known that long standing amputation may result in cortical reorganization over a distance of up to 14 mm (Pons et al., 1991; Manger et al., 1996). Similar changes in the S1 with amputation have also been demonstrated in other animal species [*see* Kaas (1991) *for review*].

In the motor system, changes in cortical representation also occur after peripheral injury. Following amputation or peripheral nerve lesions, the area from which stimulation evoked movements of the adjacent body parts enlarged and the threshold for eliciting these movements was reduced (Donoghue and Sanes, 1988; Sanes et al., 1990). In adult mammals, the somatotopic representation map of the muscle system in the M1 is not stable, but may be modified within hours to days after peripheral nerve injury (facial nerve) (Donoghue et al., 1990; Farkas et al., 1999). Closer analysis has revealed that some plastic changes develop much more rapidly, i.e. within minutes, after N7x. In particular, we have previously demonstrated an N7x-induced early disinhibition of the commissural connections between the M1s by intracortical microstimulation of the facial muscle representation field (Toldi et al., 1996; Farkas et al., 2000). Our own results and the literature (Huntley, 1997; Ziemann et al., 1998a; Hickmott and Merzenich, 2002) suggest that the N7x-induced changes take place in both somatosensory and M1s, and may develop in more than one phase, i.e. short-, medium- and long-term aspects of plasticity are defined (Calford, 2002a, b). Primary disinhibition, as an immediate change, may reduce the cortical inhibition driven by afferents, referred to by Calford and Tweedale (Calford, 2002a, b) as the "unmasking of latent inputs" in the S1.

III.2.1.2. *Human studies*

In humans, reorganization of the somatosensory and motor systems also occurs following amputation. Sensations in the phantom limb can be elicited by somatosensory stimulation of the face and upper body in upper-limb amputees (Ramachandran et al., 1992), suggesting that the somatosensory representations of the face and upper body may have expanded to occupy the arm and hand area. This was supported by magnetoencephalography (MEG) studies which revealed medial displacement of the face area toward the hand representation in the S1 (Elbert et al., 1994; Flor et al., 1995). The extent of shift in cortical representation correlated with the amount of phantom (Flor et al., 1995; Knecht et al., 1996). In the motor system, transcranial magnetic stimulation (TMS) studies demonstrated that for both upper-and lower-limb amputees, resting motor-evoked potentials (MEPs) can be elicited at lower intensities (Hall et al., 1990; Cohen et al., 1991; Chen et al., 1997) and from more scalp positions (Cohen et al., 1991; Fuhr et al., 1992) in muscles immediately proximal to the site of amputation compared to the homologous muscle on the normal side. These results suggested that the excitability

of the motor system projecting to the muscle immediately above the amputation is increased. Cortical reorganization was also demonstrated by positron emission tomography (PET) studies that showed increased regional cerebral blood flow in the contralateral M1 with movement of the amputated side compared to the normal side in congenital and traumatic upper-limb amputees (Kew et al., 1994). Cortical reorganization also occurs in humans after peripheral nerve lesions. In patients with facial palsy, TMS and PET studies reveal an enlargement of the hand representation with medial extension into the site of the presumed face area (Rijntjes et al., 1997).

III.2.2. The site of reorganization following peripheral lesions

Changes in cortical maps following a peripheral lesion may be the result of reorganization at cortical or sub-cortical levels. There is evidence that reorganization can occur at multiple levels including the cortex, thalamus, brainstem, spinal cord and peripheral nerves.

In rats, reorganization of the somatosensory neuraxis is detectable after unilateral ION lesion. The results obtained in the main brainstem trigeminal nucleus, in the ventroposteromedial nucleus of the thalamus, and in the cortex strongly suggest that the higher the station in the neuraxis, the greater the degree of plasticity after infraorbital nerve injury (Kis et al., 1999b).

The M1 of the adult rat exhibits also noteworthy capacity to react to peripheral nerve lesions, with changes in perisynaptic glia and synaptic reorganization with latencies of from 1h up to one day. In, M1, however, the interhemispheric connections between homotopic representation fields of vibrissal muscles undergo even more rapid disinhibition. In adult rats trigeminal afferents participate in modulation of the activity of M1 output neurons via S1 to M1 associational connections. As an example, minutes after unilateral transection of the N7x, the cortical responses evoked by trigeminal stimulation ipsilateral to the denervation are facilitated (both gross potentials and unit activity) in the M1s in both hemispheres (Toldi et al., 1999) *(see **Figure 2**)*.

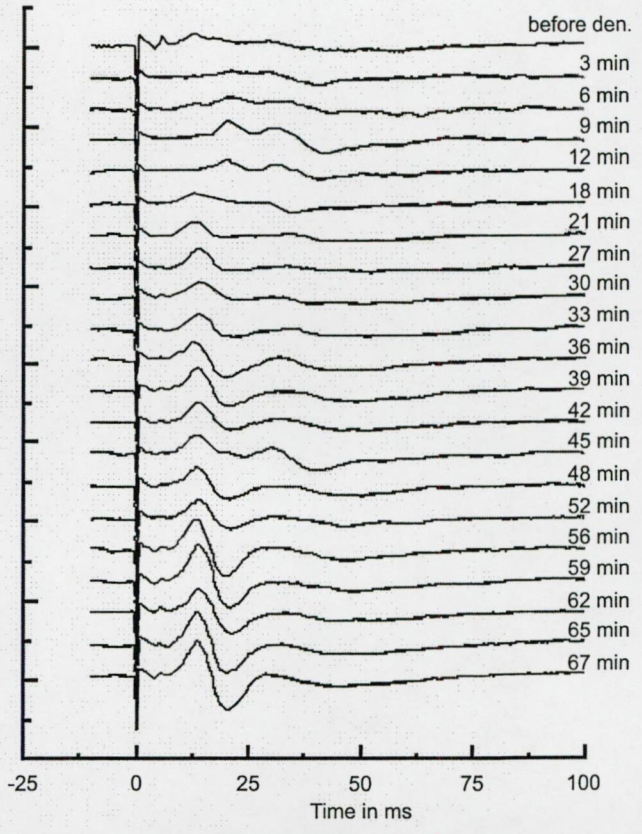


Figure 2 Appearance and development of evoked potentials in the ipsilateral M1 before and after the N VII cut. From the responses to 1Hz trigeminal stimulation, the representative potentials were collected for 2-6 min, successively. Before the N7x no evoked response could be observed in the ipsilateral M1. By 6-9 min after facial nerve transection evoked potentials appeared and increased in amplitude until the end of the registration.

Since these changes are not observed in control animals, but develop after N7x, and can be mimicked by picrotoxin, they are considered to be based on the disinhibition of pre-existing, but not mature associative and commissural connections (Toldi et al., 1999; Farkas et al., 2000).

After chronic partial digit amputation in monkeys, there was both increased branching of the severed digital nerve and increased innervation density in the stump *(see **Figure 3**)*.

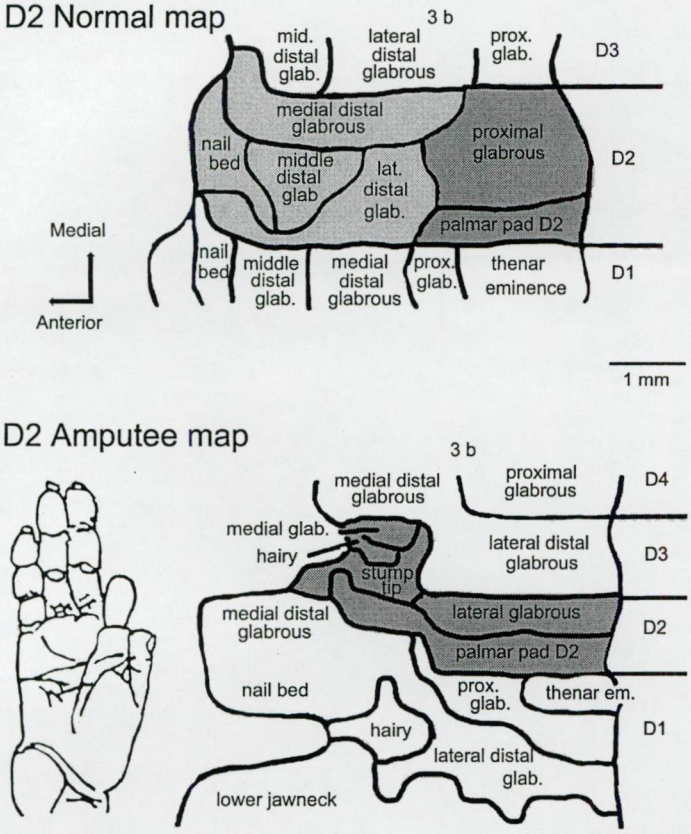


Figure 3 Somatosensory cortical map of the index finger of an undamaged hand (*upper*) and of the remaining proximal stump of an index finger (D2) from which the middle and distal phalanges had been amputated 2 years previously (*lower*). Macaque monkey. In the map of the affected hand, the representation of the index finger has become encroached on by the representations of the adjacent fingers and has become intrinsically reorganized so that the region formerly devoted to distal skin has now become occupied by an enlarged representation of the stump. *Modified from Manger et al* (1996b).

It was suggested that the stump may be innervated by neurons that previously had innervated the amputated digit, which may explain the expansion of the cortical map of the stump (Manger et al., 1996).

In monkeys with hand amputation, afferent terminations from the forearm were found to extend into the hand areas of the dorsal horn of the spinal cord and the cuneate nucleus in the medulla (Florence and Kaas, 1995), providing some evidence for sprouting at the level of the spinal cord and brainstem. Plasticity changes have also been demonstrated in the thalamus, where neuronal recordings that local anesthesia induced immediate and reversible reorganization of the ventral posterior medial nucleus (Nicolelis et al., 1993). Long-term dorsal rhizotomies in monkeys resulted in transneuronal degeneration of non-nociceptive somatosensory pathways and increased activity of thalamic cells innervated by pain afferents (Rausell et al., 1998).

In other situations, reorganization may occur mainly within the cortex. One such example is the increased receptive field size in the visual cortex after a retinal lesion, which cannot be sufficiently accounted for by changes in the lateral geniculate nucleus (Gilbert and Wiesel, 1992). These changes may arise through modifications in synaptic coupling of the extensive intrinsic cortical connections (DeFelipe et al., 1986; Huntley and Jones, 1991; Kaas, 1991; Donoghue and Sanes, 1994). It appears that plasticity changes in the somatosensory system can occur at multiple cortical and subcortical sites,

whereas in the visual system reorganization mainly is in the cortex (Kaas and Florence, 1997). Proposed mechanisms of recovery in the somatomotor system include redundancy of brain circuitry with parallel pathways performing similar functions such that an alternative pathway may take over when another has been damaged, unmasking of previously existing but functionally inactive pathways, and sprouting of fibers from the surviving neurons with formation of new synapses (Lee and van Donkelaar, 1995).

III.2.3. Reorganization associated with recovery from stroke

Stroke is the third leading cause of death in the western countries and is the main cause of long-term disability among adults. Spontaneous recovery usually occurs, although the extent is highly variable. Motor recovery occurs predominantly in the initial weeks to first three months, but can continue at a slower pace throughout the first year (Kotila et al., 1984; Kelly-Hayes et al., 1988). Recovery in the first few days may be due to resolution of edema or reperfusion of the ischemic penumbra.
Much of the recovery after the initial 2 weeks is likely due to brain plasticity, with some areas of the brain taking over the functions previously performed by the damaged regions. The mechanisms involved likely depend on the extent of injury. When damage to a functional system is partial, within-system recovery is possible, whereas after complete destruction, substitution by a functionally related system becomes the only alternative (Seitz and Freund, 1997).

III.2.3.1. *Animal studies*

Several studies examined the reorganization following small lesions of the M1 or the S1. Jenkins and Merzenich (1987) reported that after small infarcts of the S1 in owl monkeys, the skin surface formerly represented by the infarcted zone became represented topographically in the surrounding cortical region. This was associated with enlargement of the cutaneous receptive fields in the cortical areas surrounding the lesion, since the same cortical area now represented a larger skin area. However, the findings appear to differ in the motor system following a small M1 infarct in the digit representation. In monkeys not receiving post-infarct training, the movement formerly represented in the infarcted zone did not reappear in the adjacent cortical regions. In addition, the digit representation previously present in the areas adjacent to the infarcted cortex was further reduced (Nudo and Milliken, 1996). In contrast, monkeys which received 'rehabilitative training' following the infarct had a preserved hand territory, and in some cases, the hand territory expanded to the elbow and shoulder representations (Nudo et al., 1996). However, with or without post-infarct training, the monkeys recovered their hand functions. These findings showed that functional recovery after small cortical lesions may be associated with the adjacent cortical area taking over the function of the damaged cortex. The extent of reorganization may be limited by the extent of convergence and divergence of anatomical inputs and outputs of the area (Jenkins and Merzenich, 1987). However, large lesions may involve different mechanisms. Recovery of function in monkeys with an M1 lesion but no post-infarct

training demonstrated that assumption of lost functions by the adjacent undamaged M1 is not inevitable, but to some extent is use-dependent.

III.2.3.2. *Human studies*

Motor recovery following damage to the M1 or the pyramidal tract may be mediated by the use of alternative cortical areas in the damaged hemisphere that can access spinal motoneurons. Two general mechanisms are possible here: the use of parallel, redundant pathways or new regions taking over the function of the damaged area. There are several parallel motor pathways in the motor system. In addition to the M1, motor areas have been identified in the premotor cortex, supplementary motor area and cingulate cortex (Dum and Strick, 1991). All these motor areas contain somatotopic representations and all contribute to the pyramidal tract (He et al., 1993, 1995). These parallel pathways may substitute for each other in recovery from hemiparesis.

There is also evidence for the adjacent cortex taking over the function of the damaged pathways. In patients with lesions limited to the posterior limb of the internal capsule, recovered hand movement led to M1 activation that extended laterally to the face area, suggesting that the hand representation shifts toward the face area (Weiller et al., 1993). This change was not observed in patients with lesions in the anterior limb of the internal capsule (Weiller et al., 1993). This may be explained by sparing of the pyramidal tract from the face representation in patients with lesions confined to the posterior limb of the internal capsule, since the fibers destined for the face travel to more anterior locations in the internal capsule than fibers for the arm and leg (Hardy et al., 1979). Thus, the shift of cortical representation following amputation or peripheral nerve lesions may also be present with recovery from stroke. Wassermann et al. (1998) presented PET studies in patients with subcortical infarct; there was increased activation of the contralateral sensorimotor area with finger movement compared to normal subjects. This suggests that the sensorimotor area was working vigorously to compensate for the lesion or additional sensorimotor cortex was recruited.

III.3. MECHANISMS OF CORTICAL REORGANIZATION

III.3.1. Mechanisms for short-term changes

The two main mechanisms proposed to explain reorganization after peripheral lesions are unmasking of previous present but functionally inactive connections and growth of new connections (collateral sprouting). Since the growth of new connections takes time, rapid expansion of muscle representation that occurs within minutes to hours following transient deafferentation in humans (Brasil-Neto et al., 1992; Brasil-Neto et al., 1993; Sadato et al., 1995) or nerve lesions in animals (Merzenich et al., 1983; Donoghue et al., 1990; Nicolelis et al., 1993) likely involve unmasking of latent excitatory synapses. Unmasking of latent synapses can be due to several mechanisms and include increased excitatory neurotransmitter release, increased density of postsynaptic receptors, changes in membrane conductance that enhance the effects of weak or distant inputs, displacement of presynaptic elements

to a more favorable site, decreased inhibitory inputs or removing inhibition from excitatory inputs (unmasking excitation) (Kaas, 1991). Among these possibilities, the evidence is strongest for removal of inhibition to excitatory synapses, which is likely due to reduced GABAergic inhibition, in mediating short- term plastic changes.

III.3.1.1. *Role of GABAergic inhibition*

Several lines of evidence indicate that modulation of GABAergic inhibition plays a significant role in cortical plasticity. GABA is the most important inhibitory neurotransmitter in the brain (Jones, 1993). GABAergic neurons constitute 25-30 % of the neuronal population in the M1 and their horizontal connections can extend up to 6 mm or more (Gilbert and Wiesel, 1992; Jones, 1993). Following application of the GABA antagonist bicuculline to the forelimb area of the M1, stimulation of the adjacent vibrissa area led to forelimb movements, suggesting that GABAergic neurons are crucial to the maintenance of cortical motor representations (Jacobs and Donoghue, 1991).These changes are similar to the expansion of TMS maps of the involved muscles following transient deafferentation (Brasil-Neto et al., 1993) or peripheral nerve lesions (Merzenich et al., 1983; Donoghue et al., 1990). Deafferentation of the somatosensory (Welker et al., 1989) or visual cortex (Hendry and Jones, 1986) also led to a reduction in the number of neurons containing GABA or its synthesizing enzyme, glutamic acid decarboxylase.

III.3.1.2. *Intracortical inhibition in transient deafferentation*

Ziemann et al., (1998b) applied paired-TMS to the biceps muscle during transient deafferentation induced by forearm ischemia and found reduced intracortical inhibition. The TMS motor threshold remains unchanged during transient deafferentation (Brasil-Neto et al., 1993; Ziemann et al., 1998b). These changes may be related to reduced GABAergic inhibition, since drugs that enhance GABAergic inhibition such as ethanol, lorazepam and vigabatrin increase intracortical inhibition but have no effect on TMS motor threshold (Ziemann et al., 1996b, c). These findings suggest that changes in GABAergic inhibition can be rapidly induced in humans. But an increasing number of results suggest, however, that the transiently reduced inhibition (e.g. after nerve injury) is a necessary, but not sufficient condition for the development of M1 plasticity.

III.3.2. Mechanisms for long-term changes

Plastic changes that occur over a longer time likely involve mechanisms in addition to the unmasking of latent synapses. These may include axonal regeneration and sprouting with alterations in synapse shape, number, size and type (Kaas, 1991). LTP may also be involved, which requires NMDA receptor activation and increased intracellular calcium concentration, and has been demonstrated in the M1 (Hess et al., 1994). The potential for plasticity in the M1 has been closely linked to the function of NMDA receptors. Moreover, it has been shown that a component of field potentials itself evoked in the horizontal pathways of the rat M1 is mediated by NMDA receptors, and LTP can develop in these

horizontal connections (Hess et al., 1994). We recently reported that the M1 responses evoked by contralateral trigeminal stimulation, are conveyed mostly by horizontal associations from the S1 (Farkas et al., 1999). Therefore it seems probable that the NMDA receptors play a decisive role in the responses in M1 evoked by contralateral trigeminal stimulation, and especially in their potentiation.

III.3.2.1. *Mechanisms for reorganization in amputees*

Chen et al., (1998) have studied the mechanisms for reorganization in lower-limb amputees with testing of TMS motor threshold and paired-TMS studies. Both TMS threshold and intracortical inhibition were reduced for the muscle just proximal to the amputation. Therefore, it appeared that diminished intracortical inhibition, which may be related to reduced GABAergic inhibition and occurs shortly after deafferentation, persists for prolonged periods after deafferentation. Changes in motor threshold apparently required longer time to develop, because the motor threshold was unchanged with transient deafferentation(Ziemann et al., 1998a). The mechanisms underlying reduction in motor threshold are likely separate from those for intracortical inhibition, since the motor threshold is altered by drugs that change membrane excitability, whereas intracortical inhibition is altered by drugs that influence GABAergic mechanisms (Ziemann et al., 1996a).

Because the excitability of subcortical structures is unchanged, the reduction of motor threshold likely involves enhancement of cortico-cortical connections (Chen et al., 1997). Since drugs that block voltage-gated sodium channels raise the motor threshold, one possible mechanism involves changes in sodium channels. Shifts in the voltage dependence of sodium-channel activation have been implicated in motoneuron plasticity in the spinal cord (Carp and Wolpaw, 1994; Halter et al., 1995). Other mechanisms are also possible, including LTP, axonal regeneration and formation of new synapses.

III.4. ESTROGENS AND THE BRAIN

III.4.1. Genomic or non-genomic effect of estrogen?

Neuroscientists, using electrophysiological recordings, were the first to observe the rapid effects of estrogens on ion conductance, which led to the adoption of the terms non-genomic and genomic to discriminate between cell responses to estrogens induced within seconds and those requiring hours to occur (McEwen, 1981).

The long-term genomic effects have been thoroughly investigated in many estrogen target cells. It is now well established that they are mediated by the two ERs: ERα (Green et al., 1986) and ERβ (Kuiper et al., 1996) [ESR1 and ESR2 according to the nomenclature proposed by the *Nuclear Receptors Nomenclature Committee* (1999)]. These two proteins, encoded by separate genes, are structurally and functionally distinct, and both are members of the superfamily of intracellular receptors. Upon binding the cognate ligand, ERs dimerize and bind to specific elements in the promoter of target genes [estrogen responsive elements (EREs)] where they interact with coregulators,

integrators, and other proteins of the transcription machinery to regulate the synthesis of selected mRNAs and therefore the levels of estrogen-regulated proteins (1999).

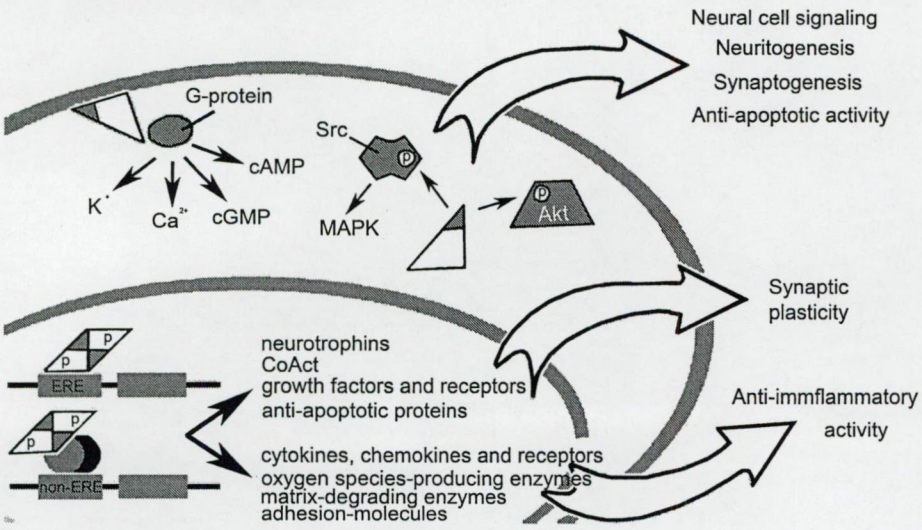
Less understood are the molecular mechanisms responsible for the non-genomic effects of estrogens. Recently, substantial evidence from a number of laboratories supports the concept that ERα and ERβ might also be responsible for the rapid effects of E2; indeed, in the cytoplasm, ER monomers can form multimeric complexes with other signaling proteins to induce rapid changes in the neuronal activity (Levin, 2001). On the other hand, engineered forms of ERs appear to associate with the cell membrane and cross-couple with the membrane receptor–signaling molecules to activate rapid hormonal responses (Levin, 2001). These receptor forms might be responsible for binding E2 and for the ER-like immunoreactivity reported by several authors also noted in nonneural cell systems (Pietras and Szego, 1977; Pappas et al., 1995). However, there is no clear evidence that ERα and ERβ associate with cell membrane in physiological settings. Alternatively, the existence of a specific form of membrane ER capable of rapid signaling has been suggested by a recent study (Toran-Allerand et al., 2002), which needs to be confirmed by the isolation and biochemical characterization of this novel E2-binding protein encoded by a gene so far not predicted by genomic studies.

Finally, several splice variants of ERα and ERβ have been found in the rodent and human brain (Price et al., 2000; Shupnik, 2002). Typically, these variant mRNAs lack one or more of the coding exons or contain base insertions within an exon; most are translated into transcriptionally active proteins (Bollig and Miksicek, 2000). The changes in expression of such variants during embryonic and postnatal development and their differential localization in adult brain nuclei (Price et al., 2000) suggest that they contribute to the complexity of estrogen signaling in the brain.

III.4.2. Estrogens and neuroprotection: potential mechanisms

The neurotrophic and neuroprotective actions of E2 might explain most of the beneficial effects on cognition, motility, and neural disorders. In several animal models, estrogens were shown to exert a trophic and neuroprotective effect and effectively prevent neuronal damage induced by neurotoxic stimuli. In OVX rats, E2 attenuates the extent of brain damage induced by permanent or transient brain ischemia (Gridley et al., 1997; Wang et al., 1999; Carswell et al., 2000), and reduces cortical lesions owing to glutamate excitotoxicity (Behl et al., 1997; Wang et al., 1999) or secondary to *status epilepticus* (Veliskova et al., 2000), or autoimmune encephalomyelitis (Ito et al., 2001; Liu et al., 2002). Thus in a variety of models leading to neural death, estrogen has a protective function. Several hypotheses can be formulated to explain the mechanism of estrogen neuroprotective action *(see next page **Figure 4**)*: (a) The trophic activities of estrogens during the maturation of the CNS may continue to exist in the adult brain and ensure that neurons maintain the synaptic connections indispensable for neural signaling and survival; (b) E2 may positively regulate the synthesis of proteins protecting neurons against apoptosis; (c) E2 may influence inflammatory responses by controlling microglia

reactivity and the vascular function; and (d) E2 may induce proliferation of stem cells to replace neurons undergoing neurodegeneration.



III.4.2.1. *Insights on neurotrophic actions of 17β-estradiol*

The trophic actions of estrogens have been known since the pioneering work carried out by Toran-Allerand, who demonstrated that E2 treatment of explant cultures of cerebral cortex and hypothalamus stimulates extensive neurite outgrowth. Since then, several studies on neuroblastoma cells or on dissociated neurons in culture showed that E2 increases cell viability, differentiation, neurite outgrowth, and spine density (Toran-Allerand et al., 1980; Murphy et al., 1998). Most likely these effects observed *in vitro* recapitulate the *in vivo* action of the hormone during the differentiation of the nervous system and in adult animals, where it affects the plasticity of cells such as hippocampal neurons. Thus E2 might control the capability of neurons to extend neurites and to form synaptic connections with other cells via dendritic spines. Depending of the model system utilized, neurite extension or spine formation was observed rapidly and continued for several days after the exposure to the hormone. Altered changes in Ca^{2+} fluxes are indispensable for the increase in dendritic spines observed in hippocampal neurons. On the other hand, estrogens were shown to modulate the synthesis of growth factors such as nerve growth factor (NGF), brain-derived neurotrophic factor, insulin-like growth factor-1, transforming growth factor beta and related receptors, tyrosine kinase A and B, in neurons and astroglia (Sohrabji et al., 1994, 1995; Garcia-Segura et al., 2001), and this *de novo* synthesis of growth factors is required for neurite formation. The existence of multiple mechanisms might be necessary for the generation of neurites and synaptic connections morphologically similar, but functionally distinct. How E2 induces neuronal cell plasticity *in vivo* is unclear. The observation that NGF stimulates astrocytes to function as substrates for axonal growth led to the hypothesis that interplay between astrocytes and neurons is indispensable for estrogen effect on neuronal plasticity.

This was subsequently demonstrated by a study in which E2, by eliciting the synthesis and release of NGF by neurons, could induce tyrosine kinase A immunoreactive astrocytes to reorganize laminin into extracellular fibrillar arrays thus supporting axonal growth, structural plasticity, and synapse formation (Kawaja and Gage, 1991). On the other hand, estrogen may also cause neurite outgrowth in the absence of astrocytes, as was shown in estrogen-dependent neuritogenesis in a neuroblastoma model. In this case, neurite extension was initiated by estrogens via ER-dependent modulation of protein synthesis. Interestingly both ER isoforms contributed to neurite outgrowth even though the mechanism of action was different because ERα, but not ERβ, required a functionally active Rac 1b small G protein (Patrone et al., 2000).

III.4.2.2. *Insights on anti-apoptotic actions of 17β-estradiol*

E2 was shown to protect neural cells against death induced by a variety of stimuli including hypoxia, oxidative stress, excitotoxicity, glucose deprivation, exposure to amyloid β-peptide (AP), or other neurotoxic agents (Goodman et al., 1996; Patrone et al., 2000). This effect has been observed in primary neuronal cultures, tumor-derived neuronal cell lines, mixed neuron/astrocyte cell culture, and organotypic explants. The mechanism responsible for this effect is still controversial. Most of the studies in which physiological concentrations (in the nanomolar range) of E2 were used support the view of an involvement of ERs and *de novo* protein synthesis; others using pharmacological concentrations of the hormone demonstrated its capability to directly alter the synthesis or metabolism of the toxic stimulus. Several laboratories (Gollapudi and Oblinger, 1999; Meda et al., 2000) demonstrated that physiological concentrations of estrogen enhances the survival of neuroblastoma cells only when transfected with ERα, thus proving the significance of this receptor in the mechanism of neuroprotection. Other studies have focused on the identification of genes modulated by estrogens in neural cells and have demonstrated that several of the known anti-apoptotic genes (Bcl-2, BclXL) are transcriptionally activated by the hormone, whereas pro-apoptotic genes are down-modulated (BNIP2), thus indicating that E2 might protect neural cells from apoptotic death by affecting the balance between apoptotic and anti-apoptotic genes (Meda et al., 2000).

III.4.2.3. *17β-estradiol as an anti-inflammatory agent*

The beneficial effects of E2 on demyelinating and neurodegenerative diseases are supported by the anti-inflammatory hypothesis, which is based upon experimental evidence on animal models and cellular systems, suggesting that estrogen suppresses the inflammatory process induced by several stimuli in the CNS. Indeed, several neural cell pathologies that benefit from hormone action, such as multiple sclerosis (MS), Alzheimer's disease (AD), and ischemia, have a different etiology but share a strong neuroinflammatory reaction that sustains disease progression. Thus the anti-inflammatory hypothesis may represent a unifying explanation for hormone action. In addition, this mechanism is viewed as a pharmacological challenge for the prevention of these pathologies because anti-

inflammatory drugs have been shown to be protective against the onset of AD (McGeer and McGeer, 1996). The anti-inflammatory properties of female steroid hormones have been observed *in vivo* in animal models with CNS inflammation, i.e., experimental autoimmune encephalomyelitis (which is the animal model of MS), brain ischemia, globoid cells leukodistrophy, and experimental brain inflammation. These reports clearly demonstrated that estrogen inhibits the CNS inflammatory reaction. In fact, treatment with physiological doses of estrogen at the time of disease induction suppressed experimental autoimmune encephalomyelitis by down-regulating inflammatory factors and inhibiting the migration of inflammatory cells into the CNS (Ito et al., 2001). Estrogen has also been shown to reduce leukocytes adhesion in the cerebral circulation of female rats subjected to transient forebrain ischemia and reperfusion (Santizo et al., 2000). That this activity occurs through the specific activation of ERα (Weaver et al., 1997) suggests that inflammation may also be a target for estrogen in brain vascular pathology. Finally, the activation of brain macrophages induced by the bacterial endotoxin lipopolysaccharide has been blocked by administration of physiological concentrations of hormone that specifically acts through the activation of ERα (Vegeto et al., 2003). These animal studies provide some hints to the specific molecules that are regulated by estrogen; these include cytokines, chemokines, and their receptors (Matsuda et al., 2001); apolipoprotein E; and other modulators of leukocyte migration such as matrix metalloproteinase-9 and complement receptor-3 (Vegeto et al., 2003). Among the cells that are targeted by hormonal anti-inflammatory activity, microglia, the resident macrophage cells of the CNS, play a central role in brain homeostasis because they are the first cellular sensors of any local mechanical or chemical injury and immediately react by mounting the inflammatory response (Vegeto et al., 2003). A state of chronic microglia activation has been observed in association with the neurodegenerative process in several CNS diseases (Vegeto et al., 2003), and it is currently believed that the production of oxygen reactive species, which inflict an oxidative stress to neighboring cells, is the major cause of neuronal cell death. Studies conducted in primary cultures of microglia cells and in microglial cell lines (Bruce-Keller et al., 2000) showed that nanomolar concentrations of E2 decrease the activation of these cells toward an inflammatory stimulus. This effect has been reconciled with the blockade of the expression of pro-inflammatory factors, such as inducible nitric oxide synthase, prostaglandin E2 and MMP-9, and inhibition of superoxide release and phagocytic activity. In addition, clearance of amyloid, which is impaired in AD, is enhanced by estrogen in microglia derived from the human cortex, a mechanism that further explains the protective role of hormone mediated by its interaction with microglia. As to the molecular mechanism of hormone action, it is clear that a reduced expression of pro-inflammatory proteins and an increased synthesis of protective factors underlie the anti-inflammatory activity of hormone. Because no estrogen responding elements were found in the promoter region of these genes, it is hypothesized that the ER interferes with the signaling pathways activated by an inflammatory stimulus, such as nuclear factor kappa B or activator protein-1, and therefore regulates their transcriptional activity.

III.4.2.4. *17β-estradiol and neural stem cells*

We now know that neural stem cells express both ERα and ERβ and respond to the treatment with E2 with increased proliferation. It has been shown (Tanapat et al., 1999) that stem cell proliferation, at least in the hippocampus, is most active in sexually mature female mice compared with male mice, and a natural fluctuation in stem cell proliferation was also noted: females produced more cells at proestrus (when plasma E2 levels are highest) compared with estrus and diestrus. These cells acquired neuronal characteristics. However, further examination demonstrated that these cells have a very short survival time. Thus these neurons generated under the influence of estrogens do not seem to be of importance for the positive effects of the hormone on cognition and neuroprotection. The estrogen-induced proliferation of neural stem cells is related more to the reproductive functions by showing that the newly formed cells from the subventricular zone migrate to the accessory olfactory bulb where they exert an important function in the acquisition of odorant cues necessary for initiating sexual behavior. Therefore, the short half-life of these cells is necessary to ensure a receptive sexual behavior only after the follicle matures and ovulation occurs. These observations would rule out a role for stem cells in the described neuroprotective actions of estrogens.

III.4.3. Neuronal plasticity and the hypothalamic arcuate nucleus

Estrogens are known to affect the number of synaptic inputs in different neuronal populations of the CNS (Raisman and Field, 1973; Matsumoto et al., 1988; Gould et al., 1990; Arnold, 1992; McEwen and Woolley, 1994; Murphy and Segal, 1996; Silva et al., 2000; Mong et al., 2001). In female rodents, there are well defined neuro-glial plastic changes that are linked to hormonal variations during the ovarian cycle (Woolley et al., 1990; Woolley and McEwen, 1992; Langub et al., 1994). In the ARC Olmos et al., (1989) described a rapid reduction in the number of axo-somatic synapses between proestrus and estrus, which subsequently returned to baseline level at metestrus. A decade ago Parducz et al., (1993) have demonstrated that the E2 induced remodeling of axo-somatic inputs to arcuate neurons is specific, because not all of the synapses are affected. A quantitative postembedding immunocytochemical analysis of the ARC revealed that the administration of a single dose of E2 resulted in a significant and specific decrease in the number of GABA-immunoreactive axo-somatic synapses in ovariectomized (OVX) rats. Glial cells play a major role in the normal neuroendocrine functioning of the ARC. In the intact female rat, the large quantities of E2 secreted from the preovulatory ovary causes synaptic separation (imposition of glial processes between the pre- and postsynaptic elements of ARC axo-somatic and astro-dendritic synapses) followed by synaptic reapplication as the estrogen surge is completed and the glial processes retract. This sequence is required for the midcycle and ovulation. It is well known, that ARC astrocytes are activated on the afternoon of proestrus (Garcia-Segura et al., 1994, 1996), putting out processes that aid the separation of buttons from their targets during synaptic separation and retracting to facilitate synaptic replacement. It was also found that the gonadotropin releasing hormone cells in the rat hypothalamus

are wrapped by astroglial processes during the peak of E2-induced decreased numbers of axo-somatic synapses (Witkin et al., 1992). In hypothalamic slice cultures, it was demonstrated that E2 induces growth and branching of astroglial processes (Garcia-Segura et al., 1989b).

IV. MATERIALS AND METHODS

The experimental procedures used in this study followed the protocol for animal care approved both by the Hungarian Health Committee (1998) and the European Communities Council Directives (86/609/EEC). All efforts were made to minimize the number of animals used.

IV.1. ANIMALS AND PREPARATIONS

Sprague-Dawley rats gave birth in our colony and the pups were randomly distributed among different mothers. The animals were weaned on postnatal day (PND) 22 and were housed in large cages (four to six rats per cage). The animals were kept under 12-h light and 12-h dark conditions, with lights on at 07.00 hours and were raised with access to water and food pellets (Altromin) *ad libitum*. The room temperature was 22±1°C.

All the surgical procedures were carried out under deep anesthesia. During the experiments, the rats were anesthetized with a mixture of Ketavet (10.0 mg/100 g) and Rompun (xilasine, 0.8 mg/100 g). The head of each rat was fixed in a stereotactic headholder (David Kopf) that provided access to the requested brain area. After surgery the animals were allowed to rest for 1 h. The core temperature was maintained at 37 ^{0}C.

Three-month-old anesthetized OVX rats (27 animals) were used in the electrophysiological experiments of the ARC. During the course of the experiments, while the activity of an arcuate neuron was recorded (at least 25±30 min), E2 was injected (100 μg/100 g in sesame oil, i.p.). Five minutes after the injection, the plasma concentration of E2 reached 60±120 pg/ml and at the completion of the experiments the values were between 150 and 250 pg/ml, which is above the physiological levels measured in intact females at the time of the proestrus morning peak. According to our experimental paradigm one arcuate cell was studied per animal.

We used also three-month-old OVX rats (23 animals, 2-5 weeks after ovariectomy) in the cortical microiontophoretic experiments. Craniotomy was performed on the left hemisphere over the posteromedial barrel subfield.

In our PRV-tracing experiments in the ARC, a total of 28 adult rats (22 females and six males) were used. Fourteen of the 22 females were OVX by means of bilateral dorsal incisions. Eight females and six males remained intact. Vaginal smears were taken daily from the control females throughout at least two consecutive 4-day estrus cycles before PRV injection. Fourteen days after OVX, the operated female rats (n = 14) were divided into three groups: (i) those (n = 4) that received a single dose of E2 (100 mg/100 g in sesame oil, i.p.) 4 h before PRV injection (OVX + E 4 h); (ii) those (n = 4) that received a single dose of E2 (100 mg/100 g in sesame oil, i.p.) 12 h before PRV injection (OVX + E 12 h); and (iii), those (n = 6) that received an injection of vehicle alone (OVX). Intact males and females received an injection of vehicle 4 or 12 h before PRV injection.

After the arcuate studies, we started to adopt the viral tracing method on peripheral nerve damage induced cortical changes. In the motor cortical PRV-tracing studies 12 adult male rats were used. In 7 of the 12 animals, the right facial nerve trunk was transected 1 h before PRV injection. The nerve cut was made near the stylomastoid foramen.

To test the influence of the central lesion on peripheral nerve injury induced cortical reorganization, a total of 30 Sprague–Dawley adult rats of either sex were operated. On the left side, above the M1, the skull was thinned with a dental drill from about 2 mm posterior to 5 mm anterior of the bregma, and from 0.5 to 5 mm lateral of the midline. In the course of the operation, we also exposed the right side facial nerve, including its postauricular branch. The cortical photothrombotic (pt) lesion was carried out by i.v. injection of Rose Bengal (1.3 mg/100 g) and cold light exposure, as described by (Buchkremer-Ratzmann and Witte, 1997). There were two main groups of animals: the controls (n=10) and the lesioned animals (n=20). In the ten controls, either illumination was applied (n=4) or Rose Bengal was given without cold light application (n=3). In three animals, neither Rose Bengal nor light exposure was applied. In three of the 20 lesioned rats, only a pt lesion was carried out, while in 17 rats, the pt lesion was combined with the transection of the facial nerve. In four of the 17 animals, the pt lesion was followed by N7x immediately, before the electrophysiological recordings. In 13 animals, the N7x was carried out with a 30-min. delay, during the electrophysiology.

For behavioral experiments with MK-801 we used only male rats. The treated animals (10 pups) received subcutaneous injections of 0.1 mg/kg MK-801 (dizocilpine; Sigma) twice daily, at 09.00 and at 17.00 hours, starting on PND 7 and lasting until PND 19. The controls received an equal volume of saline vehicle (0.9% NaCl in sterile distilled water). The MK-801 treatment resulted in a body growth reduction, which more or less compensated after the treatment was stopped. For the present study, 22 rats were used (MK-801-treated: n=10, controls: n=12). The rats were used for the water maze study between PNDs 90 and 102, and the electrophysiological study followed between PNDs 105 and 135.

After the behavioral experiments, eight of the 12 controls and seven of the ten MK-801-treated animals participated in electrophysiological studies. On both sides, the M1 was exposed by craniotomy from about 2 mm posterior to 5 mm anterior from the bregma, and from 0.5 to 5 mm lateral from the midline. In the course of the operation, we also exposed the right side facial nerve, including its postauricular branch. This was transected later, during the electrophysiological recordings.

IV.2. BEHAVIORAL EXPERIMENTS: WATER MAZE

The rats were trained in a large circular swimming pool (165 cm in diameter, 0.7 m high) filled with water to a depth of 35 cm. The water was at room temperature and was made opaque by the addition of 3 l of milk. The pool was situated in a small rectangular room. The walls were equipped with a variety of spatial cues (a picture or a lamp giving diffused light), that remained unchanged during the experiment. A video camera was mounted above the center of the pool. Animal movements were

monitored, timed, and recorded on videotape. The pool was divided into four quadrants and a removable platform (8 cm in diameter) was hidden at any of four positions in the pool exactly 25 cm from the sidewall. The platform was 1.5 cm below the water surface and not visible to the swimming rat.

IV.2.1. Experiment I (PNDs 90–97)

All animals were trained in five trials in each daily session. The intertrial interval was 30 s for each rat; the platform location was fixed in a particular quadrant. The information about the localization of the platform was available to the rat exclusively from extra-maze cues. Within a session, the rats started each swim from different entry points. Each animal was placed into the pool facing the wall at one of the four entry points and was allowed a period of 60 s to escape to the platform. The escape latencies of each trial were measured. The animals were left on the platform for 20 s and then put into an empty cage for 30 s. If an animal failed to escape onto the platform within 60 s, it was placed there for 20 s, and was awarded a latency of 60 s for that trial. Means of latency of trials were calculated in each session.

IV.2.2. Experiment II (PNDs 101–102)

Following a 3-day interval, cue training was carried out on PNDs 101–102. This experiment was designed to allow an evaluation of the sensory and motoric capabilities. For non-spatial learning, a visible black platform (situated 1.5 cm above the surface of the water) was used as a visual cue. In each trial, the platform was positioned in any of the four possible quadrants in a random sequence. The rats were placed in the water, facing the wall, always at the same entry point.

IV.3. ELECTROPHYSIOLOGY

IV.3.1. Stimulation

- electromechanical vibrissa stimulation (multiangle electromechanical stimulator, ramp-and-hold trapezoids, 1.2 mm vibrissal displacements, 500 ms duration, slope 20 ms)
- electrical stimulation of the vibrissa pad (bipolar needle electrodes,1 Hz, 0.3 ms duration, 150±200 μA, in 9 pt lesioned animals: 0.05 Hz, in case of paired-pulse stimulation protocol: 200 ms interstimulus interval)
- paired-pulse stimulation protocol (two electrical pulses, 200 ms interstimulus interval), the ratio of the amplitudes of the EPs elicited by the second versus the first stimulus was calculated and defined as the Q-value (e.g. $Q=EP_2/EP_1$), $Q<1$ means that the second response was inhibited by the first one.
- subcortical stimulation (bipolar tungsten electrodes, 2 Hz, 0.3 ms duration, 50±100 μA, accessory olfactory bulb (AOB) coordinates: 5 mm anterior to the bregma and 1.4 mm lateral to the midline)

IV.3.2. Recordings

- EP surface recording (two tungsten electrodes, M1 I, M1 C coordinates: 2 mm lateral and rostral from the bregma, averaged amplitudes of 60 trials)
- multi-channel surface recordings (16 channels, 4 × 4 mm matrix tungsten electrodes, averaged amplitudes of 3 or 60 EPs)
- extracellular unit recording (glass micropipettes, 2.5 M NaCl,15± 20 MΩ, 3±5 μm steps, Narishige hydraulic micromanipulator)
- compound recording with multibarrel microelectrodes (seven-barreled borosilicate glass capillary tubing,1.5mm o.d., 1.12mm i.d.; WPI, Sarasota, FL.; center barrel-recording electrode 7μm carbon fiber, low impedance, 0.4-0.8 MΩ at 1 kHz)
- iontophoresis with multibarrel microelectrodes 100 mM sodium L-glutamate (GLUT, Sigma) in 100 mM NaCl (pH 8.0), and 100 mM GABA (Sigma) in 100 mM NaCl (pH 4.0), and 100 mM 17β-estradiol hemisuccinate (Sigma) in 100 mM NaCl (pH 7.2), GLUT was ejected at –50 nA, 30s, E2 at –100 nA, 60s, and GABA at +50 nA, 30s. Retaining currents in the opposite direction were used in the interval 3-10 nA.

The signals were fed into an ExAmp-20KB differential amplifier (Kation Scientific, Minneapolis, MN) with 1 Hz lower and 10 kHz upper frequency limits, and visualized on a Tektronix storage oscilloscope. Amplified responses were fed into a computer via an interface (Digidata 1200, pClamp 6.0.4. software, Axon Instruments) and stored for further processing.

IV.4. HISTOLOGY

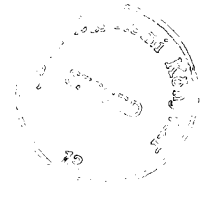
IV.4.1. Tracing experiments with recombinant pseudorabies virus

IV.4.1.1. *Cells and virus*

A porcine kidney cell line, PK-15, was used for the propagation and titration of PRV. Cells were grown in Dulbecco's modified minimum essential medium (DMEM) supplemented with 5% fetal calf serum at 37 °C in a CO_2 incubator. Aliquots of PRV (1000 μl/vial) were stored at -80 °C, and single vials were thawed immediately prior to injection. Ba-DupLac was constructed by the insertion of a pair of beta-galactosidase expression cassettes to a putative latency promoter (antisense promoter) of PRV-Ba, located in the inverted repeat of the virus (Boldogkoi et al., 2000; Boldogkoi et al., 2002).

IV.4.1.2. *Perfusion and immunocytochemistry*

After survival for 72 h, animals were deeply anaesthetized with a mixture of Ketavet/Rompun solution as described above and perfused transcardially with approximately 200 ml of phosphate-buffered saline (PBS, 0.1 mol/L, pH 7.3), followed by approximately 200 ml of Zamboni's fixative. Brains



were postfixed in fresh Zamboni's solution overnight. Coronal sections of the brain (50 μm) were obtained using a vibratome (Campden Instruments) and every fifth section was processed for PRV immunocytochemistry. The sections were blocked in 5% normal goat serum (diluted in PBS) for 1 h, and incubated with a rabbit polyclonal antibody (Rb133; 1 : 10 000, courtesy of Professor L. W. Enquist, Department of Molecular Biology, Princeton University, Princeton, USA) overnight at 4 °C. The sections were then treated with biotinylated anti-rabbit IgG (1 : 200, Vector Laboratories) for 2 h at room temperature. The immunohistochemical reaction was visualized with the avidin-biotin complex + diamino-benzidine technique; sections were mounted on gelatinized slides, dehydrated and coverslipped with Entellan® (Fluka). Only sections containing labeled cells were included in cell counting. As every fifth section was processed for PRV immunohistochemistry, four sections were used in both the ARC and SFO in each animal, and all the positive neurons were counted in these structures.

IV.4.1.3. *Injection of the virus*

The head of each rat was fixed in a stereotactic headholder (David Kopf), onto which a special Hamilton syringe (tip outer diameter 200 μm) was mounted. Fourteen days after OVX, the animals were reanesthetized. The PRV was injected with special care; the inoculations were carried out by the same person at the following coordinates: frontal, -2.5 mm (to the bregma); lateral, 0.2 mm; vertical, 9.0 mm from the cortical surface (ARC), or following coordinates: frontal: +2.0 mm to the bregma, lateral 2.0 mm, vertical 800 μm from the cortical surface (in case of M1, Paxinos & Watson, 1998). The injection of 0.1 μl PRV was achieved over 5 min by pressure (PRV concentration 10^9 pfu/ml; vehicle, DMEM + 5% fetal calf serum). After completion of the injection, the pipette remained in the tissue for an additional 5 min in order to prevent any backflow of the PRV or its spread into the surrounding areas. After the PRV injection, the incision on the head was closed and each animal was housed individually in a plastic isolation cage. The necessary precautions relating to the handling of PRV were taken throughout the experiment. All materials that came into contact with PRV during surgery were cleaned in 95% ethanol and Softa-Man solution and disposed of as biohazardous materials.

IV.4.2. Histological verification of the electrophysiology

The brains were removed and site of the recording electrode was verified in cresyl violet-stained Vibratome sections. Routine light microscopic analysis was carried out also on animals surviving for 2 h or 2 days following the pt lesion, and on the control rats. The brains were removed from the skull, postfixed in the same fixative and cryoprotected in 30% sucrose. Frozen 40 μm thick sections were cut serially in the coronal plane and processed according to the Gallyas reduced silver method for degenerating neurons (Gallyas et al., 1980).

IV.5. STATISTICAL ANALYSIS

We used parametric unpaired two-tailed t-test, one-way analysis of variances (ANOVA), repeated measures ANOVA (between-subject and within-subject effect) and in one case it was needed to use nonparametric Mann-Whitney U-test. On the basis of the estimated marginal means of ANOVA, we adopted pair-wise comparisons with Bonferroni's correction. Statistical analysis was performed with the aid of the SPSS 11.0 for Windows program. The results are expressed as means ± standard deviation (S.D.) or means ± standard error of the mean (S.E.M.). A P value less than 0.05 were regarded as significant.

V. RESULTS

V.1. USE OF PSEUDORABIES VIRUS TO ANALYZE MOTOR CORTICAL REORGANIZATION AFTER UNILATERAL FACIAL DENERVATION

V.1.1. Facial denervation influences the transcallosal spread of pseudorabies virus in a time-dependent manner

To determine whether the peripheral injury of the nervous system has a detectable effect on the tract tracing via PRV, the right facial nerve was cut or the animals were sham-operated before administration of the virus suspension. Synaptic reorganization can reveal cell surface proteins or can induce protein-protein interactions, which can modulate the entry or transmission of viral particles. We set out to determine the time course of the possible reorganization, so we applied different postinjury/preadministration times. The results obtained with PRV are shown in ***Figure 5***.

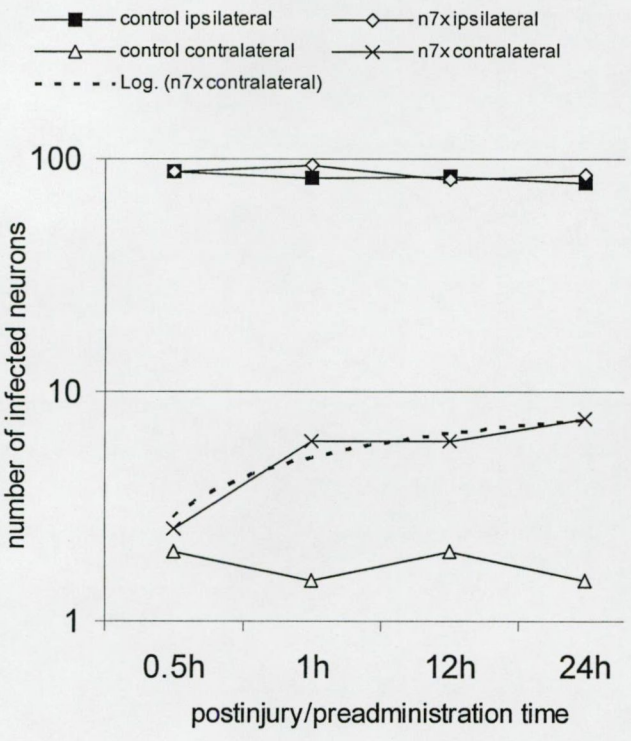


Figure 5 The number of infected neurons increased logarithmic (log) with postinjury/preadministration time only on the contralateral side of n7x animals. The other three curves are linear and parallel with the x axis.

The duration of the postinjury/preadministration time did not have a significant effect in either group on the inoculation side. On the contralateral side of the injured animals, the number of infected neurons increased in time dependent manner. It reached a plateau at around 1 h postinjury/preadministration. In the sham-operated animals, there was no significant effect of the resting time. It is likely that the changes in the M1 affect the neuronal transmission of PRV within a short period, i.e. in 2 h.

V.1.2. Analyzing the motor cortical reorganization after unilateral facial denervation with pseudorabies virus

V.1.2.1. *PRV immunoreactivity is not different on the inoculation side in controls and N7x animals*

To examine the effects of N7x on the entry and/or cell to cell spread of PRV, we compared the number and distribution of infected cells on the inoculation side of the cerebral cortex. Primarily infected neurons were found around the penetration channel in both the controls and the N7x animals *(see **Figures 6A** and **C**)*. In all cases, the primarily infected neurons were located discretely in the third lamina of the M1. The highest number of labeled neurons was found close around the injection channel, and the number decreased with increasing distance from it. These results are consistent with the diffusion of virus-containing solution around the cannula. There was no significant difference between the control and N7x groups in the number of labeled neurons (83.0 ± 1.2 vs. 86.2 ± 1.6; P= 0.119), or in the infection patterns on the injected side *(see **Figures 7A** and **C**)*. These results indicate that the entry of PRV into the motor cortical neurons is not dependent on facial denervation.

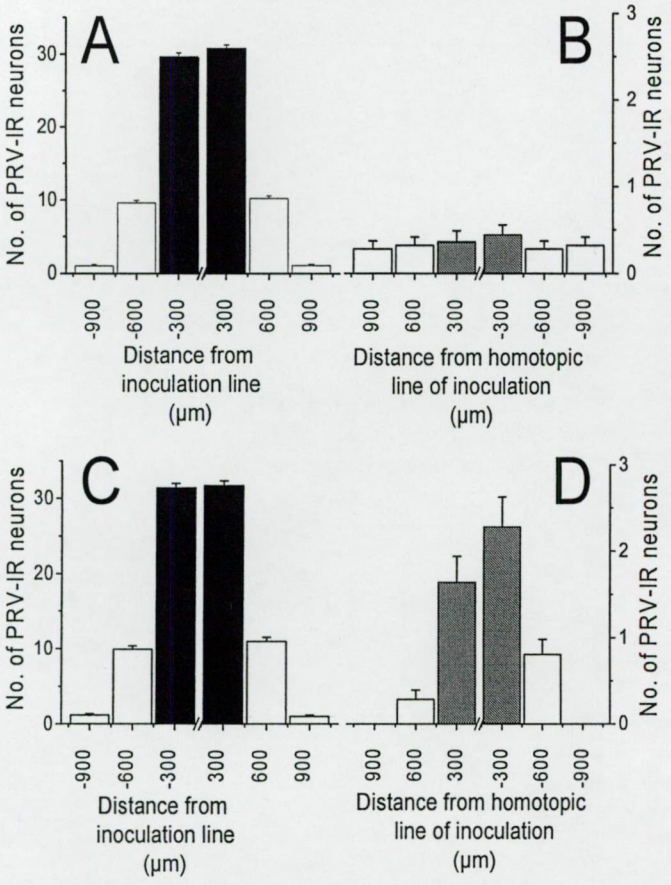


Figure 6 Schematic diagrams of the distribution of labeled cells in 2 slides of controls **A** and **B** and in 2 slides of n7x animals **C** and **D**. The average number of labeled cells per slide is given in these diagrams. The averages of the cell numbers were calculated from the corresponding data on 5 controls and 5 n7x animals, i.e. from 5 x 5 slides. The motor cortices are divided into 300-μm-wide areas. The black areas denote the 300-μm-wide cortical areas in the close medial and lateral environment of the injection channel in the left hemispheres (A and C). The gray areas are homotopic to them in the right hemispheres (B and D). Values are means and standard deviations for PRV-IR neurons (n=25).

V.1.2.2. *PRV immunoreactivity differs in the contralateral M1 in controls and N7X animals*

To test whether N7x affects the cell-to-cell spread of PRV, we compared the number and distribution of infected cells on the cortical side contralateral to the inoculation. The secondarily infected cells displayed cytoplasmic staining *(as shown in **Figure 7B** inset)* and, by 72 h following inoculation, various intensities of PRV-IR could be seen in these neurons. In the control animals, there were significantly fewer labeled neurons in the contralateral (right side) cerebral cortex than in the N7x animals (2.0 ± 0.2 *vs.* 5.0 ± 0.8, P=0.001; *see **Figures 6B** vs. **6D** and **7B** vs. **7D***).

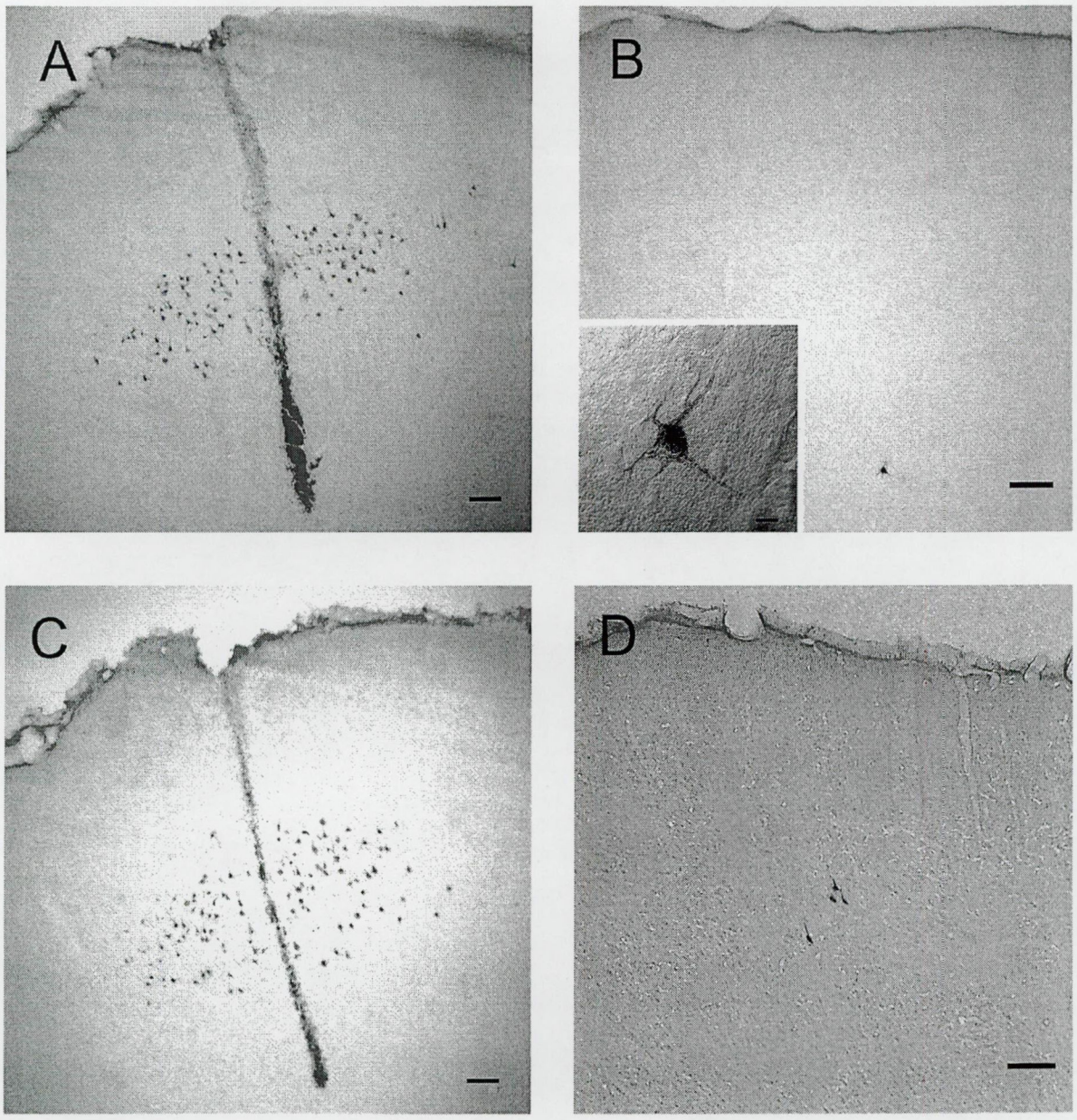


Figure 7 Labeled neurons in the left **A** and right **B** primary motor cortices (M1s) of a control animal, and in the left **C** and right **D** M1s of a rat in which the right facial nerve was transected 1 h before the PRV infection. Several PRVinfected neurons are localized around the injection channels (A, C). We could not usually observe labeling in the contralateral M1 of the controls (B), with some exceptions, where a few labeled cells were detected. Inset in B: higher magnification of the one labeled pyramidal neuron found in this control animal. (A and B are corresponding slides.) (D) PRV-IR neurons in the homotopic area of the right hemisphere of an N7x animal, after facial denervation. Calibration: 100 μm in A-D. In the inset in B, the bar is 10 μm.

In the control animals, the distributions of the secondarily infected neurons were equal in the divided cortical areas *(see **Figure 6B**)*. On the basis of the estimated marginal means of ANOVA, we adopted pair-wise comparisons of the areas with Bonferroni's correction. There was no significant difference between the divided areas in the number of labeled neurons.

However, in all the N7x animals several transcallosally labeled neurons were found in the contralateral cerebral cortex *(see **Figure 7D**)*. These neurons were located close to the homotopic line of the injection channel *(see **Figure 6D**)*. These PRV-infected cells displayed strong cytoplasmic staining. After unilateral N7x, the number of transcallosally labeled neurons was significantly higher than in the controls (5.0 ± 0.8 vs. 2.0 ± 0.2, $p=0.001$; *compare **Figures 6B** and **D***). N7x not only increased the number of transcallosally labeled neurons, but also affected their distribution. ANOVA indicated a significant three-way interaction between the facial nerve status, the cortical side and the cortical territory ($F_{5,40}=15.64$, $P<0.0001$), i.e. the mean of the PRV-IR cell number is dependent on the cortical side, the cortical area and the unilateral N7x.

V.1.2.3. *The primer motor cortex is homogenous for viral spread in both mediolateral and orocaudal directions*

To confirm that this special infection pattern exists not only in two dimensions, one animal randomly selected from each group was treated and processed as described previously and serial sections of the brains were made. The surface diagrams *(compare **Figure 8A** vs. **C** and **8B** vs. **D**)* reveal the predicted cone-like shape on the ipsilateral side both groups and also on the contralateral side in the N7x group.

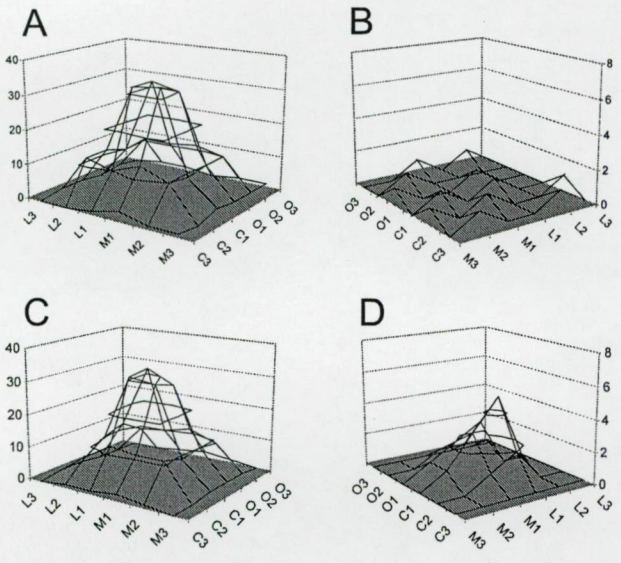


Figure 8 Surface diagrams for a sham-operated **A, B** and N7x animal **C, D**. The diagrams exhibit a cone-like shape, which demonstrates the distribution of PRV-IR neurons on the inoculation side (A, C). In contrast, the diagram of the contralateral cortex is virtually planar (B), while in the right hemisphere of the N7x animal there is an impressive peak in the surface diagram at the homotopic point of the injection channel (D). It should be mentioned that the distance of sampling was in 300 μm in the mediolateral direction, but 50 μm in the rostrocaudal direction in these animals.

On the contralateral side of the sham-operated animal, the diagram is rather uniform *(see **Figure 7B**)*. A possible explanation is the diffusion of the viral suspension on the inoculation side: the farther from the injection channel the lower the probability of infected neurons. This means that at least this part of the cerebral cortex is homogenous for viral infection. On the contralateral side of the control animal, the distribution of infected neurons was uniform, and the infection point received afferentation from all parts of the contralateral side. After N7x, this afferentation was more focused, and the surface diagram exhibited the same shape as was observed on the inoculation side *(see **Figure 7D**)*.

These results indicate that the transcallosal cell-cell spread of PRV within the M1s of both hemispheres is basically influenced on N7x.

After the anatomical follow-up of the peripheral nerve injury induced intercortical reorganization of the M1s, we try to characterize pharmacologically some of the involved neural pathways.

V.2. THE EFFECTS OF MK-801 TREATMENT ON BODY WEIGHT, MEMORY AND MOTOR CORTICAL ELECTROPHYSIOLOGY

V.2.1. Animals

Although it was not the purpose of this experiment to make a detailed behavioral study on the effects of early treatment with MK-801, from the appearance of the adult animals it was obvious that the neonatal MK-801 treatment resulted in long-term effects. The body weight of the drug-treated animals on PND 90 was somewhat, but not significantly decreased: MK-801-treated group: 293.5 ± 15.6 g, control group: 301.2 ± 9.7 g (unpaired two-tailed t-test, P=0.1718; t=1.4). The behavior of the MK-801-treated rats, however, was obviously different from that of the controls: they walked back and forth in the cage almost continuously with poor postural support.

V.2.2. Water maze performance

V.2.2.1. *Experiment I*

Even from the beginning, the performance during the eight training sessions revealed that the MK-801-treated animals needed somewhat more time to reach the platform. This tendency remained more or less unchanged during the eight sessions, though the difference in performance of the two groups was not significant in any one of the sessions *(see **Figure 9**)*.

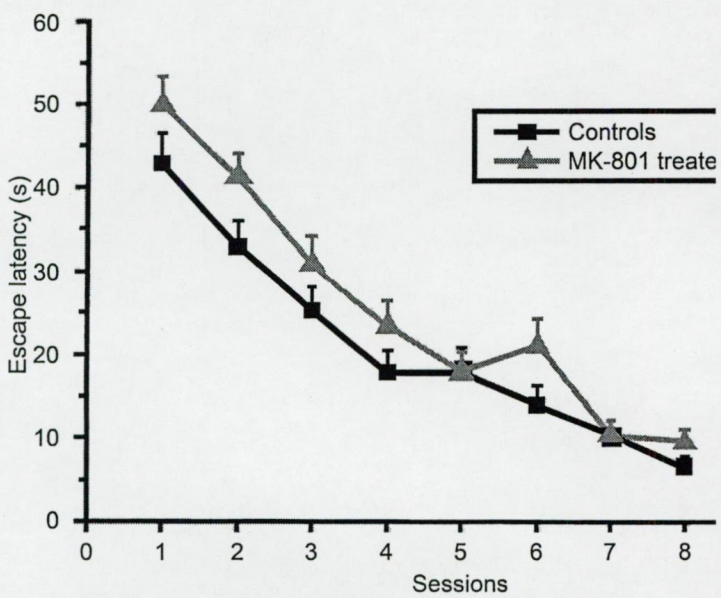


Figure 9 Escape latencies (in seconds) of adult controls and MK-801- treated rats. Times (means±SE) needed to find the hidden platform during the eight sessions in experiment I. The performance of the MK-801-treated rats (0.1 mg/kg, twice daily, postnatal days 7–19) was slightly poorer than that of the saline-treated controls.

The Repeated Measures ANOVA showed no significant difference between the groups (saline versus MK-801 treatment): $F_{1,20}$=0.9740; P=0.336, but the time as a within-subject factor was significant: $F_{7,140}$=52.811, P<0.001. We did not

find a grouptime interaction: $F_{7,140}=0.611$, P=0.746. It was interesting to observe that, in the first trial in the first session, the two groups needed approximately the same time to reach the platform. Later, the difference between the performances became larger, but did not attain the level of significance. The swimming strategies of the treated and control animals were not the same: the treated rats spent more time swimming round the pool, by the wall. The difference in performance, however, was not due to a difference in anxiety or swimming speed between the two groups. Taken together, therefore, the MK-801-treated rats were capable of learning in the course of the sessions, but their performance tended to be somewhat poorer than that of the controls.

V.2.2.2. *Experiment II*

To check the possibility of modification of the sensory and motor capabilities by MK-801 treatment, a visual cue task was given to the animals in both groups on PNDs 101 and 102. The visual task was performed equally well both by the control and the MK-801-treated animals *(see **Figure 10**)*.

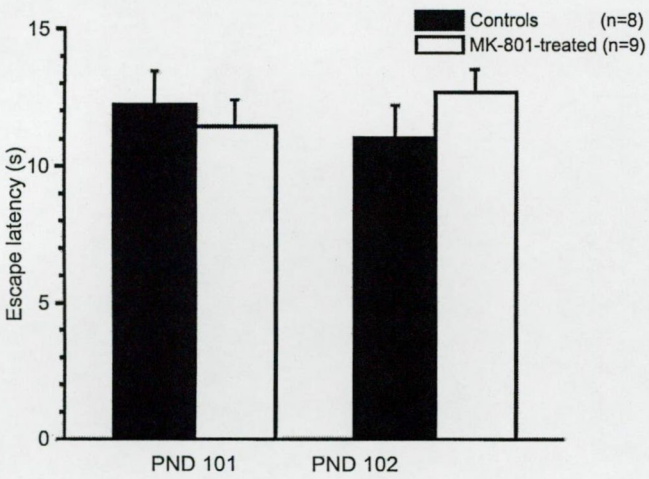


Figure 10 Escape latencies for the two sessions of the visual cue task in experiment II. The MK-801-treated rats and the controls performed equally well in this task.

Though the two-way ANOVA resulted in a significant interaction: $F_{1,15}=8.52$ P=0.022, the minimal difference (1.2 s) between the two groups on PND 102 has no meaning from a biological point of view. On the basis of the water maze analysis, it can be stated in general that the performances of the MK-801- treated animals in the spatial learning and memory task were slightly (but not significantly) poorer than that of the controls. However, the visual task was performed equally well both by the control and the treated rats.

V.2.3. Electrophysiology

Electrophysiological recordings followed the behavioral experiments. In addition to the barrel field of the contralateral somatosensory cortex (S1 C), responses to trigeminal stimulation were also observed in the M1 C. In anesthetized adult rats, both mechanical stimulation of the vibrissae and electrical stimulation of the whisker pad activate trigeminal afferents, which in turn produce complex response patterns in a subpopulation of neurons in the M1 (Farkas et al., 1999). It has been shown that a facial nerve cut induces disinhibition in an extended area of the M1 C (Farkas et al., 2000), resulting in the facilitation of evoked responses (Toldi et al., 1999). In the control rats, as described above, somatosensory evoked potentials (EPs) in the contralateral M1 C were rapidly modified by facial

nerve transection. After 1 h, the amplitude of these potentials was significantly enhanced and the latencies of all the components had shortened. Responses with enhanced amplitudes could be observed throughout the 3- to 4-h recording session *(see **Figure 11A1**)*. The same was true for the M1 I (ipsilateral to the stimulation): in the control rats, stimulation of the trigeminal nerve or parts of it induced EPs in the S1 C and M1 C, but very small if any potentials in the M1 I (*see **Figure 11B1**)*. However, a few minutes after the N7x, EPs could also be elicited with enhanced amplitude in the M1 I. Their amplitude increased considerably within 1 h and remained high until the end of the experiments (3–4 h after denervation, *as in **Figure 11B2***).

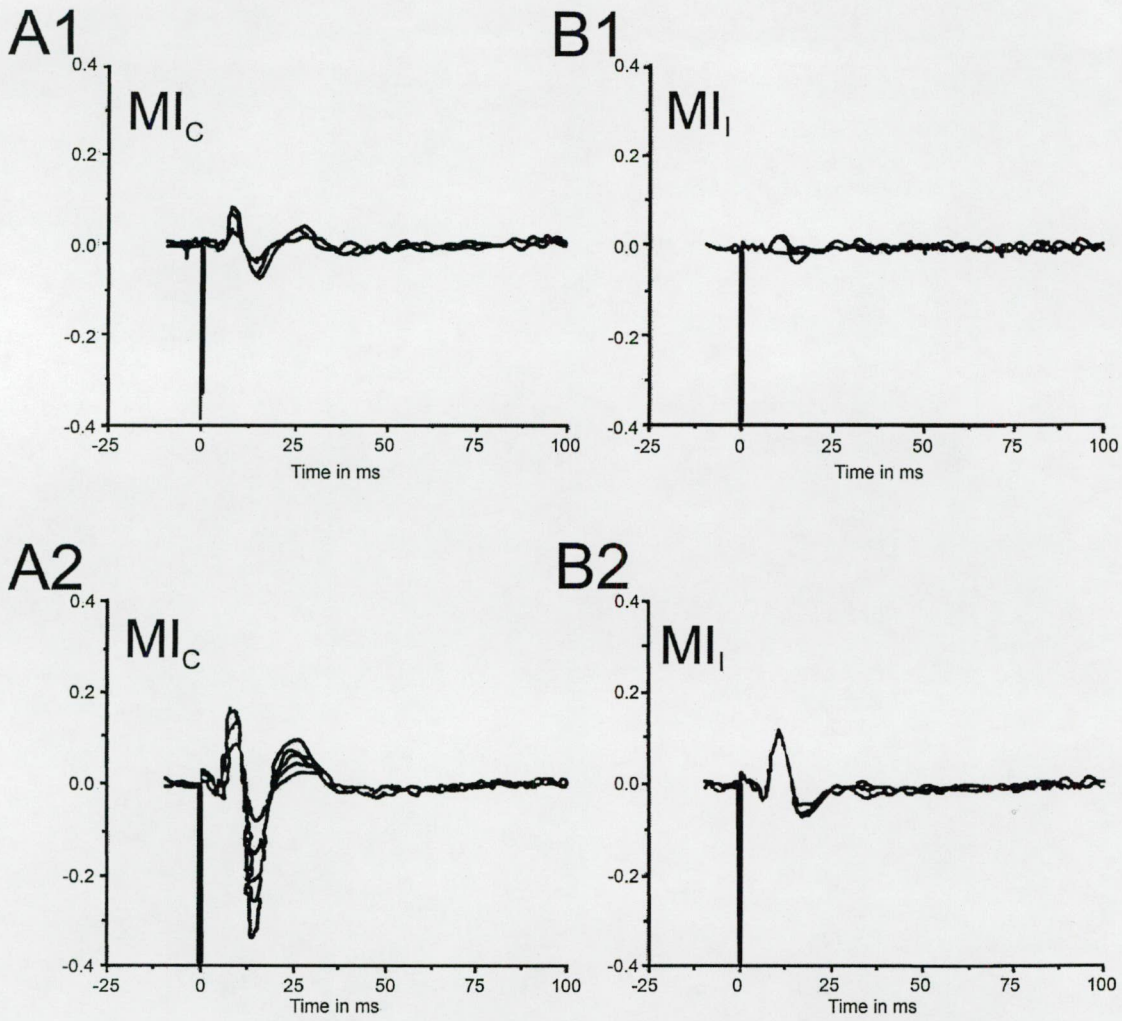


Figure 11 Responses evoked in the MIs in both hemispheres of a control rat by right side electrical vibrissa pad stimulation. Evoked potentials (*EPs*) in the contralateral primary motor cortex (*M1C*) recorded before **A1**, and 3 h after a facial nerve cut **A2**. EPs in the right side primary motor cortex ipsilateral to the stimulation (*M1I*) before **B1**, and 3 h after vibrissa pad stimulation **B2**. *Ordinates* are in mV/div. Five (of 60) successive potentials were averaged in each set.

After the facial nerve transection, the EPs in the M1s were facilitated to different degrees in all of the control animals. This was not the case in the rats treated with MK-801 as young animals. In all of these rats, trigeminal stimulation induced EPs with high amplitude in the M1 C, and with small amplitude if any in the M1 I *(see **Figure 12A1 and B1**)*. This was similar to what was observed in the control rats. The facial nerve transection, however, did not facilitate the evoked responses in the M1 in either hemisphere. In the majority of cases (60%), there was no change in amplitude or latency of the EPs following N7x *(see **Figure 12A2 and B2**)*. In fact, in 40% of the cases studied, the amplitudes of the EPs decreased or vanished from both M1s.

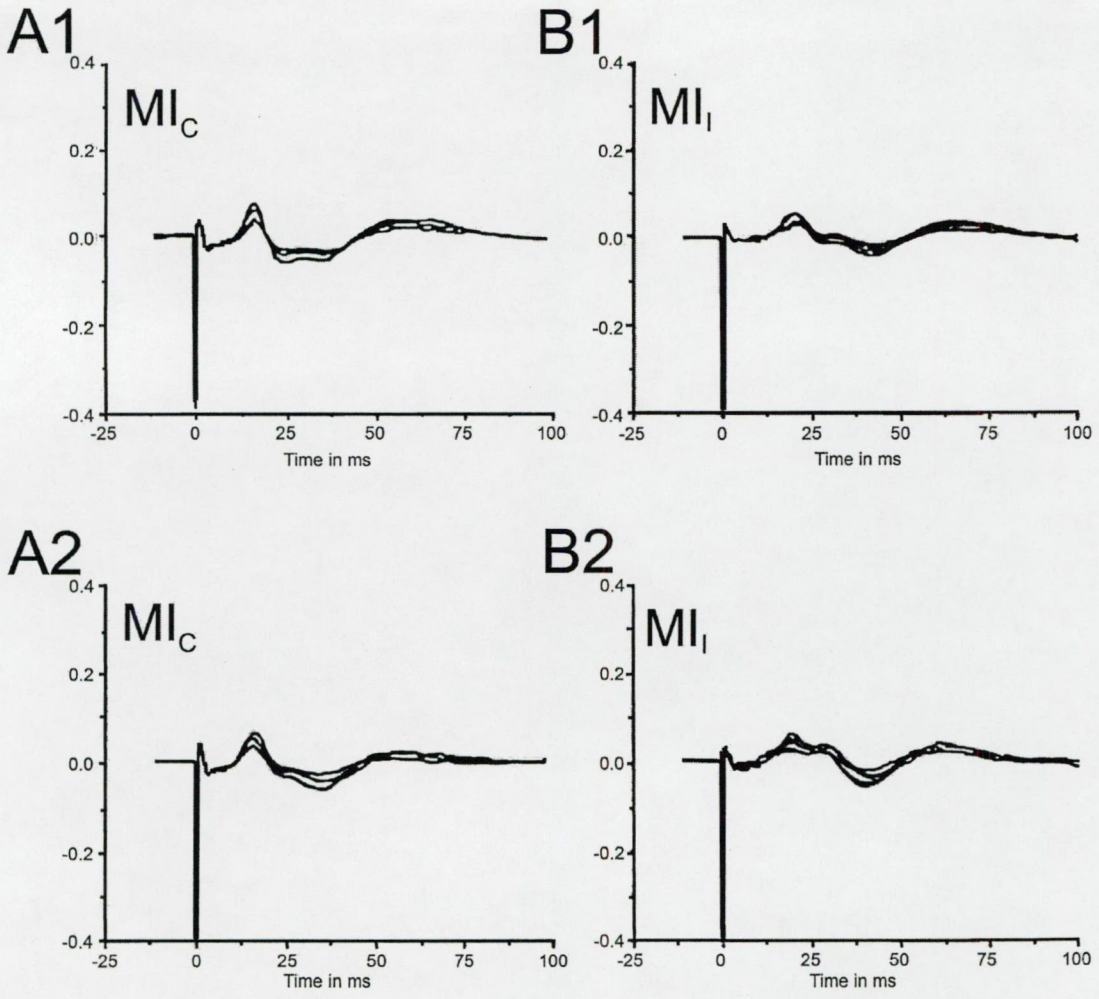


Figure 12 Responses evoked in the MIs in both hemispheres in an MK-801-treated rat by electrical vibrissa pad stimulation on the right side. Evoked potentials (*EPs*) in the contralateral primary motor cortex (*M1C*) before **A1**, and 3 h after a facial nerve cut **A2**. EPs in the primary motor cortex ipsilateral to the stimulation (*M1I*) before **B1**, and 3 h after the facial nerve cut **B2**. The EPs were not changed after the facial nerve transection. *Ordinates* are in mV/div. Five (of 60) successive potentials were averaged in each set

The Q-values were calculated in both the controls and the MK-801-treated animals. In all the controls, the studies with a paired-pulse paradigm revealed disinhibition (Q-values >1), which lasted in some cases for a short time *(see **Figure 13A**)*, but in most cases for longer periods (Farkas et al. 2000) following N7x. However, there was hardly any increase in Q-values after the N7x in the MK-801-treated animals *(see **Figure 13B**)*.

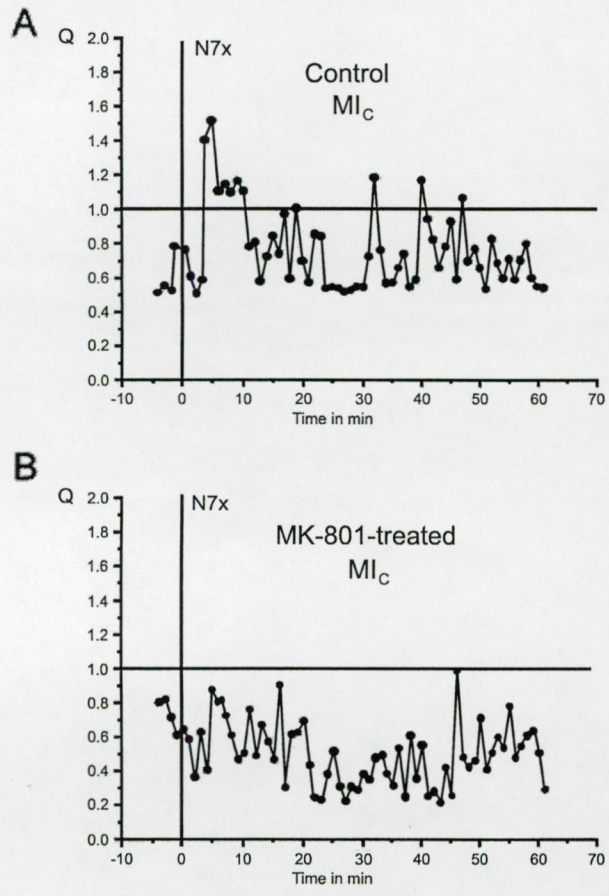


Figure 13 Q-values calculated from the paired-pulse paradigm in the contralateral MI of a control and of an MK-801-treated animal, displayed as a function of time. **A** In the control animal, facial nerve transection produced a short (~4 min) and, with some delay, a longer-lasting (~35–40 min) elevation of Q. **B** In the MK-801- treated animal, transection of the facial nerve did not cause cortical disinhibition, e.g. the Q-values remained <1

In the controls, the N7x induces a considerable disinhibition (elevation in Q-values) shortly after (5–10 min) denervation, this disinhibition lasting for a long time (40–45 min). This is not the case in the MK-801-treated animals: after N7x, moderately increased Q-values were observed in most animals (with Q<1 in all cases), while even decreases in Q-values could be detected in some animals. The Repeated Measures ANOVA revealed a significant grouptime interaction: $F_{2,26}$=8.819, P=0.001. This is an indication that the Q-values in the controls are changed significantly after N7x, but not those in the MK-801-treated animals. Minimal differences in the latencies of the EPs were observed in the controls and in the treated animals. The treated animals had EPs with somewhat longer latencies *(see **Figure 14**)*.

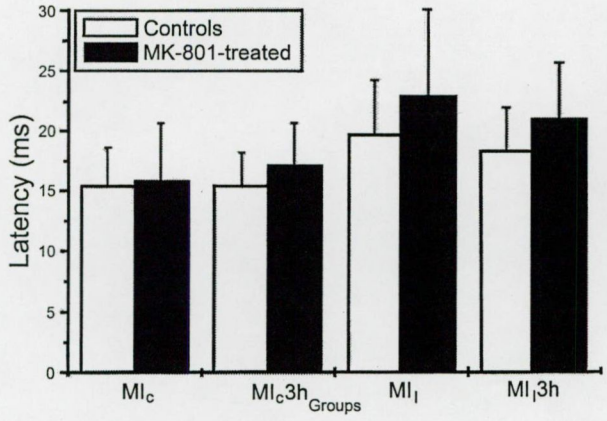


Figure 14 Mean latencies of the evoked potentials (*EPs*) (first positive peak) in the different animal groups. The mean latencies of the treated animals were somewhat longer than those for the controls, though these differences were not significant (independent *t*-test, *P*>0.05). *Bars* represent means±SD. *M1C* and *M1I* mean latencies of EPs in the primary motor cortex contralateral and ipsilateral, respectively, to the stimulation and nerve injury. *M1C3h* and *M1I3h* mean the latencies of the evoked responses 3 h after facial nerve transection

In the control animals, the mean latencies of the EPs recorded from the M1 C before and 3 h after the facial nerve cut were 15.36 ± 3.142 ms and 15.382 ± 2.774 ms, while in the M1 I they were 19.533 ± 4.669 ms and 18.210 ± 3.664 ms. In the treated animals, the mean latencies of the EPs recorded from the M1 C before and 3 h after the facial nerve cut were 15.770 ± 4.845 ms and 16.949 ± 3.658 ms, while in the M1 I they were 22.708 ± 7.384 ms and 20.816 ± 4.714 ms. For further analysis of the amplitudes and latencies of EPs recorded in the contralateral M1s of five controls and five MK-801-treated animals before and 3 h after N7x, a repeated measures ANOVA was carried out. A significant interaction was found for the amplitudes: $F_{1,8}$=5.701, P=0.044, but not for the latencies.

Intercortical disinhibition can be modified not only by pharmacological agents but also by interaction of different pathological effects. In the next set of our experiments we show an example for that.

V.3. ELECTROPHYSIOLOGY OF THE CORTICAL PHOTOTHROMBOTIC LESIONED ANIMALS

The present study focused on the early electrophysiological events that took place in the cortex during the first 2 h after ischemic lesion and the possible modificating effect of N7x on it.

The control animals showed no evidence of pathological changes in the brain tissue. No one animal from the three different control groups exhibited any histological alteration. In the pt-lesioned animals, after 2 h, the ischemia had caused well-observable damage 800–1000 μm in diameter in the brain tissue, which was typical after 2 h of survival *(see **Figure 15B**)*. The tissue of the contralateral hemisphere was never involved; normal-appearing cells and pale neurons were observed. The recorded 4 × 4 mm area in the M1 is shown in ***Figure 15A***. Right-side whisker pad stimulation (at either 0.05 or 1 Hz) evoked responses in the entire left M1, with amplitudes of 200–500 μV. The punctum maximum of the EPs in the M1 on contralateral whisker pad stimulation was localized 2–2.5 mm rostral to the bregma and 2.0–2.5 mm lateral to the midline. In the controls, and in all the treated animals before the intervention, a double pulse stimulation study [see details in (Farkas

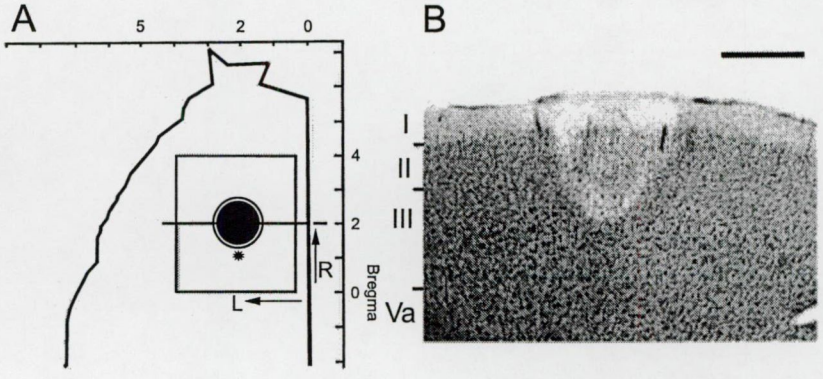


Figure 15 Exact localization of lesions and the site of electrophysiological recordings in the frontal cortex of the rat. **A** The photothrombotic lesion was produced at the following coordinates: frontal, 2–3 mm; lateral, 2 mm. Transcranial electrophysiological recordings were carried out at 16 points within the matrix shown in the figure. The filled circle represents the centre of the core, which was surrounded by the penumbra (open circle). The sizes of both (core and penumbra) changed with time. The histologically detectable penumbra developed with a delay of a few days. **B** Brain coronal section made as indicated with a horizontal line in A. The photomicrograph shows a damaged cortex region as early as 2 h after the beginning of the photothrombotic lesion. The section was processed according to the Gallyas method. L, lateral; R, rostral. Calibration: 500 μm.

et al., 2000)] indicated a rather strong paired-pulse inhibition, with Q values of 0.3–0.5 *(see **Figure 16A** and inset in **Figure 17**)*.

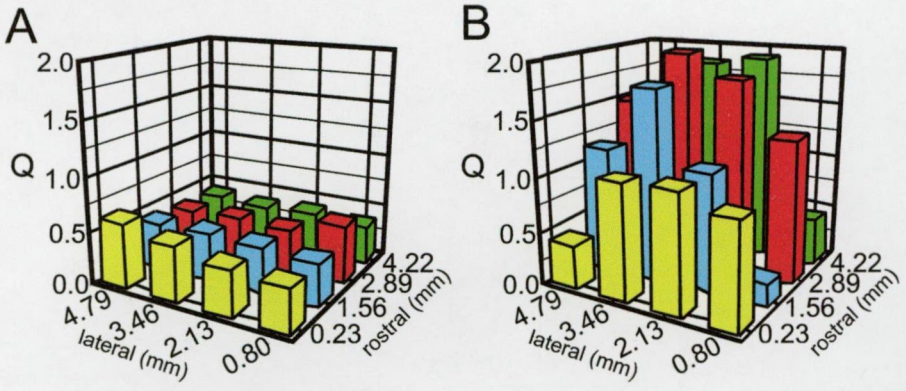


Figure 16. Q values calculated from paired-pulse stimuli-evoked averaged potentials recorded at 16 points in the frontal cortex of an animal. **A** Q values before pt lesion. **B** Q values calculated from potentials recorded 40 min after the pt lesion.

A few minutes after the pt lesion, the amplitudes of the EPs were dramatically reduced. Although the amplitudes of the EPs in the M1 were reduced 5–10 min after the pt lesion, the Q values calculated on the basis of the double pulse stimulation protocol suggested an increased level of disinhibition throughout the entire cortical area. After the pt lesion, the elevated Q values varied in time, but they were always around or above 1 *(see **Figure 16B** and the first 25 min in **Figure 17**)*, and persisted at this high level during the studied period of 2–2.5 h. Similar results were observed in all animals, independently of the stimulation frequency applied. Interesting results were obtained in previously pt-lesioned animals, in which N7x was carried out with a 30 min delay. In these animals, the Q values were observed before and after N7x, as detailed earlier. However, a "general" level of inhibition-excitation was expressed by the Q_a value, which represents the actual inhibition-excitation level in the entire 4 × 4 mm cortical area during the respective 5 min period. In these animals, the Q_a values were around 1 because of the previous pt lesion; these values were further increased by N7x *(see **Figure 17**)*. The time-dependent pattern of the changes in Q_a is similar to that of the changes induced by pure N7x (Farkas et al., 2000), except for the much higher Q_a values. Similar time-dependent patterns of the changes in Q_a were found in all 13 animals stimulated at either 0.05 or 1 Hz.

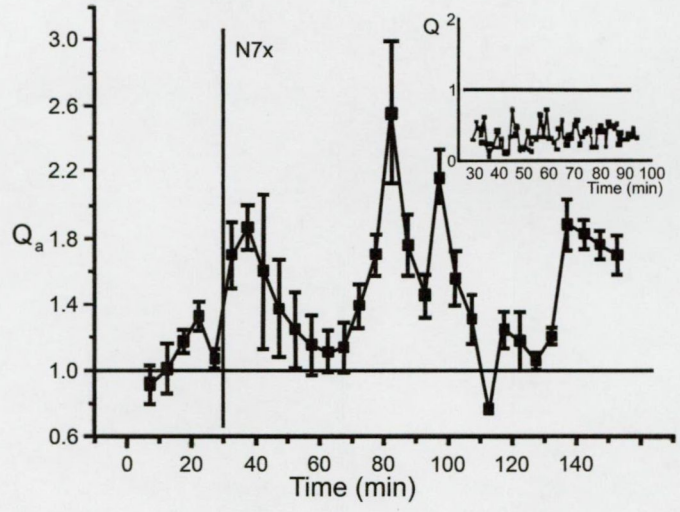


Figure 17. An example of the change in the 'averaged' Q values (Qa) in time. These Qas represent the means of Q values (with SD) calculated from the paired responses observed at 16 points of the recorded area of an animal which underwent the photothrombotic lesion previously, before the electrophysiological recording [see the high level of Qas (around 1) at the beginning, due to the photothrombotic lesion]. After facial nerve transection (vertical line with N7x), the Qa values further increased. Inset: diagram of the ratio (Q) of the paired response amplitudes evoked by right whisker pad stimulation in a control animal, in relation to time (min). It should be noted that the Q values in the primary motor cortex of a control rat were always below 1 (around 0.3–0.5).

V.4. TRACING EXPERIMENTS OF THE ARCUATE NEURONS WITH RECOMBINANT PSEUDORABIES VIRUS

V.4.1. Pseudorabies virus immunoreactivity in intact females and males

In our next experiment, we tested our PRV-based neuronal tracing method in the other well-defined plasticity model, in the hypothalamic arcuate nucleus model.

Efforts were made to determine the exact phase of the estrous cycle in females. As we could not precisely determine this, we used a larger number of intact females for inoculation with PRV. In these animals, primary infected neurons were found in the ARC, around the end of the penetration channel. In all the evaluated intact animals, PRV-IR neurons were also seen in the SFO *(see **Figures 18A-D**)*.

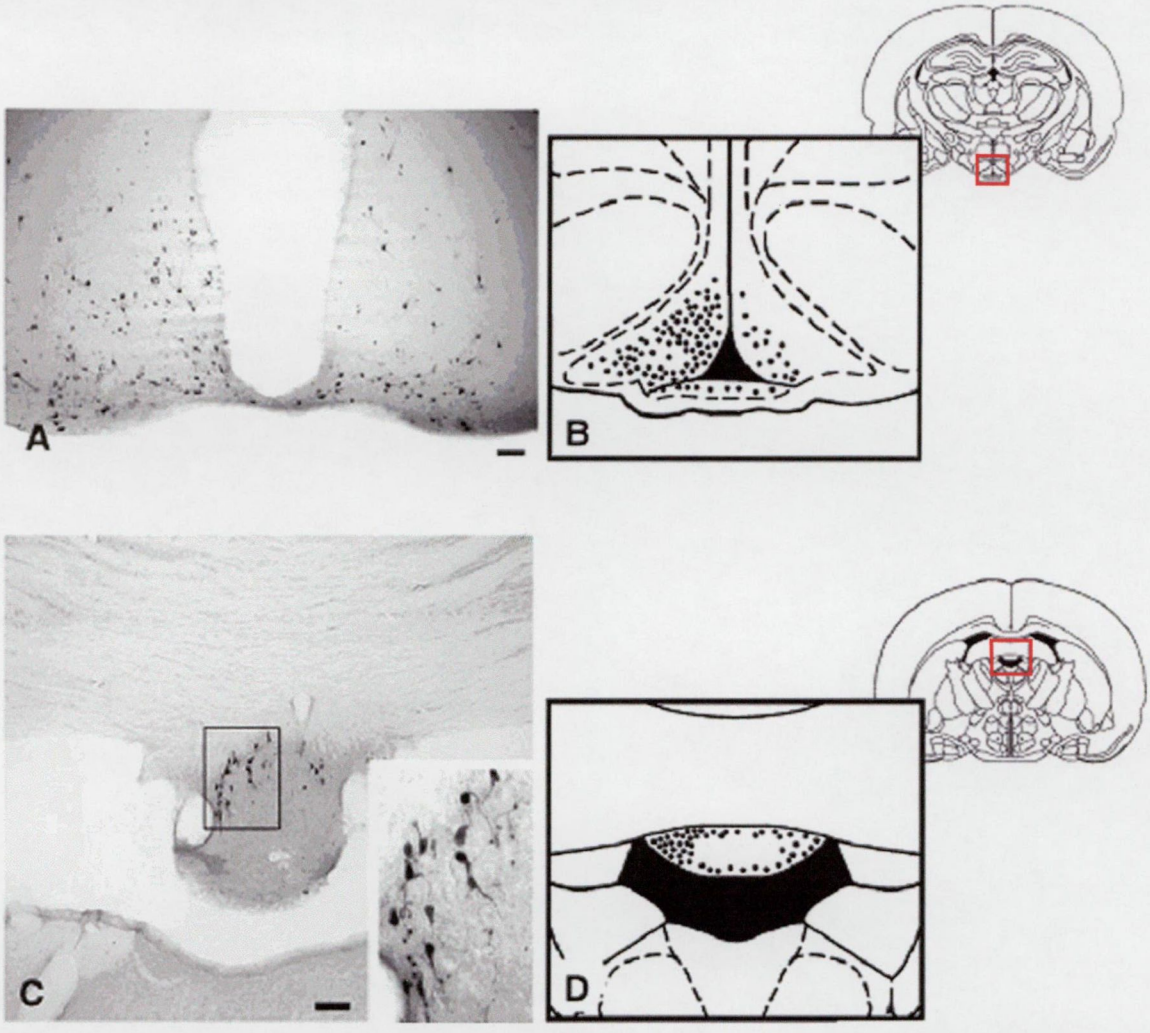


Figure 18 Primarily infected neurons in the arcuate nucleus (ARC) 72 h after the injection of PRV into the nucleus in a control animal. **A** Labelled neurons in the medial posterior part of the arcuate hypothalamic nucleus (ARC), at low magnification. Note the specificity of the PRV injection and the higher number of labelled neurons on the injected side (left). **B** Number and localization of immunoreactive neurons in the ARC are indicated, using a drawing modified after Paxinos & Watson (1998). Each dot represents one neuron; the total number of neurons labelled in this Figure indicates the total number of labelled neurons in one animal. The boxed area in the schematic diagram (top right-hand corner of panel B) shows the enlarged area in B. **C** and **D** Secondary infected neurons in the SFO following the injection of PRV into the ARC of an intact animal. The boxed area in C is shown at higher magnification in the bottom right-hand corner of panel C. The boxed area in the schematic diagram (top right-hand corner of panel D) shows the enlarged area in D, in which the number and localization of the immunoreactive neurons in the SFO of one animal are indicated, using a drawing modified after Paxinos & Watson (1998). Note the higher number of labelled neurons on the injected side (left). Scale bar, 100 μm.

The specificity of PRV injection is shown by the lateralization; a higher number of labeled neurons were found on the injected (left) side (males 66.0 ± 4.1; females 74.5 ± 7.7 labeled neurons) than on the contralateral side (males 34.8 ± 5.2; females 39.5 ± 3.5; *see* ***Figures 18A and B***). In the ARC, no significant differences were found between male and female control animals. PRV-IR neurons were also found in the SFO of both males and females. Significant differences were observed between females and males in the number of infected SFO neurons (males 4.4 ± 0.3; females 6.9 ± 2.4 on the injected side). Labeled neurons were rarely found in other hypothalamic structures, and were therefore not studied systematically elsewhere.

V.4.2. Pseudorabies immunoreactivity in ovariectomized female animals

In the OVX animals treated with vehicle, PRV-IR neurons were found only in the ARC, as a result of the primary infection *(similar to* ***Figures 18A and B****)*, but not in the SFO. These primary infected cells displayed cytoplasmic staining and, by 72 h following infection, various intensities of PRV-IR could be seen in these neurons. The number of labeled neurons in the ARC of the OVX animals did not change significantly as compared to the intact females *(see* ***Figure 19****)*.

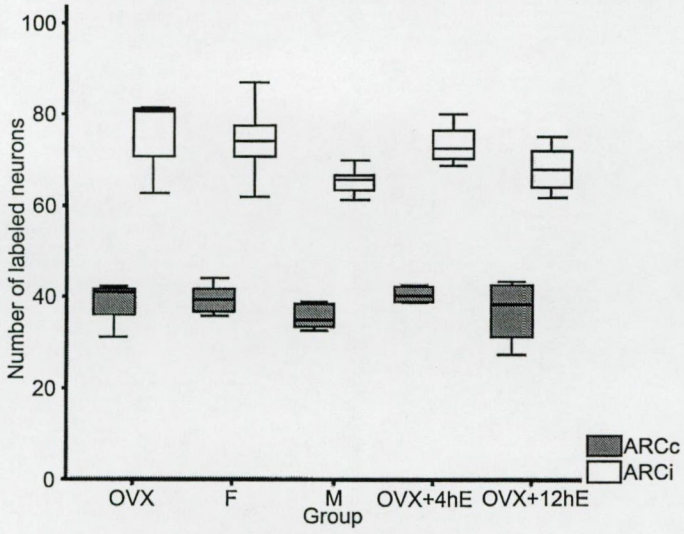


Figure 19. The diagram shows the number of labeled neurons in the ARC of the different animal groups. OVX, ovariectomized animals; F, females; M, males; OVX + E 4 h, 17β-E2 treatment 4 h before virus injection; OVX + E 12 h, 17β-E2 treatment 12 h before virus injection; ARCi, arcuate nucleus ipsilateral to the inoculation; ARCc, arcuate nucleus contralateral to the inoculation. Median values are presented by transverse lines. Boxes relate to a probability of 95%. There is no significant difference in the number of labeled neurons between the groups, neither ipsilateral nor contralateral to the inoculation.

Careful observation of sections derived from the brains of the OVX rats revealed no sign of PRV-IR in the SFO *(see* ***Figure 20A,*** *next page)*, but PRV-IR neurons appeared in the pyriform cortex in all of these animals *(see* ***Figure 20C,*** *next page)*. It was interesting that PRV-IR was observed in the pyriform cortex in the OVX rats, whereas staining was not seen at all in the SFO of these animals. However, in the controls and the OVX + E 12 h animals, which exhibited stained neurons in the SFO *(see details below)*, PRV-IR was found in the pyriform cortex in only a single neuron of one animal *(not shown)*.

V.4.3. Pseudorabies immunoreactivity in 17-β-estradiol-treated ovariectomized rats

In these animals, primarily infected neurons were found in the immediate vicinity of the penetration channel in the ARC, as described above. In the SFO of the OVX + E animals, however, PRV-IR neurons were observed in only one of the two groups. The animals which were treated with E2 4 h before inoculation (OVX + E 4 h group) did not exhibit PRV-positive neurons in their SFO *(similarly as shown in **Figure 20A**)*. However, all the animals in the OVX + E 12 h group contained PRV-IR in the neurons in the SFO *(see **Figure 20B**)*. These PRV-infected cells displayed strong cytoplasmic staining *(as in **Figure 18C, inset**)*. The lateralization in labeling could also be seen in the SFO, i.e. there was either only staining ipsilateral to the inoculation *(see **Figure 20B**)* or a higher number of labeled cells were found on the ipsilateral side: 3.1 ± 0.6 on the injected side and 1.2 ± 0.5 on the control side *(as in **Figures 18C and D**)*. The statistical analysis did not reveal a significant difference in the ARC between the groups, either ipsilateral to the inoculation ($F_{4,20}$ = 1.267, P = 0.316) or contralateral to it ($F_{4,20}$ = 2.383, P = 0.086, ***see Figure 19***).

In the SFO, however, the groups differed significantly both ipsilateral to the inoculation ($F_{2,14}$ = 8.07, P = 0.005) and contralateral to it ($F_{2,14}$ = 5.1, P = 0.0022). Bonferroni's post hoc test showed that there was not a significant difference in the number of labeled cells in the SFO between the OVX + E 12 h animals and the intact males. The numbers of labeled neurons in these groups, however, were much lower than in the intact females. The numbers of labeled neurons both ipsilateral and contralateral to the inoculation in

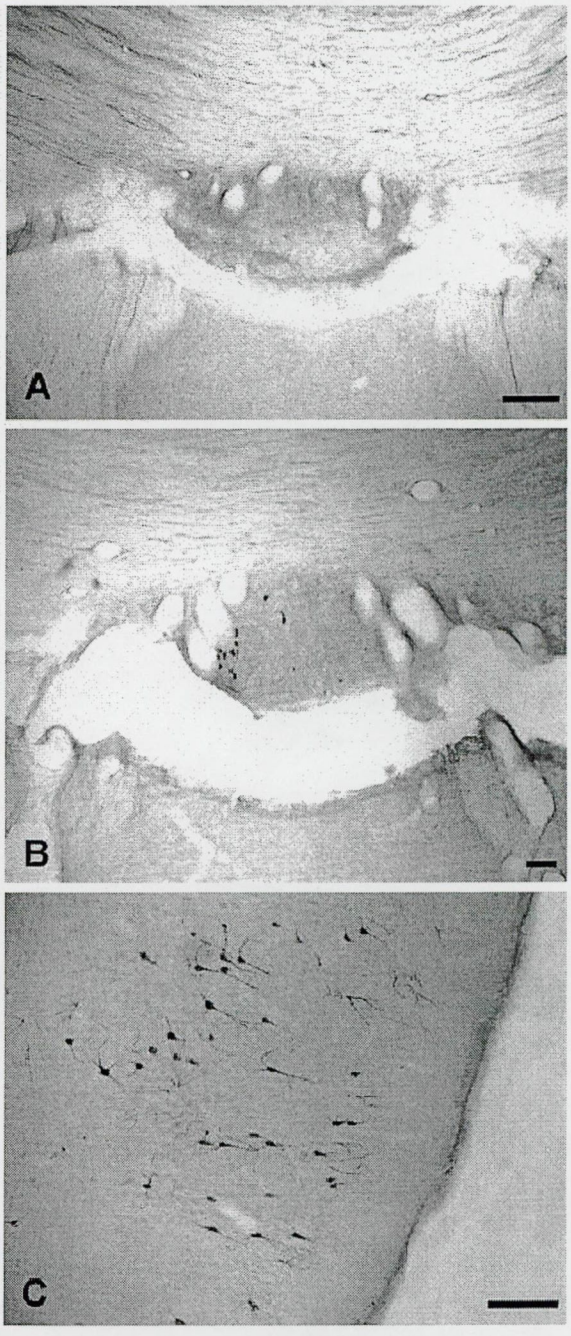


Figure 20 **A** Lack of secondarily infected PRV-immunoreactive neurons in the SFO of an ovariectomized (OVX) animal. Note the complete lack of staining 72 h after PRV inoculation. **B** Immunoreactive neurons in the SFO of an OVX animal which received E2 12 h before virus infection (OVX + E 12 h). Note the presence of PRV-immunoreactive cells in the SFO. **C** PRV-immunoreactive neurons in the pyriform cortex of an OVX animal. Scale bars, 100 µm.

the groups of females differ significantly from those found on the respective sides of the other groups *(see **Figure 21**)*.

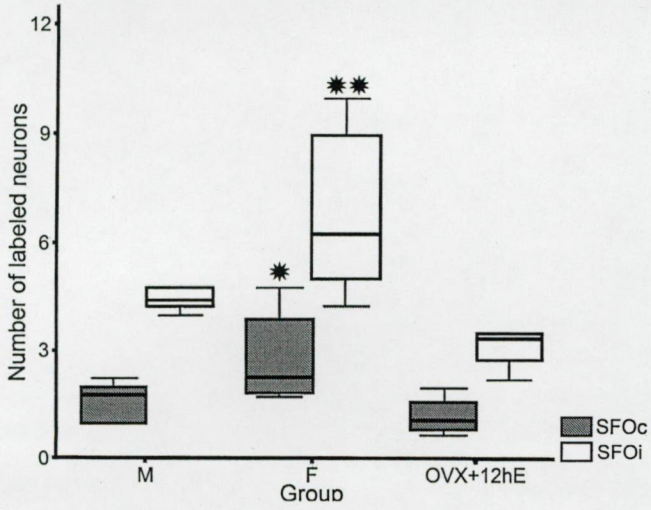


Figure 21 The number of labeled neurons in the SFO of the different groups. SFOi, subfornical organ ipsilateral to the inoculation; SFOc, subfornical organ contralateral to the inoculation. All labels are as in Fig. 19. The numbers of labeled neurons both ipsilateral (**) and contralateral (*) to the inoculation in the groups of females differ significantly from those found on the respective sides of the other groups. Note the high variability in the number of labeled neurons in the group of females. The reason for this is probably that the animals were in different phase of the etrus cycle.

The plastic anatomic changes in the ARC are quite well known but the *in situ* electrophysiological properties remain still to be elucidated. In the next experiments we try to correlate our electrophysiological recordings to the anatomical findings.

V.5. ELECTROPHYSIOLOGY OF THE ARCUATE NUCLEUS

The *in situ* study of the electrophysiological activity of the arcuate neurons is not an easy task in the mouse (Olmos et al., 1989) and it is even more difficult in the rat: this is one of the possible reasons why *in vitro* recording is frequently used in such studies (Nishihara and Kimura, 1989; Lagrange et al., 1994). Considering the complex synaptic organization of the hypothalamus and the arcuate area, this technique has serious limitations, because the slice preparation lacks functionally important input and output connections. The only way to study the effect of certain sensory inputs and systemically administered E2 on the activity of arcuate neurons is *in situ* recording; therefore we used this technique. In the course of our experiments, we measured the activity of more than 30 units, and analyzed the data when the unit activity could be followed for >25 - 30 min the effect of E2 on the activity of the arcuate neurons: in all those cases when unit activities could be followed for a sufficient time, spontaneous activity of the neurons increased after 20±25 min of E2 application. At the 25th minute the difference was highly significant: the frequency was increased from 5.5 ± 1.8 spikes/s (mean ±S.D.) to 20.2 ± 2 spikes/s *(see **Figure 22**)*.

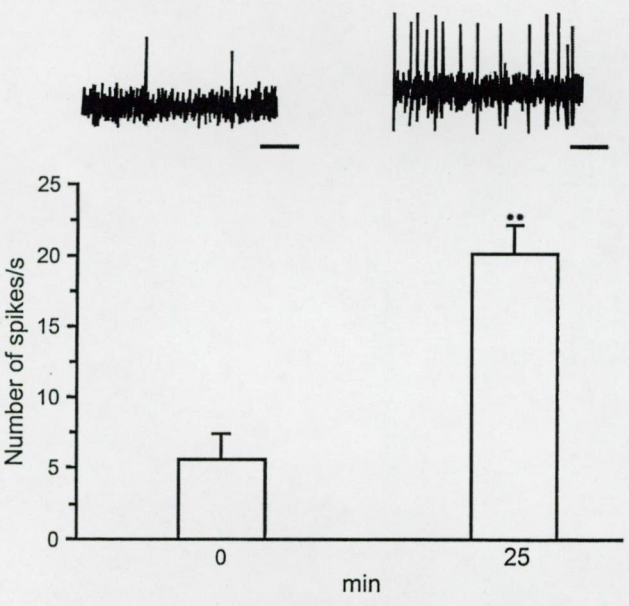


Figure 22 Spontaneous activity of arcuate neurons before (0 min) and after (25 min) E2 injection. Inset: Activity of an arcuate neuron before and 25 min after E2 treatment. Bar=100 ms. ** p.<0.01.

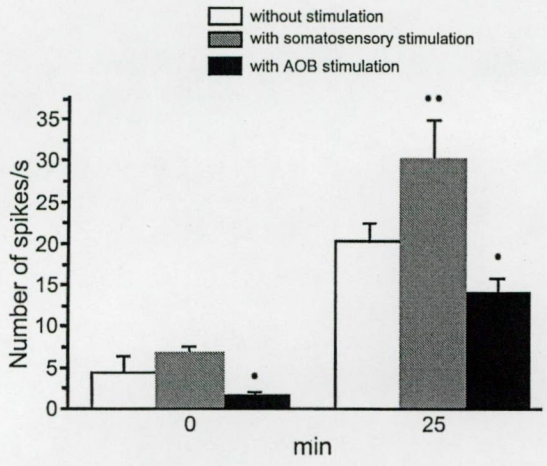


Figure 23 The modulatory effect of vibrissa pad (gray columns) and accessory olfactory bulb (black columns) stimulation on the spontaneous activity of arcuate neurons before (0 min) and after (25 min) E2 injection (i.p.). * $p<0.05$, ** $p<0.01$.

In the same experiments we tested the effect of different modulatory components on the activity of arcuate neurons. Four of the nine neurons responded to whisker pad stimulation, exhibiting enhanced activity on somatosensory inputs *(see **Figure 23**)*. The increased firing was observed both before and after E2 application (7 ± 0.8 spikes/s and 30.3 ± 4.7 spikes/s, respectively). AOB stimulation, however, which was tested on the same cells, resulted in an opposite effect, with decreased activity in both the absence and presence of E2. In control circumstances the firing frequency changed from 4.6 ± 1.8 to 1.9 ± 0.3 spikes/s, while after E2 treatment the values were 20.2 ± 2.3 and 14.1 ± 1.9 spikes/s: the differences in both cases were significant *(see **Figure 23**)*.

The above demonstrated set of experiments had led us to test the effect of estrogen in the most plastic field of the rat somatosensory cortex, namely the barrel cortex.

V.6. ELECTROPHYSIOLOGICAL STUDY OF THE BARREL CORTEX: THE EFFECT OF 17β-ESTRADIOL

Unit responses evoked at a depth of 800-1200 μm in the barrel cortex by vibrissal deflections were similar to those already described: the cells within a barrel responded best to one vibrissa, the principal vibrissa, but could commonly be driven less effectively and with a longer latency by some surrounding vibrissae (Armstrong-James et al., 1992; Kis et al., 1999b). First of all, the effectiveness of the iontophoresis was controlled by GABA and GLUT application to those neurons which seemed to be recordable reliably and for long enough. In the course of the experiments, we tested the activity of >70 neurons, but analyzed only those data when the unit activity could be followed for > 45-50 min. The *in situ* study of the electrophysiological activity of barrel cortex neurons combined with drug application by iontophoresis can be difficult task especially when a substance (such as E2) is applied which influences the neuronal activity with a long delay. We had to hold the cells for > 45-50 min. This is the reason why we fully analyzed only 14 neurons, though we started the analysis of > 70 neurons.

V.6.1. Effects of γ-amino-butyric-acid and glutamate on the activity of barrel cortex neurons

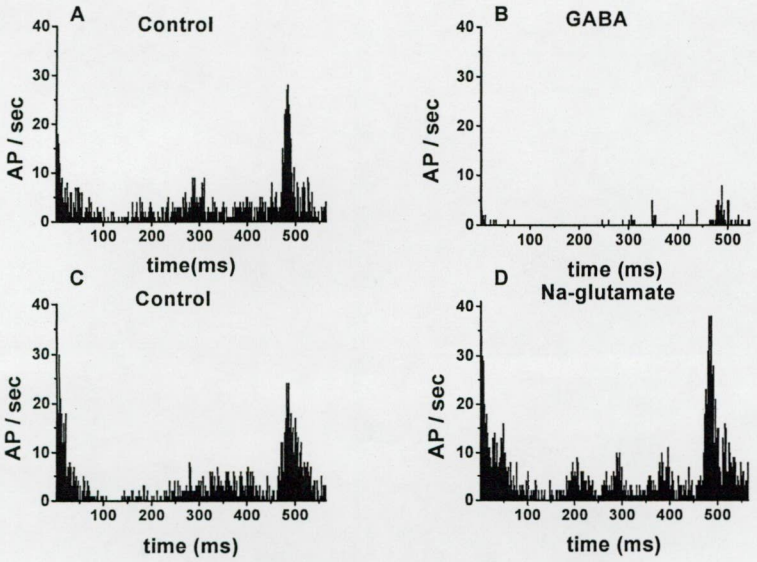


Figure 24 Peristimulus time histograms of evoked activities observed in the barrel cortex. On-off neuronal responses were evoked by contralateral vibrissal deflection before **A** and 30 s after iontophoretic application of GABA **B**. On-off responses before **C** and 30 s after Na-glutamate iontophoresis **D**.

Iontophoretic application of GABA (+50nA, 30s) resulted in immediate decreases in both spontaneous discharges (to 6-10%) and evoked unit activity *(compare **Figures 24A** and **B**)*. In contrast, the application of GLUT (-50nA, 30s) increased both the spontaneous and the evoked neuronal discharges *(compare **Figure 24C** and **D**)*. The spontaneous activity increased by 350-400% within 30s after GLUT application.

V.6.2. Effect of 17β-estradiol on the activity of barrel cortex neurons

In all those cases when unit activities could be followed for a sufficient time, the spontaneous activity of the neurons increased after E2 iontophoresis (-100nA, 60s) in five of 14 neurons. By 25-35 min after iontophoresis, in four of five responding cells, the E2-evoked excitation was increased by 208-360% over the control level. (One responding cell had an extremely long latency: 38 min, *see later*). Similarly, both on and off components of the responses evoked by vibrissa deflection were facilitated (compare **Figures 25A** and **B**).

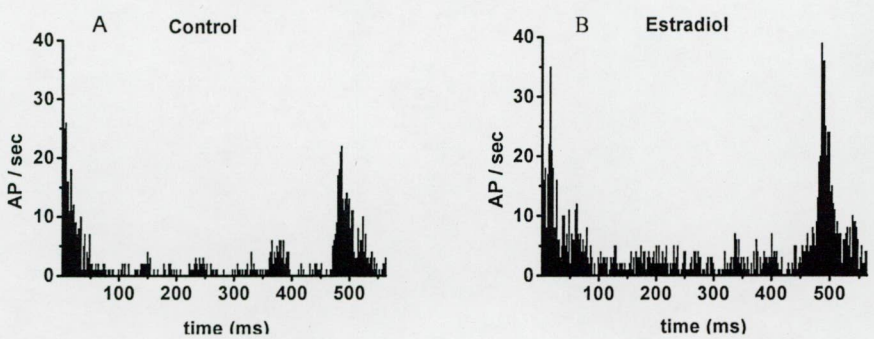


Figure 25. Peristimulus time histograms of a neuron in the barrel cortex, observed before **A**, and 38 min later after iontophoretic application of E2-hemisuccinate **B**.

The spontaneous activity increased by 214% and the evoked on and off responses by 262% and 274%, respectively. All changes were highly significant. Similar results were observed for each of five neurons, which responded to E2. It was important to determine the delay of the response to E2. As detailed above, the firing rate was regarded as the onset of the response to E2. The delays of the five responses were as follows: 17 min, 18 min, 38 min, 24 min and 23 min. On average, the delay of the responses to E2 was 24 min. As opposed to the effect of GABA or GLUT, the E2-induced increase in neuronal activity was prolonged, and lasted until we lost the cell. Unfortunately, this means that we cannot judge how long the responses lasted. We can establish only that the responses were elevated for the time of recording (for ≥ 15-25 min after the onset). The rates of responses were not uniformly high; they peaked and then attenuated but remained above the control level during the time of registration. The remainder of the fully tested neurons did not respond to E2.

VI. DISCUSSION

In our experiments, peripheral and central injury induced plastic changes were studied in subcortical structures and in the cerebral cortex. With the long-range aim of using steroids as neuroprotective agents, we examined the estrogen actions on neural activity and plasticity. The topic mentioned above is very complex, so we adopted complex, morphological, electrophysiological and behavioral experiments to gain further insight into the physiological significance of it. If we survey our results in details the following observations can be established:

During the past decade, it turned out that the transynaptic tracing with an attenuated PRV vaccine strain called Bartha is a suitable method to analyze the function of neuronal connections within the CNS (Bartha, 1961; Sams et al., 1995; Enquist, 1999). Our first question was, whether this method is sensitive enough to detect fine plastic changes in the CNS induced e.g. with N7x.

Our tracing study has demonstrated that N7x influences the transcallosal spread of PRV from the affected side M1 to the contralateral M1 in rats. In the controls, PRV injection primarily infected several neurons around the penetration channel, but hardly any secondarily infected neurons were found in the contralateral M1. These neurons were distributed almost equally from medial to lateral in the cerebral cortex. In contrast, after right N7x, PRV was transported from the primarily infected neurons in the left M1 to the contralateral side, and resulted in the labeling of several neurons due to a secondary infection. These secondarily labeled neurons were concentrated near the homotopic line on the injection side. The number of infected neurons reaches a plateau 2 h postinjury/preadministration.

These results, which are supported by statistical analysis, suggest that **N7x not only facilitated, but also concentrated the transcallosal spread of PRV from the left M1 to the contralateral side.** Unilateral N7x did not affect the entry of PRV into the primary neurons. This finding lends support to the conclusion that the extent of neuronal PRV infection is not only under the control of immunity, but also depends on the actual state of the nervous system.

To explain this result, it should be taken into account that the entry of alphaherpesviruses into the cells usually requires multiple interactions between the viral envelope and the cell surface proteins. At least two groups [heparan sulphate proteoglycans (HSPGs) and nectins] of these cell surface (glyco-) proteins are known to play roles in these processes (Mettenleiter, 2000; Spear et al., 2000). It should also be taken into account that HSPGs and nectins participate in the development and plasticity in adulthood of tissues of neuroepithelial origin (Carey, 1997; Rauvala and Peng, 1997; Suzuki et al., 2000; Mizoguchi et al., 2002).

We have shown here that N7x does not affect the entry of PRV, but increases the efficiency of its cell-to-cell spread. Thus, the N7x-dependent infection pattern appears to be related not to cellular components (HSPGs) involved in the attachment of the virus, but rather to cellular components located in the synaptic region of the membrane of presynaptic neurons.

Nectin-1 and nectin-2, components of a novel cell-cell adhesion system, and localized within the cadherin-catenin system at cell-cell adherens junction, have been shown to play important roles in synapse formation (Mandai et al., 1997; Takahashi et al., 1999; Miyahara et al., 2000; Mizoguchi et al., 2002). Moreover, recently published results suggest that these members of the immunoglobulin superfamily may serve as entry-receptors for herpes simplex viruses (HSVs) and for PRV. In epithelial cells, dissociation of the cell junctions releases nectin-1 to serve more efficiently as an entry-receptor (Yoon and Spear, 2002). In cultured rat and mouse sensory neurons, nectin-1 is the primary receptor for HSV-1 infection, and soluble, truncated nectin-1 prevented viral entry (Richart et al., 2003). Our results show that unilateral N7x does not affect the entry, but increases the transcallosal spread of PRV. Sakisaka et al. (2001) have reported that the interaction of nectin-1α with afadin does not affect the entry of HSV-1, but increases the efficiency of the cell-cell spread of that virus. The mechanism by which N7x increases the efficiency of the cell-cell spread of PRV in the cortical network *in vivo* remains to be elucidated, but one possible explanation is the denser and more focused localization of nectins and afadin at newly formed synapses.

Whatever the underlying mechanism is, **the peripheral nerve injury-induced changes in the Ba-DupLac infection pattern seem to be a suitable model for the study of injury-induced neuronal plasticity.** Thus, we have confirmed the utility of this genetically modified virus in studies of synaptic plasticity. The mechanisms by which other entry-receptors for PRV and/or HSV may modulate nectin-based synapse formation are not known, but our ongoing experiments lead us to predict their important roles.

The mechanism of plasticity probably differs depending on the time frame, but due to its kinetics, PRV tracing is only able to follow up plastic changes in a specific time-window. For this reason, electrophysiological and behavioral studies were also performed for further insight into this complex phenomenon.

From a functional point of view it is a crucial question to determine if injury induced synaptic remodeling is linked to developmental modifications also in the electrical activity of affected cortical pathways.

The effects of chronic neonatal MK-801 treatment on the spatial and non-spatial learning of adult rats have been extensively studied (McLamb et al., 1990; Gorter and de Bruin, 1992; Mickley et al., 1992; Tandon et al., 1996; Griesbach and Amsel, 1998). Recent studies support the hypothesis that MK-801 mainly influences learning and memory in a NMDA receptor-dependent manner (Bordi et al., 1996; Ahlander et al., 1999; Norris and Foster, 1999). Some authors have reported that a chronic NMDA receptor blockade during the neonatal period leads to long-lasting disturbances of the hippocampal functions, i.e. to the impairment of various learning and memory tasks (Gorter and de Bruin, 1992); in contrast, others did not find long-term effects on behavior (Tandon et al., 1996). Apart from differences in the amount of MK-801 applied, there were differences in the application methods, testing paradigms and results, and mainly in their interpretation. Despite these differences, most of the

authors agreed that, because of its crucial role in development, the perinatal blockade of NMDA receptors might have behavioral consequences when tests are made at a more advanced age. This view is particularly supported by those observations, which reveal that NMDA antagonists may increase neurodestruction in mature brain undergoing slowly progressing neurodegeneration (Ikonomidou et al., 1999; Pohl et al., 1999; Takadera et al., 1999). The neurotoxic effect of MK-801 is even more serious in the perinatal age: the blockade of NMDA receptors for only a few hours during late fetal or early neonatal life triggers widespread apoptotic neurodegeneration in the developing rat brain (Ikonomidou et al., 1999). We did not set out to perform another detailed behavioral study. Rather, we wanted to find a way to cause a minimal impairment in behavior, which was based on learning and memory functions without interfering with physical capabilities, and to test whether this influences experimentally induced cortical plasticity that is detectable by electrophysiological methods. First of all, we had to find a dose of MK-801 that resulted in a slight impairment of the performance in the water maze task.

We started the treatment with relatively high doses of MK-801 (0.5 or 1 mg/kg), but this led to impaired food consumption, a high loss in body weight, akinesia, and a high level of mortality among the young animals (only 67% and 51% of the treated animals survived PND 15, respectively). These results are not detailed here. Finally, we turned to the treatment of new-born rats with a dose of 0.1 mg/kg MK-801, which resulted in a very slight impairment of the performance in the water maze task and produced hyperactivity, but did not impair the performance of the visual cue response of the adult animals. In experiment I, the performance in the water maze was quite similar to that observed by Gorter and de Bruin (1992), though those authors used higher doses of MK-801, which resulted in greater differences between the performances of the treated and control groups, whereas we aimed for the slightest impairment in performance which could still be detected. As a result of the neonatal treatment with a low dose of MK-801, the spatial learning of the adults was slightly reduced, but the difference was not significant. The controls and the treated rats performed the visual task (experiment II) equally well. This suggests that the sensory and motor capabilities were not affected by the treatment. A deficit was found only in the spatial task performance of the adults. Gorter and de Bruin (1992) drew similar conclusions.

It has been shown that a component of the field potentials evoked in the M1 of rat, and its potentiation, are mediated by NMDA receptors (Hess et al., 1994; Hess and Donoghue, 1996). Simple recordings of EPs in the M1 cortices did not reveal significant differences between the controls and the MK-801-treated animals. Therefore, we tested a more complex phenomenon. It was interesting to test whether perinatal treatment with MK-801 interferes with spatial learning and the potentiation of cortical responses in adult rats. The plasticity of evoked responses induced by N7x can be tested by the paired pulse paradigm.

In the control animals, the responses evoked in the M1s of both hemispheres by continuous 1 Hz trigeminal stimulation were facilitated after facial nerve transection. This was not the case with the

MK-801-treated animals. In a majority of the cases studied, the evoked responses did not change, while in 40% of the cases, continuous stimulation reduced the evoked responses recordable after facial nerve transection. Under these conditions, an *in vitro* study proved the occurrence of LTP in intrinsic horizontal pathways in the M1 (Hess and Donoghue, 1996). Our experimental paradigm was different. The potentiation of evoked responses was observed *in vivo* in anaesthetized animals; we used 1 Hz continuous peripheral stimulation instead of theta-burst stimulation. In both cases, however, the potentiation indicated a transient reduction of inhibition. Hess and Donoghue (1996) achieved this by application of the GABA-A receptor antagonist bicuculline, while in our case (Farkas et al., 2000) facial nerve transection decreased the inhibition, as reported by Garraghty et al. (1991). This suggests that inhibition may act as a gate to permit modification in the efficacy of horizontal excitatory connections. However, both the literature and our own results suggest that the reduced inhibition is not a sufficient condition for the development of M1 plasticity. Although there is no direct evidence that potentiation of the evoked responses in the M1 requires the participation of NMDA receptors, it has been suggested (Hess et al., 1994) that these receptors can probably be modified by neonatal MK-801 treatment.

We have presented an example that a **slight impairment in learning and memory function may be accompanied by a hidden defect in cortical function, which can be disclosed with appropriate electrophysiological methods, e.g. paired pulse stimulation.**

In the course of our experiments, we tried to find a rare, but natural phenomenon, what yield increased, additive disinhibition which can be the cooperative result of both GABA and NMDA systems.

We performed also paired pulse stimulation to reveal the effect of central motor cortical lesion on peripheral nerve injury induced electrophysiological changes. **Both denervation and ischemia-induced cortical changes indicate that widespread remote decreases in inhibition are a common feature of these peripheral and central injuries.** Our experiments provide further support for the data obtained by means of electrophysiological studies showing that **both focal lesions in the M1** (Buchkremer-Ratzmann and Witte, 1997) **and N7x** (Toldi et al., 1996; Toldi et al., 1999; Farkas et al., 2000) **induce transient and widespread disinhibition in both, but especially in the insulted hemisphere.** In addition, it is shown that the disinhibition induced by a previous focal lesion can be further enhanced by N7x. In this study, we focused on the early electrophysiological events. However, it should be noted that the cortical lesion elicits post-lesion repetitive episodes of peri-infarct depolarization during the first 10–30 min (Mies et al., 1993), which reduce the synaptic excitation and inhibition, and therefore might have disturbed our observations. To avoid these early misleading effects, we followed the electrophysiological recordings for some hours after the intervention. Although the mechanism of injury-induced cortical disinhibition is still obscure, **the studies indicate that decreases in GABAergic inhibition are a common feature of focal ischemic cortical injuries** (Mittmann et al., 1994; Buchkremer-Ratzmann and Witte, 1997). **Similarly, peripheral nerve injury**

also led to decreased GABAergic inhibition (Sanes and Donoghue, 2000; Calford, 2002a, b). The mechanism of decrease in GABAergic inhibition is still not clear in all details, but recently published results of an *in vitro* study demonstrated that ischemia caused an adenosine-mediated inhibition of the GABAergic synaptic transmission, which was coupled with an increased paired-pulse facilitation (Centonze et al., 2001). Other mechanisms may also have roles in this phenomenon, e.g. the dysregulation of the $GABA_A$ receptor subunit (Neumann-Haefelin et al., 1998) or the reverse GABA effect: excitatory actions of GABA were found after neuronal trauma (van den Pol et al., 1996). Interestingly, Cl^- accumulating cells were observed in axotomised neurons, this finding fitting in well with the findings of a reverse GABA effect (Nabekura et al., 2002). However, besides disinhibition, strong facilitation was occasionally observed in the paired-pulse paradigm, after pt lesion and N7x. In the dentate gyrus, the paired-pulse facilitation is known to have an NMDA-mediated component (Joy and Albertson, 1993). It might well be that the elevated excitation of NMDA receptors is also involved in these processes. This appears more than probable, considering that the glutamate concentration rises both in the core and in the peri-infarct region in focal ischemia (Morimoto et al., 1996). In our experiments, disinhibition induced by previous focal ischemia could be further enhanced by N7x. This suggests that the cortical disinhibition capacity is not fully utilized after focal ischemia, and/or the mechanisms of these two processes are at least partly different. Whatever the mechanisms, alterations in **excitability in extensive cortical regions may have a significant impact on the ability of the function in the cerebral cortex to be reorganized following peripheral or central injury.**

So far, the most explored model to study a correlation of structural and functional synaptic changes is the LTP in hippocampal slices *in vitro*. Based on these studies, a definite view emerged suggesting that function-dependent ultrastructural changes and formation of new spine synapses is associated with the LTP. An important but yet not fully explored field is the problem of structural synaptic remodeling and its relation to the gonadal hormones. In the second part of our work, we investigated the effect of E2 on the morphology and electrophysiological activity of the ARC and S1.

Our results clearly show that i.p. injection of E2 is able to increase significantly the activity of the arcuate neurons within 20±25 min. Our data are in agreement with earlier findings which indicated that a number of steroid hormones exert marked electrophysiological effects in the CNS (Orsini et al., 1985). Concerning the sites and mechanisms of hormone actions, however, conflicting opinions exist on the hypothesis if genomic or non-genomic factors play a role in this phenomenon. Many of the effects are believed to be mediated by interactions with intracellular receptors that result in gene-controlled changes in protein synthesis (McEwen et al., 1978), but on the other hand they are often interpreted as direct membrane effects of hormones. The main argument in favor of the direct effect is the fast onset of action of the steroid observed in slice preparations, but these data are also contradictory. Li et al. (1989) reported increased activity but found no difference between estrogen-treated and control animals for any of the electrophysiological characteristics which should reflect a change in excitability of neurons. Yeoman and Jenkins (1989) demonstrated that the arcuate neurons

maintained their diurnal pattern of activity (i.e. increased firing rate during proestrus afternoon) in slices if the animals were pre-treated with estrogen. These data can not be explained as a direct membrane effect of estrogen, they rather indicate that estrogen might act on other parts of the brain, thereby increasing the amount of excitatory information transmitted to the arcuate neurons. In their experiments, Li et al. (1989) and Yeoman & Jenkins (1989) used slice preparation from animals that were chronically treated with E2; therefore they have no data about the time course of the E2 effect. This information, however, is very important for the understanding of the possible molecular mechanisms and the result of our *in situ* experiments show that the enhanced activity of arcuate neurons occurs within 25 min. Although the mechanism of the effect of intraperitoneally applied estrogen on the arcuate neurons is still obscure, some genomic effects of steroids can be very rapid. According to Mosher et al. (1971), it can be within the range of 10 or 20 min, i.e. the 20-25 min latency reported here can be explained by this mechanism (McEwen, 1990). After 5 min of i.p. injection the E2 concentration in the plasma reaches a level that is higher than that of following the proestrus morning surge. This concentration is maintained for a prolonged period and it was also shown that the circulating E2 is delivered to the target cells in a few minutes. The fact that during the phases of increased neuronal firing (Garcia-Segura et al., 1993) demonstrated increased gene transcription and nucleo-cytoplasmic transport may also indicate genomic action. According to our working hypothesis **E2 acts on the GABAergic system and the observed increase in activity is the consequence of the disinhibition of arcuate neurons.**

We have found that the majority of the evaluated units did not respond to different sensory stimuli. There is a population, however, in which the spontaneous activity could be influenced by stimulation of both the somatosensory and the olfactory systems i.e. these cells are receiving inputs from both directions. Although Li et al. (1989) found that the AOB acts to enhance the activity of a subpopulation of arcuate neurons, and that neural transmission could be modulated by estrogen, as far as we know, **our observation is the first demonstration of the modulatory effect of trigeminal activation on arcuate neurons.** The two types of sensory modulation resulted in different effects, the AOB and whisker pad stimulation caused a decreased and increased firing, respectively. The somatosensory and olfactory stimuli are important in the sexually differentiated behavioral and neuroendocrine functions; therefore further studies are needed to elucidate the role of arcuate neurons in processing these inputs.

Our previous PRV tract-tracing study showed that it is a suitable method to analyze some plastic changes in the rat CNS. So we tried to reveal some of the possibly plastic afferent projections to the ARC with the help of this method.

Recent studies have shown that PRV inoculation into the estrogen-dependent organs (e.g. the uterine cervix) results in the widespread PRV infection of CNS structures (Lee & Erskine, 2000; Weiss et al. 2001). To test whether estrogen really does influence the susceptibility of CNS structures to PRV

infection, we examined the estrogen-dependent spread of PRV infection between the neurons of the ARC and the SFO, the neurons of both of which possess ERs (Shughrue et al., 1997).

Our viral tract-tracing study has demonstrated an estrogen-dependent transneuronal spread of PRV from the ARC to the SFO and pyriform cortex of female rats. The main observations were as follows: in the presence of estrogen (intact and OVX + E 12 h animals), PRV was transported from the primarily infected neurons in the ARC to the SFO, but not to the pyriform cortex (with the exception of a single neuron labeled in one animal). By contrast, in the absence of estrogen or before the estrogen had had time to exert its effect (OVX and OVX + E 4 h animals), we never observed PRV infection in the SFO cells following ARC inoculation, whereas strongly labeled neurons appeared in the pyriform cortex of these animals. The reverse labeling of the pyriform cortex, rather than that of the SFO, is surprising, and we have no definite explanation for this phenomenon at present. We could not observe a correlation between the labeling (in the SFO *vs.* the pyriform cortex) and the phases of the estrus cycle. Although we made efforts to determine the distinct phases of cycle, we did not observe the cycling clearly in each case. Therefore, we argue that although the females were in different phases (e.g. in estrus or pro-estrus) of their cycle, the methods we use did not allow an exact evaluation of the cycle. Because of this uncertainty, we used more control females than the number of animals in the OVX groups (seven animals vs. four animals, respectively).

The hormonal influence of the different phases of the estrus cycle in the intact females used in this study was reflected by the relatively high standard deviation for the number of labeled cells. These results, which are supported by the statistical analysis, suggest that relatively low levels of estrogen are able to produce labeling in the SFO both in males (due to the activity of the enzyme aromataze) and in females (independently of the actual phase of the estrus cycle). However, this was not the case in the OVX and OVX + E 4 h animals, where the estrogen levels were likely to be so critically low as not to produce labeling. Zhu & Pfaff (1998) reported that a relatively short length of time (60 min) was sufficient to induce changes in the binding of an AP-1 nuclear transcription factor (such as c-fos or c-jun) in the rat hypothalamus after estrogen application. Nuclear ultrastructural changes were observed in the hypothalamic neurons 2 h after estrogen treatment in OVX animals (Jones et al., 1985). In our experiments, however, no transneuronal labeling was produced 4 h after estrogen application. These findings suggest that the estrogen dependence of PRV labeling be based on long-latency changes involving gene expression rather than on transcriptional mechanisms. **As far as we know, the present study is the first to demonstrate estrogen-dependent tracing with PRV in the CNS.** Our working hypothesis is based on the assumption that, by selecting a PRV strain with a highly attenuated phenotype and specific spreading characteristics, we can create a system based on the "all or none" principle, i.e. transneuronal PRV labeling does not work below a critical level of estrogen. The results of this study suggest that, in the absence of estrogen, the connections between the ARC and the SFO do not permit the transmission of PRV to the SFO, but this situation is altered in the presence of estrogen. **This estrogen-dependent labeling of the SFO could possibly be induced by**

the estrogen-dependent synaptic plasticity, which has been well documented in several regions of the CNS (Chung et al., 1988; Parducz et al., 1993; Langub et al., 1994; Naftolin et al., 1996; VanderHorst and Holstege, 1997; Woolley, 1998; Flanagan-Cato et al., 2001). However, if estrogen plays a key role in the regulation of synaptic remodeling in some brain structures, then the action of estrogen on estrogen-sensitive neurons within these areas might be expected to influence both the incidence and the rate of PRV infectivity by altering the synaptic connections between these structures. It is well established that neurons in the SFO send axons directly to the ARC (Gruber et al., 1987). The anatomical data and our own **results suggest that the existence of the synaptic contacts between the axon terminals of the neurons in the SFO and the ARC neurons is estrogen-dependent.** Other studies have demonstrated that the densities of dendritic spines and axo-dendritic synapses in the hypothalamus (Frankfurt et al., 1990) and in the hippocampus (Woolley, 1998) are estrogen-dependent.

An increase in the number of synaptic contacts between the supraspinal presynaptic neurons located in the nucleus retroambiguus of the caudal medulla and the spinal postsynaptic lumbar motoneurons in cats in estrus and in estrogen-treated cats has been reported by VanderHorst and Holstege (1997). However, as reported by Naftolin et al. (1996), there are a reduced number of synaptic contacts between the cells in the ARC on the morning of estrus. Parducz et al. (1993) found that this reduction was mainly due to a decrease in the number of GABAergic synapses, whereas the number of non-GABAergic axo-somatic synapses did not change significantly. Taken all together, as the number of synaptic connections between the ARC and the SFO is reversely influenced by estrogen, this mechanism cannot provide a direct explanation for the increased labeling by PRV due to estrogen treatment. A second, more viable mechanism that could underlie the phenomenon is that estrogen modulates the infectivity of neurons. Weiss et al. (2001) found that PRV injections into either the uterine cervix or the kidney produced an estrogen-dependent infection in specific CNS structures. The estrogen-dependence of transneuronal viral tracing from the kidney suggests that estrogen influences the spread of PRV between CNS structures and not the uptake of virus from the site of injection. By contrast, Lee and Erskine (2000) found that PRV infectivity was not altered by estrogen treatment. The reason for that result might be that they applied the PRV peripherally (uterine cervix) and not into the CNS.

The different labeling patterns in the SFO and pyriform cortex lead us to suggest that the different types of neurons (different structures in the CNS) may be susceptible to virus infections in different ways, thereby resulting in the occurrence or lack of transneuronal viral labeling. The results could also suggest that not only did the direct injection of PRV into the ARC infect the ARC neurons and then, by transneuronal spreading of the virus, the SFO neurons, but the terminals of the SFO neurons could have taken up the virus and also become primarily infected. Although this possibility cannot be excluded, the high number of primary infected ARC neurons suggests that neurons in the SFO were mainly infected by transneuronal spread of the virus. These data and our own observations indicate

that transneuronal PRV labeling depends on the effects of estrogen on the CNS structures and connections, and that synaptic remodeling influenced by estrogen is less likely to play a key role in the spread of PRV within the CNS. Though little is known of the relation between the ERs and the estrogen-dependent synaptic plasticity or neuronal infectivity, this is presumably also an important factor. From this point of view, it might be interesting that different types of ERs are expressed on the neurons in these structures, e.g. only ER α was found in the SFO, whereas both ER α and ER β exist in the ARC (Shughrue et al., 1997). These results demonstrate that **PRV tracing is a suitable method for the evaluation of estrogen-dependent labeling in the CNS.**

Several groups, including us *(see our previous two experiments)*, have demonstrated effects of estrogen on the neuronal plasticity in both hypothalamic and extrahypothalamic structures (Frankfurt et al., 1990; Parducz et al., 1993; VanderHorst and Holstege, 1997; Woolley, 1998). It has also been shown that estrogen plays a key role in many other processes of the CNS, including neuroprotection (Garcia-Segura et al., 2001; Liao et al., 2001). Great efforts have recently been made to elucidate the mechanisms of the multiple actions of steroid hormones, with particular focus on a differentiation between its genomic and non-genomic effects (Zakon, 1998; Falkenstein et al., 2000).

The latency of the neuronal excitation induced by estrogen was found in this study to be 25-35 min. In cerebellar Purkinje cells, the potentiations of responses were earlier observed as soon as 5-10 min post-E2 (Smith et al., 1987; Smith, 1989). It is suggested that the 20-25 min latency of the effect of E2 be based not so much on membrane mechanisms as on genomic mechanism (McEwen, 1991). However, it is probably an over-simplification to categorize the effects of estrogen into genomic and non-genomic mechanisms merely on the basis of latency. First, some genomic effects of steroids can be very rapid: within 10-20 min (Mosher et al., 1971). Second, the ability of the liganded intracellular E2 receptor to stimulate membrane processes suggests cross-talk between the genomic and membrane receptor pathways (Zakon, 1998; Rupprecht and Holsboer, 1999). In spite of these possibilities, the long latency observed for the estrogen effect in this study suggests that is based on genomic mechanisms.

Another important question is the **function of estrogen in the barrel cortex.** If it is presumed that estrogen has any role in the S1, the presence of ERs must be proved. This was done recently (Shughrue et al., 1997). The next step was to demonstrate the functioning of estrogen in that cortical area. This has been done in the present study. We found only five out of the fully analyzed 14 cells (36%) that responded to estrogen. This fits well with the relatively low density of ERs in the cortex (Shughrue et al., 1997). Apart from the latency, the facilitatory effect of estrogen on cortical neurons demonstrated here is comparable to findings in the hippocampus (Wong and Moss, 1991) and Purkinje cells (Smith et al., 1987; Smith, 1989). Unfortunately, at the moment we do not know the site of action of estrogen within the cortex. It could well be that estrogen does not act directly on the recorded barrel cortical neuron responding with short latency to the principal vibrissa stimulation, but on a GABAergic interneuron, for instance. This mechanism is known in the hypothalamic ARC, where

inhibitory interneurons having ERs respond to estrogen by withdrawing their presynaptic axon terminals, resulting in decreased GABAergic axo-somatic synapses (Garcia-Segura et al., 1994), and consequently, in the increased firing of principal arcuate neurons (Kis et al., 1999a). There is another possibility: neuractive steroids are potent positive allosteric modulators of $GABA_A$ receptors because they increase the frequency or duration of openings, or both, of the GABA-gated Cl^- channels (Zakon, 1998). If this takes place on a GABAergic interneuron, it could result in disinhibition of the recorded cortical neuron. Thus is not probable, however, because of the relative long delay of estrogen-induced facilitation. Whatever the mechanism, as far as we know, **the observation presented here is the first demonstration of an estrogen-induced change in excitability of neurons in rat cortex.**

These findings make the study of estrogen actions in neural tissue an exciting and challenging topic and target for pharmaceutical development. Together with understanding the mechanism of neuronal plasticity will help to develop treatment programs to improve functional outcome of patients with neuronal injuries.

VII. ÖSSZEFOGLALÁS, KÖVETKEZTETÉSEK

A disszertációmban ismertetett kísérletek a patkány központi idegrendszer plasztikus változásaival foglalkoztak. Munkatársaimmal a munka egy részében azt vizsgáltuk, hogy perifériás idegi sérülés hatására milyen plasztikus folyamatok játszódnak le a központi idegrendszerben. Elektrofiziológiai, biokémiai, hisztokémiai, molekuláris biológiai és elektronmikroszkópos technikák alkalmazásával kimutatták idegi sérülést követően az extracelluláris proteáz-szint emelkedését (Landgrebe et al., 1998), az asztrociták fokozott aktiválódását (Rohlmann et al., 1994) a szinaptikus vezikulák fúzióját a külső axonterminális membránjával valamint a lizoszómák degradációját a szinapszisokban (Wolff et al., 1995). Ezek a neuron-glia elemek közötti változások a szinaptikus kapcsolatok újrarendeződésére utalnak. Mindezek arra engednek következtetni, hogy a perifériás idegi sérülés összetett változást eredményez, pl. kérgi idegsejtek egyes génjeinek aktiválódását, vagy sejtfelszíni molekuláinak újrarendeződését, amely a neuronok közti funkcionális kapcsolatot megváltoztatják. Pálya-jelölő módszerként a pseudorabies (PRV)-immunhisztokémiát alkalmaztuk. A megszokott transz-szinaptikus jelölő anyagokkal szemben a herpes vírusoknak az az előnye, hogy a sejtről-sejtre történő terjedést követően a jel nem gyengül (Card et al., 1993). A Bartha vírustörzs (PRV-Ba) (Bartha, 1961) a PRV gyengített változata, amelyről kimutatták, hogy elsősorban szinaptikus kapcsolatok mentén, kizárólag retrográd irányba terjed, pl. az axon végződés felől a sejttesten keresztül a preszinaptikus afferens felé [*Lásd.* (Enquist et al., 1998)]. Mi a PRV-Ba egy genetikailag pályajelölésre módosított változatát, Ba-DupLac-ot használtuk.

Első kísérletsorozatunkban azt a feltevésünket vizsgáltuk meg, hogy az arcideg (*nervus facialis*) átvágása (N7x) olyan mértékű változást eredményez a két agyfélteke primer motoros kérge (M1) között, amely a PRV transzkallózális jelölési mintázatában is megjelenik. Kísérleteink igazolták feltevésünket. A főbb megfigyelések az alábbiak: a kontroll állatokban, a beadási szúrt csatorna körül számos elsődlegesen fertőzött sejtet találtunk, míg az ellenoldali M1-ben csak nagyon nehezen találtunk egy-két másodlagosan fertőződött idegsejtet. Ezen idegsejtek csaknem egyenletesen oszlottak el mediolaterális irányban az agykéregben. Ezzel ellentétben, a jobb oldali N7x eredményeként, a PRV átterjedt az elsődlegesen fertőzött sejtekről az ellenoldali M1 számos idegsejtjére. Ezek a másodlagosan fertőzött sejtek a beadással homotóp kérgi terület körül koncentrálódtak. A fertőzött sejtek száma abban az esetben volt a legmagasabb, amikor a sérülés és a vírus beadása között két óra telt el.

Számos neurotranszmitter plaszticitásban játszott szerepét bizonyították már eddig is. Egyre növekszik azonban azoknak az eredményeknek a száma, amelyek azt igazolják, hogy a gátlási szint átmeneti csökkenése (pl. perifériás idegi sérülést követően) szükséges, de önmagában nem elegendő a primer motoros kéreg plaszticitásának kialakításához. Az M1-ben kialakuló plasztikus változások szorosan kapcsolódnak az N-metil-D-aszpartát (NMDA) receptorokhoz. Kimutatták továbbá, hogy a

horizontális pályákon keresztül terjedő mezőpotenciálok kialakításáért is NMDA receptorok tehetők felelőssé, illetve ezekben a horizontális kapcsolatokban kialakulhat hosszan tartó erősödés (LTP). Feltételezték, hogy ha MK-801-et (egy nem-kompetitív NMDA receptor antagonista) adnak a fejlődés NMDA receptor szenzitív szakaszában, akkor az, szerkezeti változásokat fog eredményezni az idegi fejlődésben (Griesbach és Amsel, 1998). Így jutottunk mi is arra, hogy megvizsgáljuk az újszülöttkorban adott MK-801 hatását a felnőttkori térbeli tanulásra és kérgi plaszticitásra. Mindezt a viselkedés legkisebb károsodása mellett próbáltuk megtenni, amelyet tanulási és memória tesztekkel ellenőriztünk. Fő kérdésünk az volt, hogy ez a kis mértékű korai behatás befolyásolja-e később, felnőtt korban a mesterségesen kiváltott kérgi plaszticitást elektrofiziológiai módszerekkel mérhető mértékben. A water maze vizsgálatok alapján azt állíthatjuk, hogy az MK-801 kezelt állatok kis (nem szignifikáns) mértékben rosszabbul teljesítettek a térbeli tanulási és memória tesztekben. A vizuális tesztekben még ekkora eltérés sem volt, itt egyformán jól teljesítettek mind a kontroll, mind az MK-801 kezelt állatok.

Nem volt bizonyítékunk arra, hogy a primer motoros kérgi kiváltott potenciálok növekedése közvetlenül NMDA függő folyamat. Hess és társai (1994) mindössze annyit feltételeztek, hogy ezek a receptorok módosíthatók újszülöttkori MK-801 kezeléssel. Ezért egy olyan vizsgálati rendszert kellett beállítanunk, amely alkalmas a gátlási szint időbeli változásának nyomon követésére. Páros impulzusokkal ingereltük a M1-et közvetlenül, illetve a háromosztatú idegen (*nervus trigeminus*-on) keresztül, s a kiváltott aktivitásokat mindkét oldali M1-ről elvezettük. A sérülésnek megfelelő oldalon és azzal ellentétes primer motoros kéregben (M1 C) regisztrált első és második kiváltott potenciál hányadosát (Q=fEPSP1/fEPSP2) vettük a kérgi gátlás mértékének alapjául. Az MK-801-gyel kezelt állatok M1 C-ben nagy amplitúdójú kiváltott potenciálok jelentkeztek, míg az ellentétes oldali kérgi területen jóval kisebbek voltak, sőt gyakran hiányoztak. Ez hasonló volt a kontroll állatokban megfigyeltekkel. Az MK-801-gyel kezelt állatokban azonban, N7x-et követően nem növekedett meg egyik oldali M1-ben sem a kiváltott potenciálok nagysága, ellentétben a kontroll állatokkal, ahol jelentős mértékű amplitúdónövekedés volt megfigyelhető. A kontroll állatok Q-értéke szignifikánsan megnőtt N7x után, míg az MK-801 kezelt állatoké nem.

Mind a denerváció, mind az iszkémia által kiváltott kérgi változások arra mutatnak, hogy a gátlás kiterjedt, távoli területeket is érintő megváltozása közös jellemzője e perifériás és centrális sérülések által kiváltott kérgi folyamatoknak. Vizsgálataink során a fokális iszkémia által kiváltott gátlásoldás tovább növekedett N7x hatására. Mindez azt mutatja, hogy fokális iszkémiát követő gátlás szintje nem teljes mértékű, ez pedig arra utal, hogy e két folyamat mechanizmusa legalább részben különbözik (mind a GABA, mind az NMDA transzmitterrendszer szerepet játszhat). A nagy kérgi területek ingerelhetőségének változása lényegesen befolyásolhatja az agykéreg perifériás, vagy centrális sérülést követő újrarendeződését.

Az utóbbi évtized kutatásai hozták felszínre, hogy a 17β-ösztradiolnak (E2) szerepe lehet a felnőttkori plasztikus folyamatokban is, hiszen az ontogenezis során a E2-nak meghatározó a jelentősége az agy

ivari dimorfizmusának kialakulásában is. Az irodalomból ismert pl., hogy a E2 hatására megváltozik az asztroglia növekedése és alakja, mind fejlődő, mind felnőtt állatban (Garcia-Segura et al., 1995), és ösztrogén hatására a szinaptikus plaszticitás fokozódását írták le a *hypothalamus*-ban (Garcia-Segura, 1997). Garcia-Segura és munkatársai immuncitokémiai módszerekkel azt is kimutatták, hogy E2 egyszeri beadása a GABA immunoreaktív axo-szomatikus szinapszisok számának szignifikáns csökkenését okozza ovariektomizált állat *nucleus arcuatus*-ban (ARC) (Garcia Segura et al., 1994). Ez az eredmény is megerősíti azt a feltételezést, hogy az ösztrusz ciklus során a szinaptikus kapcsolatok folytonos újrarendeződése történik a ARC-ban, amit a változó E2 szint hoz létre (Olmos et al., 1989).

Anatómiai vizsgálatok kimutatták, hogy két cirkumventrikuláris szerv, az ARC és a szubfornikális szerv (SFO) kölcsönös kapcsolatban állnak egymással, az SFO közvetlenül kapcsolódik az ARC-hoz (Gruber et al., 1987), és az ARC is küld efferenseket az SFO-hoz (Rosas-Arellano et al., 1993). Újabb keletű vizsgálatok azt mutatják, hogy ha ösztrogénfüggő szervet (pl. méhnyakat) fertőzünk meg PRV-vel, akkor a központi idegrendszer nagy részén találunk fertőzött idegsejteket (Lee and Erskine, 2000; Weiss et al., 2001). Ezek alapján megvizsgáltuk, hogy a E2 tényleg befolyásolja-e a PRV terjedését a központi idegrendszerben. Vizsgálati területünk az ARC és az SFO volt, részben anatómiai kapcsolatuk, részben pedig ösztrogén receptor tartalmuk miatt (Shughrue et al., 1997). Kísérleteink igazolták feltevésünket: ösztrogén jelenlétében (intakt és OVX+E állatok) a PRV az ARC elsődlegesen fertőzött sejtjeiről az SFO sejtjeire transzportálódott, ugyanakkor (egyetlen állat egyetlen kérgi idegsejtjének kivételével) a *pyriform* kéreg sejtjei nem jelölődtek. Ezzel ellentétben, ösztrogénhiányos állapotban, vagy amikor az ösztrogén nem fejthette ki hatását (OVX és OVX + E 4 h állatok), egyetlen esetben sem találtunk PRV által megjelölt sejtet az SFO-ban az ARC-ban történő fertőzést követően. Ezekben az esetekben, azonban, erőteljesen jelölődött sejteket találtunk a *pyriform* kéregben. A *pyriform* kéreg SFO-val ellentétes jelölődése rendkívül izgalmas, de általunk ismeretlen a jelenség háttere.

A E2 által befolyásolt szinaptikus változásoknak természetesen funkcionális következményei is vannak. Kísérletünkben azt vizsgáltuk, hogy a E2 által befolyásolható-e az ARC sejtjeinek elektrofiziológiai aktivitása? Munkánk során *in situ* preparátumokon tanulmányoztuk a ARC sejtjeinek spontán aktivitásában bekövetkező változásokat E2 i.p. beadása után. Eredményeink szerint 20-25 perccel a beadást követően a ARC neuronjainak spontán aktivitása szignifikánsan fokozódik. A hatás viszonylag késői megjelenése azt a feltételezést valószínűsíti, hogy genomikus úton játszódik le a folyamat, és a gátló axo-szomatikus bemenetek csökkenése eredményezi a fokozott aktivitást, amit morfológiai adatok is alátámasztanak (Parducz et al., 1993). Ezen kísérleteinkben azt is kimutattuk, hogy a ARC sejtjeinek bizonyos populációja szenzoros ingerekre is reagál. A járulékos szagló gumó (AOB), valamint a szomatoszenzoros rendszer ingerlése befolyásolja a ARC sejtek tüzelési aktivitását, tehát a ARC inputokat fogad mindkét szenzoros rendszer felől. Bár már korábban leírták azt, hogy AOB-ingerlés hatással van a ARC neuronokra (Li et al., 1989), és ez a folyamat E2 által szabályozott, tudomásunk szerint munkacsoportunk megfigyelése az első olyan bizonyíték, amely a *trigeminalis*

rendszer moduláló szerepét igazolja a ARC neuronjain. A kétféle szenzoros ingerlés eltérő hatást eredményezett. Az AOB ingerlésére csökkent, míg a bajuszpárna elektromos stimulálására fokozódott a sejtek spontán aktivitása.

A ARC-on végzett munkáink során vetődött fel, hogy a E2 neuronális aktivitásban játszott szerepének vizsgálatát a szomatoszenzoros kéregre is kiterjesszük, hiszen ez a terület a központi idegrendszer legplasztikusabb részeihez sorolható. Az elmúlt évtizedben számos kutatócsoport vizsgálta a E2 plasztikus folyamatokra gyakorolt hatását, azonban ezek a vizsgálatok jórészt kéreg alatti struktúrákra korlátozódtak. A legtöbb e témában végzett munka a *hypothalamus*-hoz kapcsolódik (Frankfurt, 1994; Naftolin et al., 1996). Az utóbbi években azonban több olyan közlemény is megjelent, amelyek *extrahypothalamikus* területeken, pl. a *hippocampus*-ban is bizonyították a E2 plaszticitásban játszott szerepét (VanderHorst and Holstege, 1997; Woolley, 1998). Mivel az ösztrogén receptor mindkét fajtáját kimutatták a szomatoszenzoros kéregben (Shughrue et al., 1997), kézenfekvő volt azon kérdés feltevése, hogy vajon a E2 mint az egyik legjelentősebb biológiai hatású ösztrogén, szerepet játszik-e a kéreg funkcionális plaszticitásában? Első lépésben azt kellett megvizsgálnunk, hogy találunk-e olyan sejteket, amelyekre hat valamilyen módon a E2. A finomabb analízis miatt a E2-t nem i.p. adagoltuk, hanem iontoforézissel juttatuk a vizsgált barell-kérgi neuronok közvetlen közelébe.

Kimutattuk, hogy E2-hemiszukcinát iontoforézisének hatására a barrel kérgi neuronok egy részének mind a spontán aktivitása, mind a perifériás ingerléssel kiváltott aktivitása megváltozott. A regisztrált sejtek egyharmadánál változás volt megfigyelhető, és ez a változás aktivitás-fokozódásban nyilvánult meg. Eredményeink szerint a E2-hemiszukcinát iontoforézisét követően átlagosan 24 perc elteltével jelenik meg a tüzelési aktivitás fokozódása, ami kizárja a közvetlen membránhatás valószínűségét. Azt, hogy az ösztrogének milyen módon változtatják meg a sejtek aktivitást, még nem tudjuk pontosan. Elképzelhető, hogy hasonló folyamat játszódik le, mint ami a ARC-ban: a GABAerg interneuronokon található ösztrogén-receptorok megkötik a E2-t, a sejtek „visszahúzzák" preszinaptikus axon termináljukat, s ezzel lecsökken a GABAerg axo-szomatikus szinapszisok száma. Ez a folyamat pedig, a gátló hatások csökkenése miatt fokozott tüzelési aktivitást eredményez (Garcia-Segura et al., 1994). Másik lehetséges elképzelés szerint a neuroaktív szteroidok pozitív allosztérikus modulátorai a $GABA_A$ receptoroknak, mivel fokozzák a GABA által szabályozott klorid csatornák nyitva tartásának idejét, illetve frekvenciáját, s ha ez GABAerg interneurokon játszódik le, akkor a regisztrált kérgi sejt diszinhibíciójához vezet az adott kérgi neuronon (Zakon, 1998).

A disszertációban ismertetett munkák a perifériás idegi sérülések okozta központi idegrendszeri plasztikus változások vizsgálatával foglalkoztak. További munkáinkban a szteroidok idegrendszeri plaszticitásban játszott szerepét, esetleges neuroprotektív hatásukat kívánjuk tanulmányozni. Ezen munkák első lépéseit ismertettem disszertációm második részében.

VIII. SUMMARY

The experiments performed in this study relate to plastic changes in adult rat. In a part of the investigations, we examined the physiological consequences of peripheral nerve transection in the central nervous system (CNS). Electrophysiological, histochemical, biochemical, molecular biological and electronmicroscopic methods were combined to follow changes in the cerebral cortex and demonstrated an increase in extracellular protease (Landgrebe et al., 1998), an enhanced activation of the astrocytes (Rohlmann et al., 1994), fusion of the synaptic vesicles with the outer axon terminal membrane and lysosomal degradation of the synapses after nerve injury (Wolff et al., 1995). These changes in neuron-glia interactions ultimately lead to structural remodeling of certain synapses. These findings lead us to suppose that peripheral nerve injury induces complex changes, e.g. surface molecule redistribution or the gene activation of cortical neurons, which lead to alterations in the functions of their connections. Pseudorabies virus (PRV) was utilized as a transneuronal tract-tracing tool. The major advantage of herpesviruses over traditional transynaptic tracers is that they can self-amplify after crossing synapses (Card et al., 1993). The transport of an attenuated PRV variant, strain Bartha (PRV-Ba) (Bartha, 1961), between CNS neurons has been reported to occur predominantly at points of synaptic contact, and to proceed in the retrograde direction, i.e. from axon terminals through the cell body to the presynaptic afferents (for a review, see (Enquist et al., 1998). We used Ba-DupLac, a genetically modified PRV-Ba derivative that was developed for tract-tracing studies.

In the first series of experiments, we tested the hypothesis that facial denervation (N7x) induces changes in the neuronal connections of the primary motor cortices (M1s) in both hemispheres, which influence the transcallosal PRV labeling pattern. Our tracing study has demonstrated that N7x influences the transcallosal spread of PRV from the affected side M1 to the contralateral M1 in rats. The main observations were as follows: in the controls, PRV injection primarily infected several neurons around the penetration channel, but hardly any secondarily infected neurons were found in the contralateral M1. These neurons were distributed almost equally from medial to lateral in the cerebral cortex. In contrast, after right N7x, PRV was transported from the primarily infected neurons in the left M1 to the contralateral side, and resulted in the labeling of several neurons due to a secondary infection. These secondarily labeled neurons were concentrated near the homotopic line on the injection side. The number of infected neurons reaches a plateau 2 h postinjury/preadministration.

Several neurotransmitters play important roles in the plasticity. An increasing number of results suggest, however, that the transiently reduced inhibition (e.g. after nerve injury) is a necessary, but not sufficient condition for the development of M1 plasticity. The potential for plasticity in the M1 has been closely linked to the function of N-metil-D-aspartate (NMDA) receptors. Moreover, it has been shown that a component of field potentials itself evoked in the horizontal pathways of the rat M1 is mediated by NMDA receptors, and LTP can develop in these horizontal. It has been suggested that MK-801 applied during a period in which the NMDA receptors are involved in developmental

changes has effects such as a learning impairment which may be related to structural alterations took place during neuronal development (Griesbach and Amsel, 1998). So, we investigated the long-term effects of neonatal treatment with MK-801 on spatial learning and cortical plasticity in adult rats. We wanted to find a way to cause a minimal impairment in behavior, which was based on learning and memory functions without interfering with physical capabilities, and to test whether this influences experimentally induced cortical plasticity that is detectable by electrophysiological methods.

On the basis of the water maze analysis, it can be stated in general that the performances of the MK-801- treated animals in the spatial learning and memory task were slightly (but not significantly) poorer than that of the controls. However, the visual task was performed equally well both by the control and the treated rats.

Though, there is no direct evidence that potentiation of the evoked responses in the M1 requires the participation of NMDA receptors, it has been suggested (Hess et al., 1994) that these receptors can probably be modified by neonatal MK-801 treatment. So, it was necessary to develop a protocol to investigate the changes in the inhibition level in time. Cortical excitability was studied by pair-pulse electrical stimulation, applied peripherally to the trigeminal nerve, while the responses were recorded bilaterally in the M1. The ratio of the amplitudes of the second and the first evoked potentials (EPs) (Q=fEPSP1/fEPSP2) was taken as a measure of the inhibitory capacity in the M1 ipsilateral or contralateral to the nerve injury. In the MK-801 treated rats, trigeminal stimulation induced EPs with high amplitude in the M1 C, and with small amplitude if any in the M1 I. This was similar to what was observed in the control rats. The facial nerve transection, however, did not facilitate the evoked responses in the M1 in either hemisphere, unlike in control rats, where it was facilitated to different degrees. The Q-values in the controls are increased significantly after N7x, but not those in the MK-801-treated animals.

Both denervation and ischemia-induced cortical changes indicate that widespread remote decreases in inhibition are a common feature of these peripheral and central injuries. In our experiments, disinhibition induced by previous focal ischemia could be further enhanced by N7x. This suggests that the cortical disinhibition capacity is not fully utilized after focal ischemia, and/or the mechanisms of these two processes are at least partly different (both the GABAergic and the NMDAergic system can be involved). Alterations in excitability in extensive cortical regions may have a significant impact on the ability of the function in the cerebral cortex to be reorganized following peripheral or central injury.

For many years, it has been known that steroids may play important roles in plastic changes in the CNS, since 17β-estradiol (E2) is definitely of importance for development of the sexually dimorphic brain. It has been reported that E2 can alter the growth and shapes of the astroglial cells in both the developing and the adult brain. Quantitative post-embedding immunocytochemical analyses in the arcuate nucleus (ARC) revealed that the administration of a single dose of E2 resulted in a significant decrease in the number of GABA-immunoreactive axo-somatic synapses in ovariectomized (OVX)

rats (Garcia Segura et al., 1994). It has also been claimed that there is a continuous synaptic remodeling in the ARC during the estrus cycle, which is driven by the change in E2 level in the plasma (Olmos et al., 1989).

Anatomical studies have revealed that the ARC and the subfornical organ (SFO) are mutually connected; the SFO projects directly to the ARC (Gruber et al., 1987), and the ARC sends efferents to the SFO (Rosas-Arellano et al., 1993). Recent studies have shown that PRV inoculation into the estrogen-dependent organs (e.g. the uterine cervix) results in the widespread PRV infection of CNS structures (Lee and Erskine, 2000; Weiss et al., 2001) To test whether estrogen really does influence the susceptibility of CNS structures to PRV infection, we examined the estrogen-dependent spread of PRV infection between the neurons of the ARC and the SFO, the neurons of both of which possess ERs (Shughrue et al., 1997). Our viral tract-tracing study has demonstrated an estrogen-dependent transneuronal spread of PRV from the ARC to the SFO and pyriform cortex of female rats. The main observations were as follows: in the presence of estrogen (intact and OVX + E 12 h animals), PRV was transported from the primarily infected neurons in the ARC to the SFO, but not to the pyriform cortex (with the exception of a single neuron labeled in one animal). By contrast, in the absence of estrogen or before the estrogen had had time to exert its effect (OVX and OVX + E 4 h animals), we never observed PRV infection in the SFO cells following ARC inoculation, whereas strongly labeled neurons appeared in the pyriform cortex of these animals. The reverse labeling of the pyriform cortex, rather than that of the SFO, is surprising, and we have no definite explanation for this phenomenon at present

The E2-induced synaptic remodeling may have functional consequences, and we wished to gain further insight into the physiological significance of this. To address this question, in the present studies we set out to investigate whether the E2-induced decrease in inhibitory synaptic inputs on the arcuate neurons results in a change in their electrophysiological activity.

We used an *in situ* recording technique to study the effect of systemically administered E2 on the activity of the arcuate neurons. The results clearly showed that i.p. injection of E2 significantly increases the activity of the arcuate neurons within 20-25 min. The late onset of the action of E2 makes it unlikely that the changes are a consequence of a direct membrane effect, and we propose that the enhancement of firing is a result of the decrease in inhibitory synaptic inputs on the arcuate neurons. We demonstrated that somatosensory (trigeminal) and olfactory inputs also modulate the activity of these neurons. Although it has been reported the accessory olfactory bulb (AOB) acts to enhance the activity of a subpopulation of arcuate neurons, and that neural transmission could be modulated by estrogen (Li et al., 1989), as far as we know, our observation is the first demonstration of the modulatory effect of trigeminal activation on the arcuate neurons. The two types of sensory modulation resulted in different effects: AOB stimulation and whisker pad stimulation caused a decreased and an increased firing, respectively.

The experiments on the ARC led us to investigate the effects of E2 in the neuronal activity of the S1, since this is one of the most plastic areas in the CNS. In the past decade, several groups have investigated the effects of E2 in plastic changes, but most of these observations related to the hypothalamus (Frankfurt, 1994; Naftolin et al., 1996). There have also been reports on the neuroplastic effects of E2 in the hippocampus and in the other structures of the CNS (VanderHorst and Holstege, 1997; Woolley, 1998). Since both kinds of ER mRNAs have been demonstrated by means of *in situ* hybridization histochemistry in the cerebral cortex (Shughrue et al., 1997) the obvious question arose of whether the E2 plays a role in the functional plasticity of the cortex. The first step was to find cells on which has E2 some effect. The chosen method was to apply E2-hemisuccinate on the cortical neurons by means of iontophoresis.

We demonstrated that both the spontaneous and the peripherally-evoked activity of the cortical neurons were altered after E2-hemisuccinate iontophoresis. About one-third of the tested neurons displayed facilitation. On average the delay in the responses to E2 was 24 min. The late onset of the action of E2 makes it unlikely that the changes are consequences of direct membrane effects. Unfortunately, at the moment we do not know the site of action of estrogen within the cortex. It could well be that estrogen does not act directly on the recorded barrel cortical neurons responding with short latency to the principal vibrissa stimulation, but on GABAergic interneurons, for instance. This mechanism is known in the hypothalamic ARC, where inhibitory interneurons having ERs respond to estrogen by withdrawing their presynaptic axon terminals, resulting in decreased GABAergic axo-somatic synapses, and consequently in increased firing of the principal arcuate neurons (Garcia-Segura et al., 1994). There is another possibility: neuroactive steroids are potent positive allosteric modulators of $GABA_A$-gated Cl^- channels. If this takes place on GABAergic interneurons, it could result in disinhibition of the recorded cortical neuron (Zakon, 1998).

The study presented here relates to plastic neuronal changes evoked by peripheral nerve injury. In our further investigations we intend to study the effects of steroids on neuronal plasticity, and the possible role in neuroprotection.

IX. KÖSZÖNETNYILVÁNÍTÁS

Sokáig tanácstalan voltam abban, hogy milyen nyelven mondjak köszönetet mindazoknak, akiknek hálával tartozom dolgozatom megszületéséért. Több érv is szólt mindkét nyelv mellett, de végül, természetesen a magyart választottam, mert ez az anyanyelvem, és ezen tudom leginkább kifejezni mindazt, amit érzek.

Első helyen Toldi József professzor úrnak szeretnék köszönetet mondani, Mindenért.

Neked, Jóska, aki atyai szeretettel terelted türelmesen érdeklődésemet, majd lehetőséget biztosítottál saját tudásom kipróbálására, és most aggódva nézed útkeresésemet. Ötleteimet végighallgatva mindig elmondtad véleményedet, de soha nem próbáltad rámerőltetni azt.

Van, akinek a tudományos mérőszáma magasabb (bár a Tiéd is tiszteletet parancsoló), de iskolateremtő munkád, kivételes emberséged a legnagyobb tudósok közé emel.

Köszönettel tartozom volt munkatársaimnak dr. Kis Zsoltnak, dr. Farkas Tamásnak, Német Hajnalkának és rendkívül tehetséges szakdolgozómnak, Prandovszky Emesének barátságukért, segítségükért, türelmükért. Köszönet az Összehasonlító Élettani Tanszék összes dolgozójának, akik segítsége nélkül e dolgozat nem jöhetett volna létre.

Biztos családi háttér nélkül nincs siker. Ezért szeretném családomnak megköszönni mindazt a szeretetet, türelmet, amit nyújt nekem.

Szüleim különleges helyet foglalnak el életemben (ennek már csak a biológiai okai is megvannak) – még, ha ezt időnként nem is érzik. Nekik ajánlom ezt a dolgozatot. Hiszem és remélem, hogy ez, további szavak nélkül is mindent elárul irántuk táplált érzéseimről.

Nagyon nagy örömmel tölt el, hogy korábbi rivalizációm bátyámmal kölcsönösen testvéri segítséggé változott. Ő nagyobb előrelátással, biológia iránti érdeklődését és tehetségét még időben feladva a megélhetés reális útját választotta.

Utolsó helyen, de természetesen nem utolsó sorban szeretnék köszönetet mondani Neked, Ildi, hogy mellettem vagy, és közösen "bonyolítjuk" kutyás életünket, amely sokszor normálisnak korántsem mondható. Természetesen köszönöm szüleidnek is a támogató segítséget, bár tudom, hogy Nekik sem könnyű.

Domaszék, 2004.szeptember 14.

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XI. CONTENTS