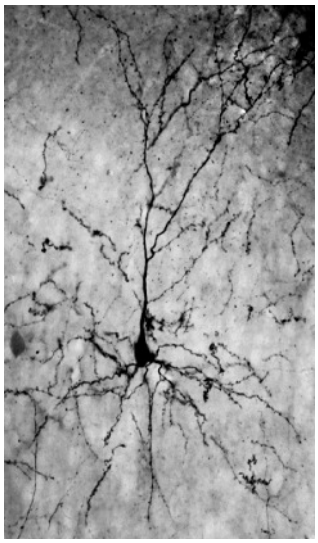
Dynamic changes in the development of prefrontal networks –neuroanatomical findings

Review of quantitative tracer- and immunohistochemical studies in the Mongolian gerbil (*Meriones unguiculatus)* rounded out by an epidemiological study in humans



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1 Summary

This essay of the cumulative doctoral thesis is based on four papers from which three result from my animal-experimental research in 2004-2006 at the university of Bielefeld and one from the clinical human research in 2006-2007 at the university medical centre of Münster. The purpose of the experiments was to examine the development and maintenance of plastic, environmental-adaptive cortical networks and in particular to analyse for the first time the postnatal maturation of the exzitatory pyramidal cells in the prefrontal cortex (PFC) on a structural level.

The PFC guarantees as phylogenetic latest area of the central nervous system higher functions like purposive actions and complex social behaviours. The prefrontal integration of multimodal inputs at highest level is processed by very selective inter- as well as intralaminar circuits between anatomical and functional different glutamatergic pyramid cells and local interneurons in higher modules.

The findings of our first long-time study (Witte et al., 2007a) admit for the cortex first detailed conclusions about the basic developmental events in the output system of the PFC. In five juvenile age-groups, efferents of the prefrontal pyramidal cells were labelled with the anterograde tracer biocytin and quantitatively evaluated in the ipsilateral frontal (FC), parietal (PC), agranular insular (AIC), and dysgranular insular (DIC), as well as in the contralateral prefrontal cortex (CON). Cortical fibre densities originating from prefrontal layer III- and layer V/VI-pyramids appeared to follow a separate and highly dynamic course during postnatal development: The growth of layer III-efferents paused at a low level up to sexual maturity, after which fibre densities exhibited a significant increase in the FC and DIC to adult values at young adulthood on day 90. In contrast, layer V/VI-efferents showed a decline of initial high fibre densities between the time of eye-opening and day 19-24, statistically significantly in the AIC. Afterwards, fibre densities described a re-growth around sexual maturity, significantly in the AIC, FC and DIC. The findings were discussed in relation to morphogenetic effects of ingrowing thalamic glutamatergic and mesenzephalic dopaminergic afferents during selective time windows.

The second biocytin study (Witte et al., 2007b) provided first-time evidence regarding the callosal prefrontal system that this selective maturation is severely impaired due to epigenetic interventions (1. an early methamphetamine-intoxication, 2. socially isolated rearing). Each intervention led to reduced efferent layer V/VI-fibre densities in the CON compared to control animals from enriched rearing. The combined treatment however, consisting of methamphetamine-intoxication *and* isolated rearing, inverted these single effects in a turn-

over fashion so that layer V/VI-efferents showed highly increased fibre densities in the CON. No alteration was to be recognised regarding layer III-efferents. These findings extend former knowledge from our two-hit animal model describing a pathological "*dys*connection" of the two prefrontal output systems as one neuro-anatomical correlative of the malfunctions in patients with schizophrenia (Bagorda et al., 2006), which was postulated hypothetically by Weinberger and Lispka (1995): The normal decline of layer V/VI-fibre densities in the third postnatal week is presumably missing due to massive disturbances by the combined intervention.

In another long-time study (Brummelte, Witte et al. 2007), I participated in evaluating the maturation of inhibitory GABAergic interneurons in limbic areas. By means of immunohistochemical staining methods, it has been shown that after a first strong growth up to weaning the GABAergic fibre densities in the PFC increased even further until old adult ages on day 520. This steady fibre-rebuilding could account for the key role in the establishment and lifelong maintenance of environmental-related functional networks ascribed to local GABAergic circuits (Hensch, in 2005).

The fourth study (Flöel, Witte et al., submitted) questions whether the powerful meaning of the environment is also transferable to cognitive functions in humans. The investigation of novel cost-efficient strategies which help to prevent age-related cognitive decline is of immense clinical importance. In this cross-sectional study we detected by means of neuro-psychological testings in 420 healthy elderly people a significant positive association of a healthy lifestyle (documented by regular physical activity, a low-calorie diet, non-smoking, as well as moderate consumption of alcohol) and a better memory performance even after adjusting for age, education and gender.

These results of the applied human research underline the strong influence of the environment on cortical plasticity. In addition, the animal-experimental studies point out that external environmental factors can considerably affect - as a consequence of the long continuing prefrontal maturation not only regarding dopaminergic afferents but also, as found, regarding glutamatergic efferents - this prolonged development of the PFC.

2 Zusammenfassung

Diese zusammenfassende Abhandlung der kumulativen Promotion basiert auf vier Publikationen, von denen drei aus meiner tierexperimentellen Forschungsarbeit von 2004-2006 an der Universität Bielefeld resultieren und eine aus der klinischen Humanforschung 2006-2007 am Universitätsklinikum Münster. Ziel der experimentellen Arbeiten war, die Entwicklung und Aufrechterhaltung von plastischen, umwelt-adaptiven kortikalen Netzwerken zu untersuchen und insbesondere erstmalig die postnatale Reifung der exzitatorischen Pyramidenzellen im präfrontalen Kortex (PFC) strukturell zu analysieren.

Der PFC gewährleistet als phylogenetisch jüngster Bereich des zentralen Nervensystems übergeordnete Funktionen wie zielgerichtete Handlungen und komplexes Sozialverhalten. Die präfrontale Integration von multimodalen Eingängen auf höchster Ebene wird durch eine sehr selektive inter- sowie intralaminäre Verschaltung zwischen anatomisch und funktionell unterschiedlichen glutamatergen Pyramidenzellen und lokalen Interneuronen in höhergestellten Modulen ermöglicht.

Die Befunde unserer ersten Langzeitstudie (Witte et al., 2007a) lassen für den Kortex erstmalig detaillierte Rückschlüsse über die zugrundeliegenden Reifungsgeschehnisse im Ausgangssystem des PFC zu. In fünf juvenilen Altersgruppen wurden die Efferenzen der präfrontalen Pyramidenzellen im ipsilateralen frontalen (FC), parietalen (PC), agranulär insulären (AIC) und dysgranulär insulären (DIC) sowie im kontralateralen präfrontalen Kortex (CON) mittels des anterograden Tracers Biocytin angefärbt und quantitativ ausgewertet. Es zeigte sich, dass die Faserdichten aus den Lamina III- und aus den Lamina V/VI-Pyramiden einen separaten und hoch dynamischen Verlauf in ihrer postnatalen Entwicklung nehmen: Das Wachstum der Lamina III-Efferenzen verharrte auf einem niedrigen Niveau bis zur Geschlechtsreife, wonach die Faserdichten signifikant im FC und DIC auf adulte Werte im jungen Erwachsenenalter am Tag 90 anstiegen. Die Lamina V/VI-Efferenzen zeigten dagegen einen Rückgang der anfangs hohen Faserdichten zwischen dem Zeitpunkt der Augenöffnung und Tag 19-24, statistisch signifikant im AIC. Im Folgenden beschrieben die Faserdichten ein erneutes Wachstum um die Geschlechtsreife, signifikant im AIC, FC und DIC. Die Befunde wurden im Hinblick auf die morphogene Wirkung der einwachsenden thalamischen glutamatergen und der mesenzephalen dopaminergen Afferenzen während selektiver Zeitfenster diskutiert.

In einer zweiten Biocytin-Studie (Witte et al., 2007b) konnte für das callosale präfrontale System erstmals nachgewiesen werden, dass diese selektive Reifung durch umweltbezogene Interventionen (1. eine frühe neurotoxische Gabe von Methamphetamin, 2. sozial isolierte

Aufzucht) maßgeblich beeinträchtigt wird. Jeweils eine der Aufzuchts-Interventionen führte zu einer Absenkung der efferenten Lamina V/VI-Faserdichten im CON verglichen mit Kontrolltieren aus Gehegeaufzucht. Die kombinierte Behandlung dagegen, bestehend aus Methamphetamin-Intoxikation *und* deprivierter Aufzucht, invertierte diese Einzeleffekte in besonderem Maße, so dass die Lamina V/VI-Efferenzen stark erhöhte Faserdichten im CON aufwiesen; keine Beeinträchtigung war für die Lamina III-Efferenzen zu erkennen. Diese Befunde erweitern frühere Erkenntnisse an unserem zwei-Phasen Tiermodell zur pathologische „*Dys*konnektion" der zwei präfrontalen Ausgangssysteme als neuro-anatomisches Korrelat der Funktionsstörungen bei Patienten mit Schizophrenie (Bagorda et al., 2006), was hypothetisch von Weinberger und Lispka (1995) postuliert war: Vermutlich bleibt der normale Rückgang der Lamina V/VI-Faserdichten in der dritten postnatalen Woche aufgrund der massiven Störungen durch die kombinierte Intervention aus.

In einer weiteren Langzeitstudie (Brummelte, Witte et al. 2007) habe ich die Reifung von inhibitorisch aktiven GABAergen Interneuronen in limbischen Arealen mituntersucht. Mittels immunhistochemischer Färbemethoden konnte gezeigt werden, dass die GABAergen Faser-dichten im PFC nach einer ersten starken Zunahme bis zur Entwöhnung noch weiter bis ins hohe Erwachsenenalter am Tag 520 anstiegen. Dieser stetige Faserumbau könnte eine Grund-lage für die Schlüsselrolle sein, die den lokalen GABA-Netzen in der Entstehung und lebens-langen Aufrechterhaltung funktioneller Netzwerke zugeschrieben wird (Hensch, 2005).

In der vierten Studie (Flöel, Witte et al., submitted) geht es um die Frage, ob die große Bedeutung der Umwelt auch auf Gehirnleistungen beim Menschen übertragbar ist. Die Erforschung von neuen, kostengünstigen Strategien, die den altersbedingten kognitiven Abbau verhindern helfen, ist von immenser klinischer Bedeutung. In dieser Querschnittsstudie konnten wir bei 420 älteren, gesunden Personen mittels neuropsychologischer Testverfahren einen signifikanten positiven Zusammenhang von einem gesunden Lebensstil (dokumentiert durch regelmäßige körperliche Aktivität, eine kalorienarme Ernährung, Nichtrauchen sowie moderater Alkoholgenuß) und einer besseren Gedächtnisfunktion unabhängig von Alter, Bildung und Geschlecht aufzeigen.

Diese Ergebnisse der angewandten Forschung beim Menschen unterstreichen den starken Einfluss der Umwelt auf die Plastizität des Kortex. Die tierexperimentellen Studien zeigen außerdem, dass bedingt durch die besonders lang andauernde präfrontale Reifung nicht nur der dopaminergen Afferenzen sondern, wie gefunden, auch der glutamatergen Efferenzen äußere Umweltfaktoren diese verzögerte Entwicklung des PFC erheblich beeinträchtigen können.

3 Introduction

One of the most fascinating and likewise fundamental claims of neural science is that the activities of the brain generate our behaviour. Only a purposive, orchestral interaction of millions of individual nerve cells that comprise the nervous system can assure such diverse functions as sensory perception and motor coordination, but also more complex tasks like motivation and memory. Considering the molecular basis of how nerve cells interact, namely via electrophysical signals from one to another, a proper cognitive processing truly depends on precise intercellular connections. Like in the sensory-motor system, a cardinal feature of higher-order cortex organization in mammals including humans is the specific arrangement and selective inter- and intralaminar wiring of integrative networks and modules. Here, highly integrative processes are provided by a selective functional architecture of distinct subgroups of excitatory pyramidal output cells and numerous types of inhibitory interneurons.

Now the main question is, how these superior cytoarchitectonic patterns may emerge and become established during ontogeny, and in particular, how they are modified by the external environment. My studies were conducted to identify the particular role of the juvenile period and related epigenetic impacts in the generation and maintenance of well-functioning higher-order networks. Therefore, I investigated the structural maturation of prefrontal pyramidal cells and interneurons in gerbils (*Meriones unguiculatus*), which are known to have a very small genetic variability and a broad wild-type like behavioural repertoire (Rosenzweig and Bennett, 1969; Thiessen and Yahr, 1977). The prefrontal cortex (PFC) enables advanced functions like purposive and reflective behaviours in time and space, which obviously require an interactive attainment and highly integrative computation of multimodal, pre-processed sensory-motor and limbic information (Fuster, 1985; 1991; 2000; Lewis et al., 2002). To establish such experience-related functions, this phylogenetic youngest part of the cortex is naturally characterized by a very slow and prolonged developemtn. By means of persistent neuronal progression and reorganisation, structural organization and functional maturation continues even beyond adolescence in both rats and primates including humans (Diamond, 2002; Goldman-Rakic 1987; Chugani et al., 1987; Giedd et al., 1999 Crone et al., 2006; Goldman, 1971; Kolb, 1984; Kolb and Nonneman, 1976).

Since the maturation of this highest brain centre, the PFC, is embedded in several main other, successive and preceding processes, common aspects of brain development are briefly summarized in the following part.

3.1 Differentiation of neurons during development

Interconnections between specific nerve cells depend on the individual type and position of each cell within the nervous system. Intriguingly, mature neurons have to develop these diverse and highly specialized properties out of equal stem cells. Thus, both genetic and epigenetic programs are supposed to underlie a complex cortical development, which can be divided into multiple, sequential steps.

First, the neuronal plate, a columnar epithelium, derives from a sheet of ectodermal cells located along the dorsal midline of the embryo at the gastrula stage (Altman and Bayer, 1985). The formation of neuronal tissue is largely dependent on signalling molecules secreted by other non-neural neighbouring cells, which direct in a complex program the expression pathway of particular genes in the individual precursor cells. First evidence to this general developmental principle was found by Spemann and Mangold (1923) in amphibian embryos, who transplanted specific "organizer cells" close to other ectodermal cells, which led to dramatic changes in the fate of these host cells. More recently several inducing factors which occur also in humans could be identified not only by studies of frogs but also by flies and worms, underlining the remarkable similarity of nerve tissue development within phylogeny (Crutcher, 1986; Inan and Crair, 2007; Wisniewski, 1979; Wodarz and Nusse, 1998).

In vertebrates, the neuronal plate begins soon after to fold into the neural tube. Hereof uniform progenitor cells proliferate rapidly, migrate to their final positions and differentiate into immature neurons or glia cells in a well-defined, heterogeneous pattern along the neural tube (Bayer, 1989). At rostral position, three brain vesicles initiate the forebrain (later segmented into telencephalon and diencephalon), midbrain, and hindbrain (later segmented into metencephalon and myelencephalon), which are the major regions of the brain. The then forming cortical plate acts as progenitor for further cortical differentiation and lamination (Super et al., 1998). In the PFC, cortical lamination and immature neurons, with pyramidal cells being visible slightly earlier than non-pyramidal cells, become obvious during the first postnatal week in rats (van Eden and Uylings, 1985), and between the 26^{th} and 29^{th} week of gestation in humans (Mrzljak et al., 1988). However, adult morphology is not present before late in adolescence (Mrzljak et al., 1988; van Eden and Uylings, 1985).

The appropriate position of a nerve cell within the cortical plate is determined by the timing of its cell cycle, reflecting the frequently described inside-first, outside-last maturation pattern (Miller, 1985). Early newborn cells migrate out of the ventricular zones along radial glia cells to settle in the deep layers of the immature cortex, whereas cells that exit the cell cycle at later stages settle in more superficial layers (for review, see Aboitiz et al., 2001). Notably,

excitatory pyramidal cells migrate from dorsal aspects of the lateral ventricles (Tan et al., 1998), whereas inhibitory interneurons originate from rather ventral proliferative zones and travel therefore a considerable distance before entering the cortex proper (Anderson et al., 1997; Yung et al., 2002). Cortical cells generate in rats between embryonic day (ED) 15-19 (Berry and Rogers, 1965; Ignacio et al., 1995), in monkeys by the 6th week of gestation (Rakic and Nowakowski, 1981), and in humans by the 8th week of gestation (Molliver et al., 1973). It is known that at least in rats, prefrontal neurons do not appear more than two days later in the cortex than occipital neurons: a prominent neurogenetic peak occurs in the PFC on ED 17 for layer V- and on ED 18 for layer II/III-neurons, respectively (Van Eden and Uylings, 1985a), whereas occipital neurons appear between ED 15 and 17 (Bruckner et al., 1976). Therefore specific programs that control especially the start of prefrontal neurogenesis appear to be less likely. However, the end of neuronal differentiation in the PFC seems to differ from that of other brain areas, since it is highly prolonged in rats and monkeys including humans (Mrzljak et al., 1988; Van Eden and Uylings, 1985a).

The individual fate of a neuron also depends on its localisation within the nervous system, since adjacent cells and integrated systems serve as inducing factors. Thus in the PFC, the excitatory glutamatergic projection from the medial thalamus (MD) is suggested to control local cell differentiation (van Eden et al., 1990). This phenomenon has clearly been described e.g. in the rodent somatosensory cortex, whose characteristic barrel fields develop soon after birth even if prospective visual cortex is transplanted at this position (Schlaggar and O'Leary, 1991). Moreover, other systems that carry the neurotransmitters dopamine (DA), γ-amino-butyric-acid (GABA), serotonin (5-HT), acetylcholine (ACh), and noradrenalin (NA) are likewise supposed to act as morphogenetic factors during development (Ben Ari et al., 1997; Kalsbeek et al., 1987; Lauder, 1988; 1993; Nguyen et al., 2001; Owens and Kriegstein, 2002a; Owens and Kriegstein, 2002b; Sodhi and Sanders-Bush, 2004). Figure 1 schematically depicts mediators of brain organization in both rodents and man, which contribute essentially in the course of development to successive processes like neuronal progression, compensation, and reorganisation. Thus, the linkage between neuronal structure and functional outcome is a key aspect in understanding the establishment of systemic interactions mediated by higher-order prefrontal networks (see e.g. Lewis et al., 2002).

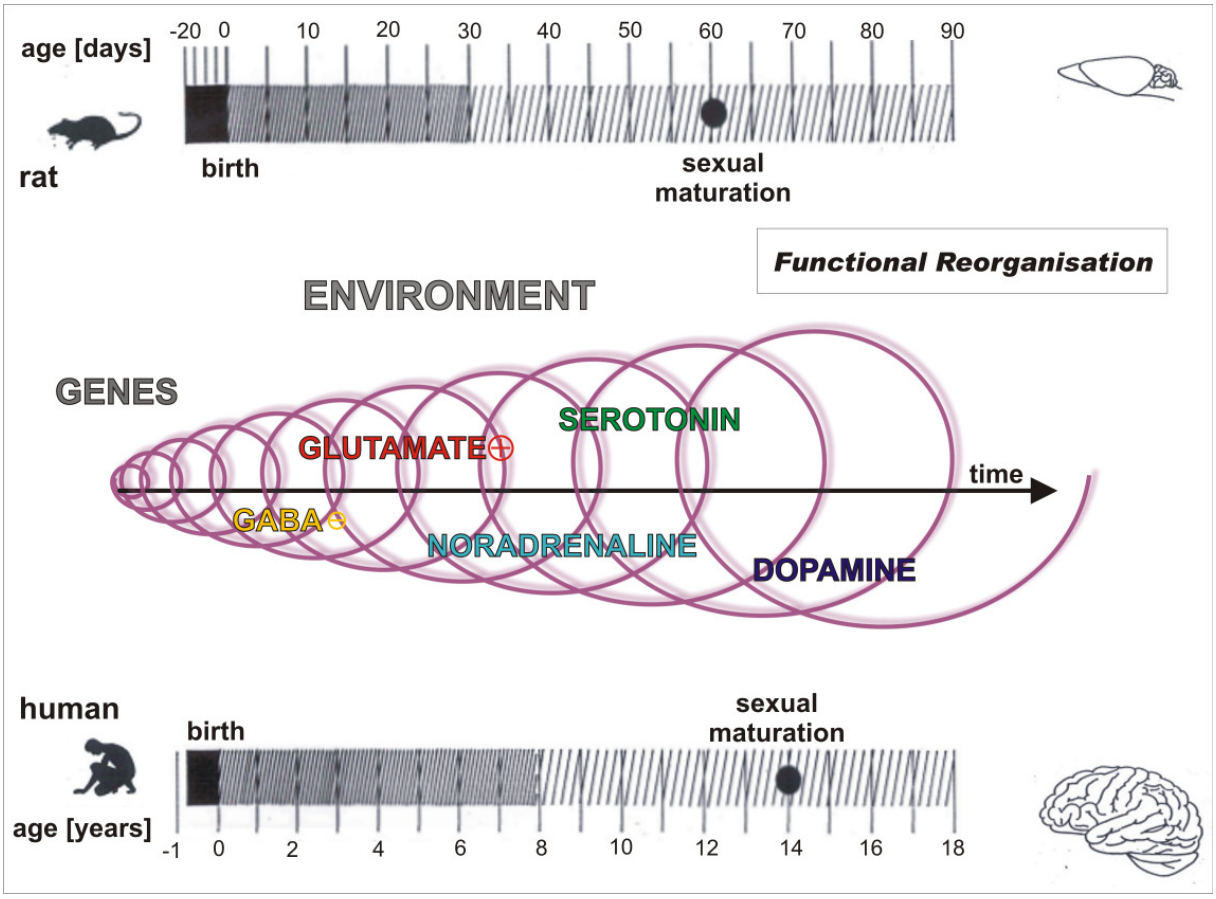

Figure 1: Beside genetic factors, morphogenetic transmitters like glutamate, GABA, noradrenaline, serotonin and dopamine, as well as the external environment contribute to the establishment of neuronal networks. Growing systems and epigenetic factors permanently induce functional reorganisation processes, thereby contributing to higher-order prefrontal networks in the course of a prolonged postnatal maturation.

As described in the compensatory theory by Wolff and Wagner (1983), ingrowing fibres in combination with experience-dependent input-changes of the periphery lead permanently during development to the perturbation of transient networks and according reorganisation processes within neuronal networks. This self-organizing principle allows neuronal circuits via stabilising mechanisms to establish functionally integrated, efficient contacts and to enable life-long plastic capacities as well as adequate and purposive behaviours (Huether, 1996; Schnupp and Kacelnik, 2002; Wolff et al., 1995; Wolff and Missler, 1992; Wolff and Wagner, 1983). Consistently, neuronal homeostasis has been described by electrophysiological studies (Turrigiano et al., 1998), and the morphogenetic potential induced by maturing transmitter systems and hormones has become well-analyzed in the last decades (Azmitia, 1999; Herlenius and Lagercrantz, 2001; Herlenius and Lagercrantz, 2004; Lauder, 1993; Sodhi and Sanders-Bush, 2004). In addition, it is supposed that especially the morphogenetic role of the transmitter DA (Lauder, 1988; 1993) contributes to the exceptional status of the PFC: DAergic afferent fibres arising from the ventral tegmental area were found

to mature at least in rodents and monkeys considerably fast during early postnatal life, but to establish their final termination patterns not earlier than late in adolescence or even in young adulthood (Fig. 2; Dawirs et al., 1993; Kalsbeek et al., 1988; Lewis et al., 1998).

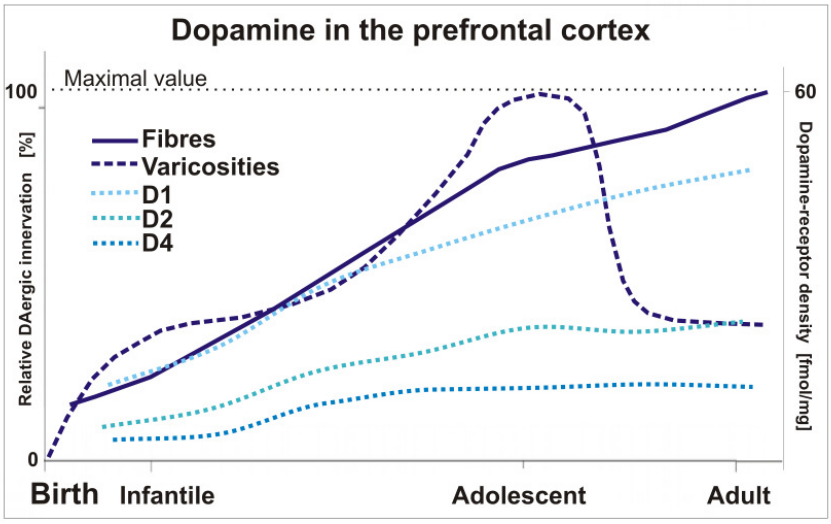

Figure 2: Aspects of the postnatal maturation of dopaminergic innervation and receptors in prefrontal areas in the gerbil (fibre densities, solid dark blue line), in the monkey (varicosities, broken dark blue line), and in the rat (dopamine-D1, -D3, and -D4 receptors, broken light blue lines). Note the extended fibre growth even beyond adolescent and the prolonged increase in receptor densities, adapted from Dawirs et al. (1994), Kaalsbeek et al. (1998), Lewis (1998), and Tarazi and Baldessarini (2000).

Thus, particularly the prefrontal system is exposed to changing conditions at a structural level even until late in development. On the one hand, this underlines a considerable prolonged vulnerability of prefrontal circuits. On the other hand, this prolonged development might also reflect an essential mechanism, which enables a more advanced level of cortical organization (Kostovic, 1990). Accordingly, the PFC obtains the exceeding possibility to re-act on and to integrate a broad variety of different, successive and activity-dependent neuronal inputs during maturation. As a result, prefrontal networks are able to generate environmentally adaptive, higher-order functions, which might also require specific neuronal properties and molecular pathways.

3.2 Generation of specific neuronal properties

Aside from their proper local morphogenetic surrounding, provided e.g. by glutamatergic, GABAergic or DAergic inputs, nerve cells have to be equipped with highly specialized, target-matched molecular properties. Evidence that inducing factors of synaptic targets may modulate for example the respective presynaptic transmitter phenotype rose from studies in sympathetic motor neurons (Doupe et al., 1985). Moreover, it is supposed that distinct

subclasses of parvalbumin-containing GABAergic interneurons get their specific molecular features first at juvenile age, which might be induced by factors of their local surrounding (Miller et al., 1991). Signals of the neuronal environment were also found to exert critical influence on the individual survival of a nerve cell, as first described in the amphibian dorsal root ganglion (Detwiler, 1937), and in the spinal chord of chicken embryos (Cohen et al., 1954; Hamburger and Levi-Montalcini, 1949). In addition, the latter studies observed that 50% of spinal motor neurons die even under normal conditions, which was termed as apoptosis (Hamburger and Levi-Montalcini, 1949). Since then, selective cell death has frequently been reported as a crucial event during normal brain (for review, see Buss et al., 2006).

Simultaneously, a broad variety of so-called neurotrophic factors were identified to promote neuronal survival. These proteins are secreted in a limited amount by target cells in neurons of the basal forebrain, the hippocampus, and the neocortex; for example neurotrophins including nerve growth factor, brain-derived neurotrophic factor (BDNF), neurotrophin 3, and neurotrophin 4/5; interleukin 6-like cytokines, transforming growth factors, fibroblast growth factors, hepatocyte growth factors, as well as BDNF- and neurotrophin 3-selective receptors (Katz and Shatz, 1996; Lessmann, 1998; Lykissas et al., 2007). Recent studies provide evidence that neurotrophic factors might inhibit the endogenous “apoptosis pathway” of a neuron, e.g. via suppressing the activation of particular proteolysis-inducing enzymes such as caspases (Jellinger, 2006). Moreover, BDNF is supposed to regulate the expression of important transmitter-related DAergic proteins, such as different NMDA-receptor subunits and DA-D3 receptors (for review, see Bustos et al., 2004). Neurotrophins might also operate as locally released feedback modulators of synaptic transmission, which could be a cellular correlate for certain aspects of higher prefrontal networks (Bustos et al., 2004).

Thus, it was found that a higher BDNF synthesis (e.g. induced by antidepressant agents) leads to an increased DAergic signalling in hippocampal and also in prefrontal areas (Castren et al., 2007).

3.3 Growth of proper axonal projections

Another synaptic target-related feature during neuronal development is the purposive growth of axons to create highly specific connections between distinct cortical areas and cells. Early experiments in the visual system of frogs showed that retinal axons orientate towards intrinsic anatomical conditions when regenerating, even if the behavioural outcome is inadequate (Sperry, 1943). Therefore, the initial (embryonic) wiring of axons is supposed to depend

rather on chemical matching than on experience-validated random connectivity (Sperry, 1945). However, functional activity is still thought to play a major role in modifying neuronal circuits (for review, see e.g. Waites et al., 2005).

The underlying molecular processes of how axons could find their discrete targets throughout the nervous system was considered first by Ramon y Cajal (1890a; 1890b): He presumed that the axonal growth cone, a protuberance of the elongating axon, combines both sensory and motor functions to find its appropriate path. Thus, already existing axonal fibres could serve as a template for the growth of later born axons to the same target. Moreover, a variety of other guiding signals in the complex environment should be necessary not only for the initial axon to avoid obstacles and to find its specific way (for review, see de Castro et al., 2007). Indeed, several molecular cues classified as short- or long-range, axon-attracting or -repelling factors have been characterized in the last decades (Benarroch, 2007; de Castro, 2003; Dickson, 2002; Kennedy and Tessier-Lavigne, 1995; Skene, 1989; Tessier-Lavigne and Goodman, 1996). For instance, heterotrimeric laminins are components of the basal laminae of the extracellular matrix, which interact with matrix-binding proteins on the motile filopodium of the axonal growth cones, e.g. the integrins (Nakamoto et al., 2004). Both laminins and integrins comprise discreet heterogenous groups of slightly different molecules, which contribute to a selective recognition during axonal growth (Sefton and Nieto, 1997).

For the sensory input to the cortex, it is supposed that limbic-associated membrane protein, cadherins, ephrins and Eph receptors, neurotrophins, netrin 1 and semaphorins guide thalamocortical axons on their way (for review, see Lopez-Bendito and Molnar, 2003). Yet, the molecular mechanisms that underlie higher cortical differentiation are widely speculative (Price et al., 2006). Moreover, the direction and polarity of a guidance cue may also vary between different sets of nerve cells and guiding structures, pointing to a rather complex than simple pathfinding process (de Castro, 2003; Kennedy, 2000). Consistently, it is supposed that cue-binding proteins in the growth cone membrane initiate a variety of different second messenger cascades, which are likely to contribute to the attraction or retraction procedure of the axon (Tessier-Lavigne and Goodman, 1996). For instance, calcium acts as an important second messenger, since it promotes growth cone motility at most on a specific local concentration (Henley and Poo, 2004). Therefore, different concentrations of calcium within the growth cone (induced by cue-binding receptors in its membrane) should guide the cytoskeleton to advance in the calcium-rich direction (Dent et al., 2003). Thus, regionally different intrinsic programs, which should exist since very early in development, might

contribute to the later regional specialization of the cortex, e.g. via selective expression of particular receptor genes in specific areas (Cohen-Tannoudji and Babinet, 1994).

In summary, several smart and even highly potent molecular mechanisms are supposed to underlie axonal guidance during development, which to date lack at least regarding different cortical areas their specific discrimination. Huffman et al. (2004) suggested as possible candidates for the segregation of cortico-cortical connections the transcription factor Id 2 and the orphan receptor RZRb. However, further research is needed to discover guiding molecules that modulate e.g. the specific growth of prefrontal afferent and efferent fibres. Therefore, one main precondition might be a better understanding of structural pattern changes during the maturation of prefrontal networks, which gave rise to the idea of my studies.

3.4 Specification of neuronal connections

Well-functioning neuronal processes depend even at a submicron level on highly precise intercellular contacts. Therefore, the forming and modifying of prefrontal synapses play an essential role in enabling higher order networks and plastic capacities during the whole lifetime. Although synaptic components can be independently formed by axons and target structures (Varoqueaux et al., 2002; Verhage et al., 2000), synaptogenesis is thought to be a highly interactive process (Knott et al., 2002; Rajan and Cline, 1998; Schmidt et al., 2004; Trachtenberg et al., 2002). To enable sufficient functionality and stability in time and space, both pre- and postsynaptic elements exchange diverse molecular and electric signals, which in turn induce further differentiation of the opposite structure. Yet, the absence of specific cues inhibits the organisation of the synaptic contact. For example, motor nerve terminals secrete so-called neuroregulins and agrins, which induce the synthesis and clustering of acetylcholine-receptors in the postsynaptic muscle membrane, whereas laminins and soluble trophic factors attract the presynaptic terminals (for review, see Waites et al., 2005). Regarding cortical neurons, detailed studies about specific molecular cues that induce for example DAergic synapse forming in the PFC are still missing.

After establishing these specifications, transmission activity is indispensable for the permanence of a synapse. Since it is known that the elimination of exuberant synapses is a crucial phenomenon during normal brain development, it appears to be likely that only effective synapses persist and prevail over rather inactive contacts (Hua and Smith, 2004; Huttenlocher et al., 1982; Lichtman and Colman, 2000; Miyazaki et al., 2004; Rakic et al., 1986). Consistently, early studies showed that the activity-dependent pruning of synapses underlies the organisation of ocular dominance columns in the visual cortex (LeVay et al.,

1980; Shatz and Stryker, 1978). This emphasizes the importance of stimulus-triggered competition and cooperation between different sets of nerve cells within a neuronal network. Regarding visual development, only a synchronous firing of neighbouring thalamocortical afferents should lead to an intense and therefore sufficient depolarisation of the target cell to activate its NMDA-receptors. As a consequence, it seems likely that the target cell releases via NMDA-coupled second messenger cascades retrograde neurotrophic factors. These molecules are then taken up exclusively by the active terminals during endocytic processes (Constantine-Paton et al., 1990). Subsequently, the inactive presynaptic terminals should retract due to a lack of neurotrophic substances, revealing an insightful mechanism how growing axonal terminations become specialized.

Likewise in prefrontal areas, Anderson et al. (1995) observed a substantial decrease in dendritic spine densities of pyramidal layer III-neurons even beyond puberty in the monkey, which points to a pruning of postsynaptic contacts. In addition, recent studies of the rodent barrel cortex provide evidence that activity-dependent regulation of synapse formation and elimination occurs also in the mature brain (Knott et al., 2002; Trachtenberg et al., 2002). Regressive events beyond synapse pruning, such as the elimination of transient axonal connections, have been shown to occur in a variety of species and in diverse brain areas exclusively during development (Innocenti and Price, 2005). For example, Price and Blakemore (1985) described that a considerable withdrawal of associational projection fibres originating from deeper cortical layers occurs in visual areas of three weeks old kittens using retrograde tracer methods. As a major outcome of my studies (Witte et al., 2007a), I found also prefrontal efferents to be subjected to structural changes and regressive events during development.

In summary, it seems likely that progressive as well as regressive events play an essential role during maturation and regeneration of the CNS (Cowan et al., 1984; Murakami et al., 1992; Price et al., 2006). Thus, activity-dependent structural and neuron (re-)organisation processes account for the remarkably adaptive features of developing brains. Together, these findings underline the above mentioned compensatory theory of ongoing neuronal reorganisation as a basic principle. The study of transient patterns should help to elucidate the complex relationships between progression, regression, and reorganisation (Cowan et al., 1984; Kostovic, 1990) and might also provide evidence for detecting selective time windows, in which the functionally integrated development takes critical epigenetic stimulations into account.

3.5 Epigenetic factors and sensitive periods

The establishment and fine-tuning of for instance visual synapses appear to happen in predefined time windows during development, after which appropriate activity is less essential for the formation of well-functioning networks (LeVay et al., 1980). Behavioural evidence for sensitive periods during development arose from the phenomenon of imprinting, which was described most impressively by Konrad Lorenz in parent recognition of grey geese (Lorenz, 1937), but also by others in a broad variety of species and different behaviours (for review, see Hess, 1972; Knudsen, 2004). For example, it was found in birds that song melodies (Marler, 1970) and prospective mating objects (Immelmann, 1972) become imprinted at infantile age. In humans, language acquisition (Oyama, 1976) and parent bonding (Leidermann, 1981) relate also to early sensitive periods. Moreover, it is suggested that later social and emotional behaviours crucially depend on early learning processes (Bischof, 2007; Immelmann, 1972; Jones et al., 2000; SCOTT, 1962).

The underlying mechanisms are again related to the structural maturation, since various studies found dendritic, axonal and/or synaptic changes to occur during sensitive periods in the respective brain areas (Antonini and Stryker, 1993; Goldman-Rakic, 1987; Hensch and Stryker, 2004; Horn, 1998; Horn, 2004; Hubel and Wiesel, 1964; Scheich, 1987; Teuchert-Noodt et al., 1991). The visual critical period, for example, is supposed to depend on the (stimulus-triggered) thalamic input to cortical layer IV (Antonini et al., 1998; Daw et al., 1992; Trachtenberg and Stryker, 2001) and also on local GABAergic circuits (Hensch and Stryker, 2004). As to the human PFC, the morphogenetic potential of DA might induce these essential structural changes during a prolonged postnatal development (Kuboshima-Amemori and Sawaguchi, 2007). Consistently, recent longitudinal imaging data found that high-level cognitive abilities in young adults are crucially related to long-lasting dynamic properties of cortical maturation in the PFC (Shaw et al., 2006). Since DA is supposed to mediate primarily experience-dependent learning processes (Bao et al., 2001), the required plasticity of prefrontal networks seems very likely to depend on somewhat higher-order epigenetic stimulation (Kuboshima-Amemori and Sawaguchi, 2007).

Thus, sensitive phases should be controlled by intrinsic structural factors, which might be crucially modulated by the environmental offer (Kandel, 2001; Mower and Christen, 1985; Zhou et al., 2003). Also abnormal experience can appallingly affect the establishment of neuronal networks during these sensitive periods, which is likely to cause maladaptive and even pathologic behaviours. For instance, it was found in the kitten that visual deprivation during the critical postnatal period causes visual de-structuring (Hubel and Wiesel, 1964) and

an irreversible loss of sensory abilities (Crair et al., 1998). Moreover, a lack of adequate environmental stimulation during juvenile development in rodents leads to alterations in working memory (Winterfeld et al., 1998) and stereotypic or aggressive behaviours also in primates including humans (Ridley and Baker, 1982; Rosenzweig and Bennett, 1996). Together, numerous disadvantages in cognitive and social behaviours depend on environmental deprivation and traumatic experience (Hall, 1998; Lapiz et al., 2003). Traumata are also assumed to act as developmental risk factors for later psychopathologies (Lehmann et al., 2002; for review, see Sanchez et al., 2001).

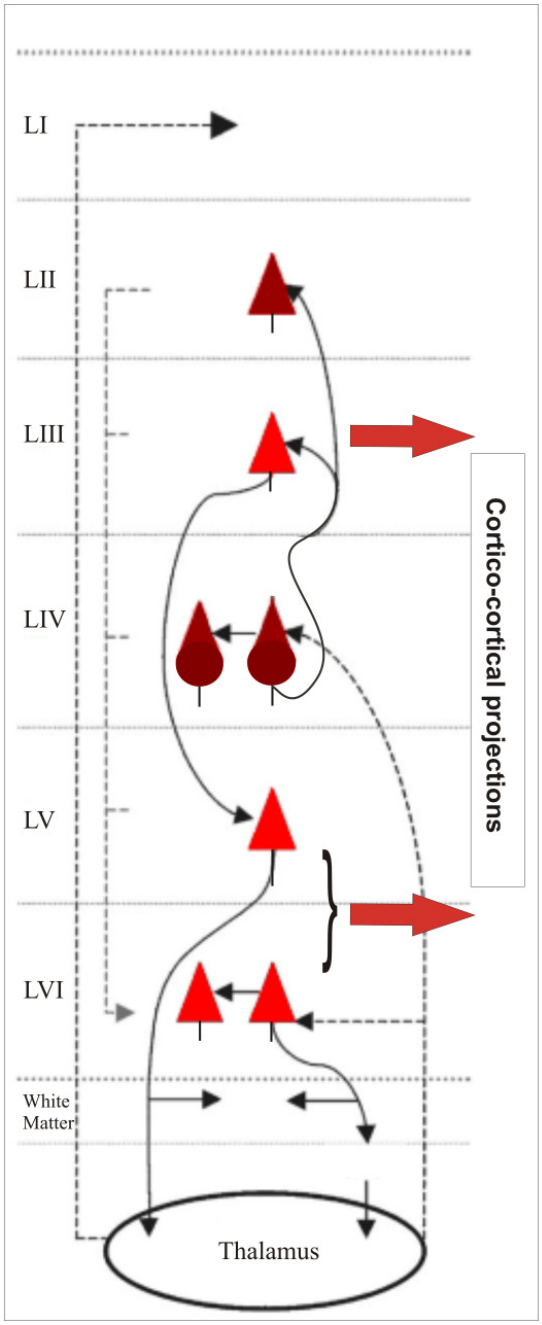

Figure 3: Principle of information flow within a cortical column. Cortico-cortical projections arise from glutamatergic layer III- and layer V/VI-pyramidal cells. Adapted from Bannister, 2005.

Even most dramatic cognitive disorders like schizophrenia, which are supposed to be somewhat genetically predisposed, are presumably triggered or provoked by adverse epigenetic circumstances (Kendler and Eaves, 1986; Raedler et al., 1998; Tienari et al., 2004; Weinberger and Lipska, 1995). Those disorders should be related to mal-development especially in the PFC (Jones, 1997; Levin, 1984; Lewis et al., 2003; Lewis and Moghaddam, 2006; Shelton et al., 1988; Weinberger et al., 1986). In the so-called glutamate-hypothesis, it is further assumed that either a glutamatergic hypo- and/or hyperfunction might evoke psychotic symptoms (for review, see Stephan et al., 2006). Glutamate is the main excitatory transmitter in the brain, expressed primarily in the pyramidal cells which comprise the layers of the cortex (Cavanagh and Parnavelas, 1988; Jones, 1984). Figure 3 pictures a simplified scheme of the cortical integration and “feed-forward” processing of information as realized by pyramidal cells (see e.g. Bannister, 2005). Thus, thalamic

inputs enter the cortical column in layer IV at spiny stellate-like pyramids and are then relayed to layers II and III. Layer III and layers V and VI represent the principal pyramidal output layers. Originating in layers II and III, axon collaterals signal to layers V and VI. After additional processing via fascilitation or inhibition of interneurons and crucial monoaminergic modulations, highly integrated orders are eventually transferred to other cortical and subcortical targets. Layer V- and VI-fibers form mainly (but not exclusively) long-range projections to distant cortical and subcortical targets, whereas layer III-projections rather innervate adjacent and distant ipsilateral and corntralateral areas of the cortex. Interestingly, layer III- and layer V/VI-projections comprise not only anatomically but also functionally distinct output layers. Moreover, these hierarchically different systems are suggested, yet on a speculative level, to develop by seperate rules of maturation in time and quality (Price et al., 2006).

3.6 Previous findings and key questions of the present work

Our research group has extensively studied in an animal model of psychosis the cytoarchitectonic changes of glutamatergic pyramidal output neurons and other transmitter systems such as GABA in limbic and prefrontal networks after early traumatic experiences (Bagorda et al., 2006; for review, see Brummelte et al., in prep.). Therefore, Mongolian gerbils (*Meriones unguiculatus*) received either an early neurotoxic methamphetamine-challenge on PD 14 and/or were exposed to chronic social deprivation after weaning (PD 30) up to young adulthood (PD 90). As previously shown, these adverse rearing conditions led in adult animals to dramatic structural alterations in the DA innervation of the PFC (Dawirs et al., 1994; Neddens et al., 2001; Teuchert-Noodt and Dawirs, 1991; Winterfeld et al., 1998). In addition, the suppressed prefrontal DA maturation is supposed to cause further extensive changes not only in other mesolimbic and mesocortical DAergic projections (Busche et al., 2004; Lehmann et al., 2002; Neddens et al., 2002), but also in the serotonin (Busche et al., 2002; Lesting et al., 2005; Neddens et al., 2003; Neddens et al., 2004), the acetylcholine (Busche et al., 2006), and the GABA system (Brummelte et al., 2007a; Nossoll et al., 1997). As another and even more direct consequence, the early non-invasive interventions were found to evoke a miswiring of prefrontal efferents to frontal, parietal, and dysgranular insular cortices in the ipsilateral hemisphere (Bagorda et al., 2006): Layer III-axonal fibres adapt considerably different to epigenetic challenge than layer V/VI-axons, which was most evident in a highly imbalanced increase in layer V/VI-fibre densities of distant insular target areas. At closer glance, it seems likely that these findings point to a dysfunctional cognitive processing

which might also account for the dysfunctions described in humans, depending on a structural prefrontal "*dys*connection" (Bagorda et al., 2006).

Now, my studies focused on different aspects concerning prefrontal network (mal-) development to complete the previous results and to provide potential explanations of underlying processes:

(1) How and at what developmental time windows could adverse postnatal interventions affect the maturation of the prefrontal glutamatergic output system?

In addition, is it possible to identify vulnerable steps during the postnatal development of the two major prefrontal projection systems?

To what extent do possible structural changes occur after early interventions in contralateral target areas of prefrontal output fibres?

(2) What are possible causes and consequences of a maladaptive glutamatergic development in relation to other transmitter systems?

In more detail, is the postnatal development of the main inhibitory transmitter, the GABAergic system, linked with the pyramidal maturation in the PFC?

(3) Is it possible to transfer the powerful influence of environmental factors on cortical development even to the aging brain in humans?

Thus, is a healthy epigenetic surrounding, provided by regular physical activity, a low-caloric diet, moderate alcohol drinking, and non-smoking related to better higher-order cognitive performance in the elderly?

4 The postnatal development of prefrontal networks

To answer these questions, a large part of my work dealed with the maturation and mal-adaption of the excitatory pyramidal output system in gerbils (Witte et al., 2007a; 2007b), and I investigated the postnatal development of inhibitory GABAergic interneurons (Brummelte, Witte et al. 2007). Thus, I quantitativly evaluated those relevant aspects of the prefrontal system, which provided further insights into how and when epigenetic challenge might crucially affect higher-order prefrontal networks. Since environmental interventions should also lead to structural changes in the human brain and throughout life, I subsequently focused on the relation between a healthy epigenetic lifestyle and better cognitive performance in the elderly in a subsequent observational clinical study (Flöel, Witte et al., submitted).

4.1 Maturation of pyramidal efferents (Witte et al., 2007a)

I conducted the first tracer study to examine seperately the development of layer III- and layer V/VI-corticocortical connections in gerbils from infantile until adult age, subsequent to previous experiments (see also Bagorda et al., 2006; Witte et al., 2007b). Therefore, 148 male animals were used for investigation, covering convincing life-span periods at the age of PD 15-19 (infantile, n = 31), PD 20-24 (early juvenile, n = 29), PD 25-29 (pre-weaning, n = 18), PD 30-49 (post-weaning, n = 45), around sexual maturity on PD 60-79 (adolescent, n = 13) or PD 90 (adult, n = 12). Axonal fibres of pyramidal cells situated in prefrontal layer III and layers V and VI were labelled with the anterograde tracer biocytin and stained by standard 3.3-diaminobenzidine (DAB) reaction. For details of the materials and methods used, please see Bagorda et al. (2006) and Witte et al. (2007b).

4.1.1 Distribution of pyramdial efferents in the PFC

Prefrontal efferents in juvenile gerbils appeared to innervate somewhat similar cortical areas as has been previously described in adult gerbils (Bagorda et al., 2006), rats (Sesack et al., 1989), and in monkeys (Selemon and Goldman-Rakic, 1988). Yet, layer III-labelling led to a similar pattern compared to layer V/VI-labelling, although slightly fewer fibres were observed following layer III-labelling. Figure 4 gives a scheme of prefrontal layer III- (fine red line) and layer V/VI-efferents (thick red line) in the gerbil. As a supplement to the report and the illustrations in the publication (see Fig. 2-6, Witte et al., 2007a), some additional observations are documented below.

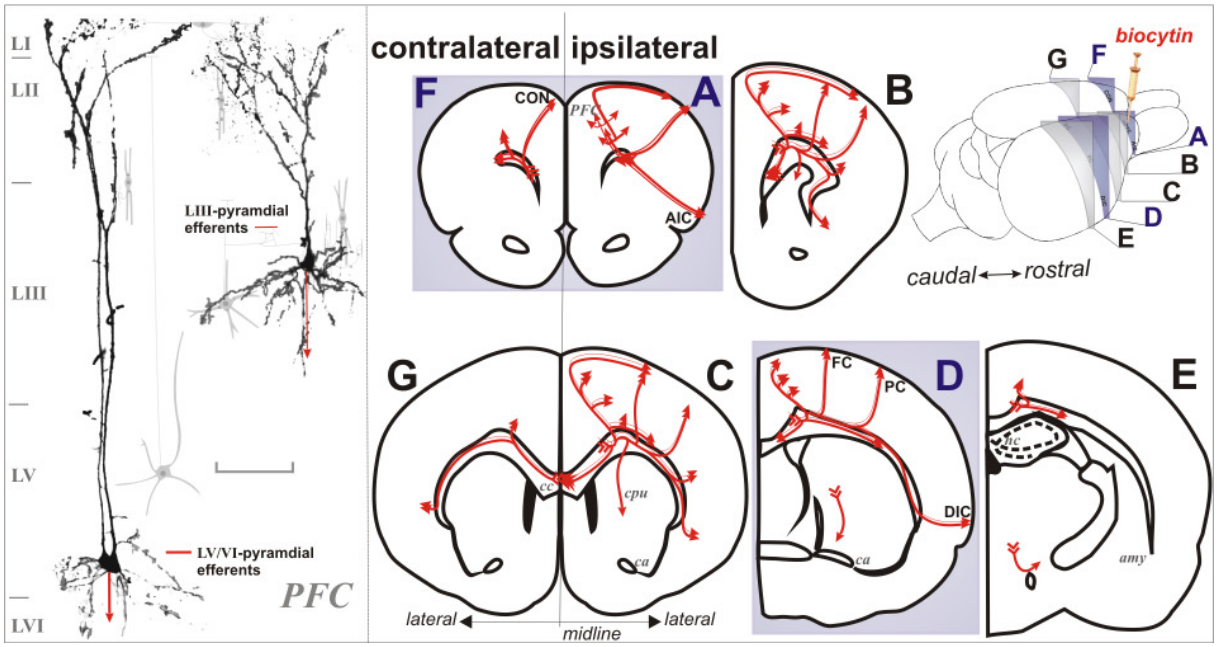

Figure 4: **Distribution of prefrontal output fibres in the gerbil.** Axons were labelled by an injection with biocytin into pyramidal cells situated in layer III and layer V/VI of the right prefrontal cortex (PFC, left panel). Given are selective coronary slices through the ipsilateral and contralateral hemisphere (**A-G**, see miniature, right corner). At the injection site, axon collaterals (red lines) ascend to layer I, spread horizontally, and descend through layer VI to rise in a lateral column or target more ventrally in the agranular ipsilateral cortex (AIC, **A**). Collaterals travel caudalwards, enter the corpus callosum (cc) and exhibit several bifurcations into the cortex (**B**). Somewhat more caudal, axons run through the cc and deeper layers to terminate in a columnar manner in rostral frontal areas (**C**). One main fibre bundle descends subcortically through the caudatus putamen (cpu). At the level where the anterior commissure (ca) crosses to the lateral ventricle, prefrontal efferents arise in distinct cortical columns in the frontal (FC), parietal (PC), and distant dysgranular insular cortex (DIC, **D**). Beside prominent subcortical projections at the level of the hippocampus (hc) and amygdala (amy), only few axonal fibres target in caudal frontal areas (**E**). More rostral, collaterals cross to the opposite hemisphere and run along the cc (**F**). Corresponding to the injection site, fibres terminate in a contralateral prefrontal column (CON) in the left prefrontal cortex (**G**). Highlighted in blue are levels of quantitative evaluation. Scale bar = 100 µm.

In all age-groups, both layer III- and layer V/VI-axons spread horizontally from layer of origin, ascended to layer I or run through deeper layers and the corpus callosum (Fig. 4A-E). Via these travelling collaterals, specific rostral cortical areas in the ipsilateral hemisphere were innervated in a column-like manner, namely in the agranular insular cortex (AIC, Fig. 4A; see also Fig. 5) and in frontal areas (Fig. 4C; see also Fig. 6C). More caudal, target-columns were found to appear in the frontal (FC), parietal (PC), and dysgranular insular cortex (DIC, Fig. 4D; see also Fig 7), as well as in caudal frontal regions (Fig. 4E; see also Fig. 8B). Some commissural fibres ascended in another cortical column in the contralateral prefrontal cortex (CON, Fig. 4F) after crossing to the opposite hemisphere via the corpus callosum (Fig 4G; see also Fig. 6A). In addition, one main fibre bundle projected via the striatum (Fig. 6B) to subcortical targets after layer V/VI-injections (Fig. 8A), where it incidentially also reached parts of the amygdala (Fig. 4E; see also Fig 9).

For further images, please see Witte et al. (2007a; 2007b), and Bagorda et al. (2006).

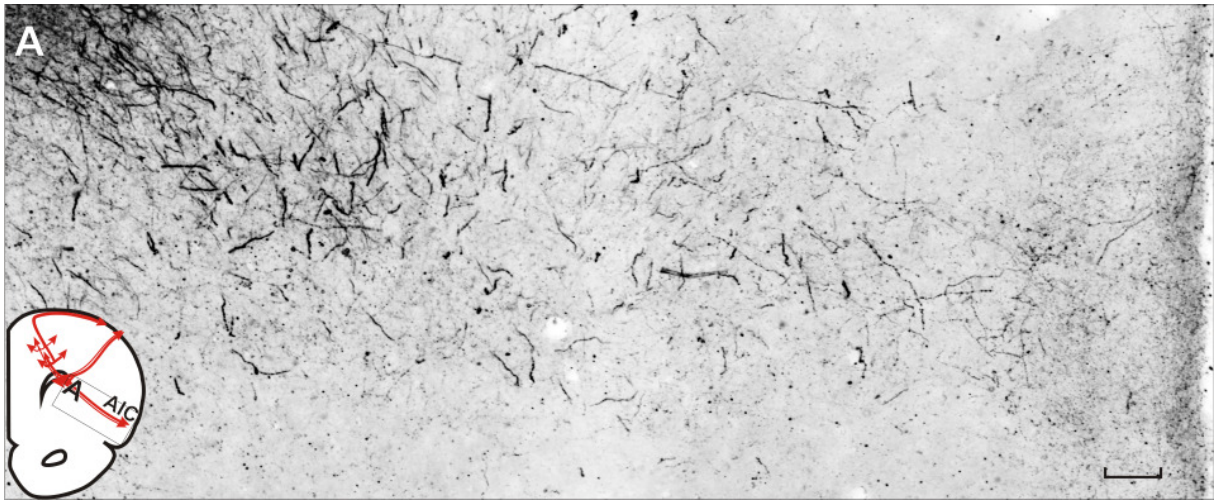

Figure 5: At the level of the injection site, prefrontal efferent fibres descended to the agranular insular cortex (AIC, **A**). Note the laminated organization of thinner fibres in the upper layers. Scale bar = 100 µm.

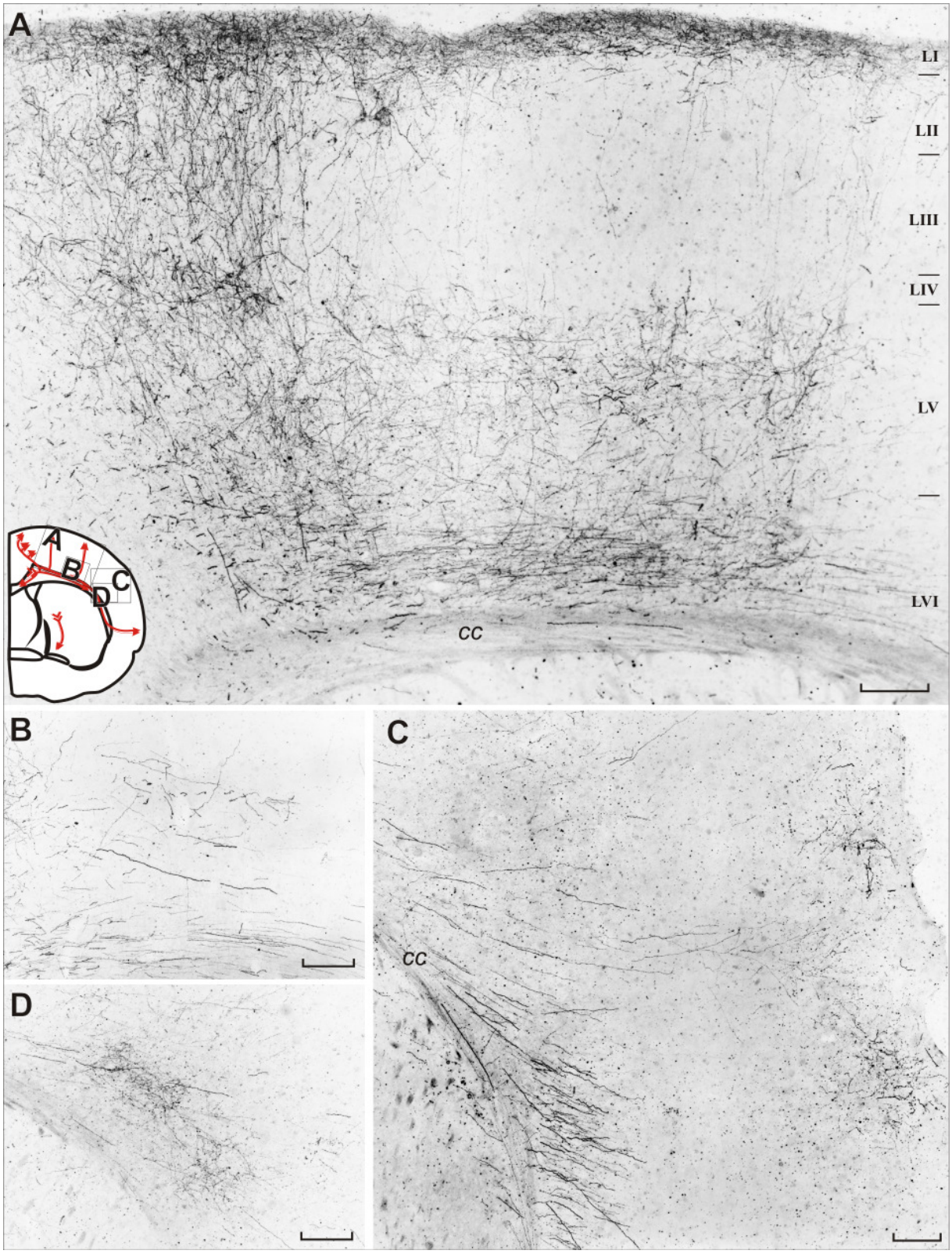

Figure 6: At the level of the crossing anterior commissure, prefrontal efferents run along and above the corpus callosum (cc) through deeper layers to arise in distinct cortical columns in the frontal and parietal cortices (**A**). Note the characteristic innervation at the parietal barrel cortex in another slice (**B**). Collateral fibres also innervated lateral parietal areas (**C**, **D**) and reached via the cc the dysgranular insular cortex. Scale bar = 100 µm.

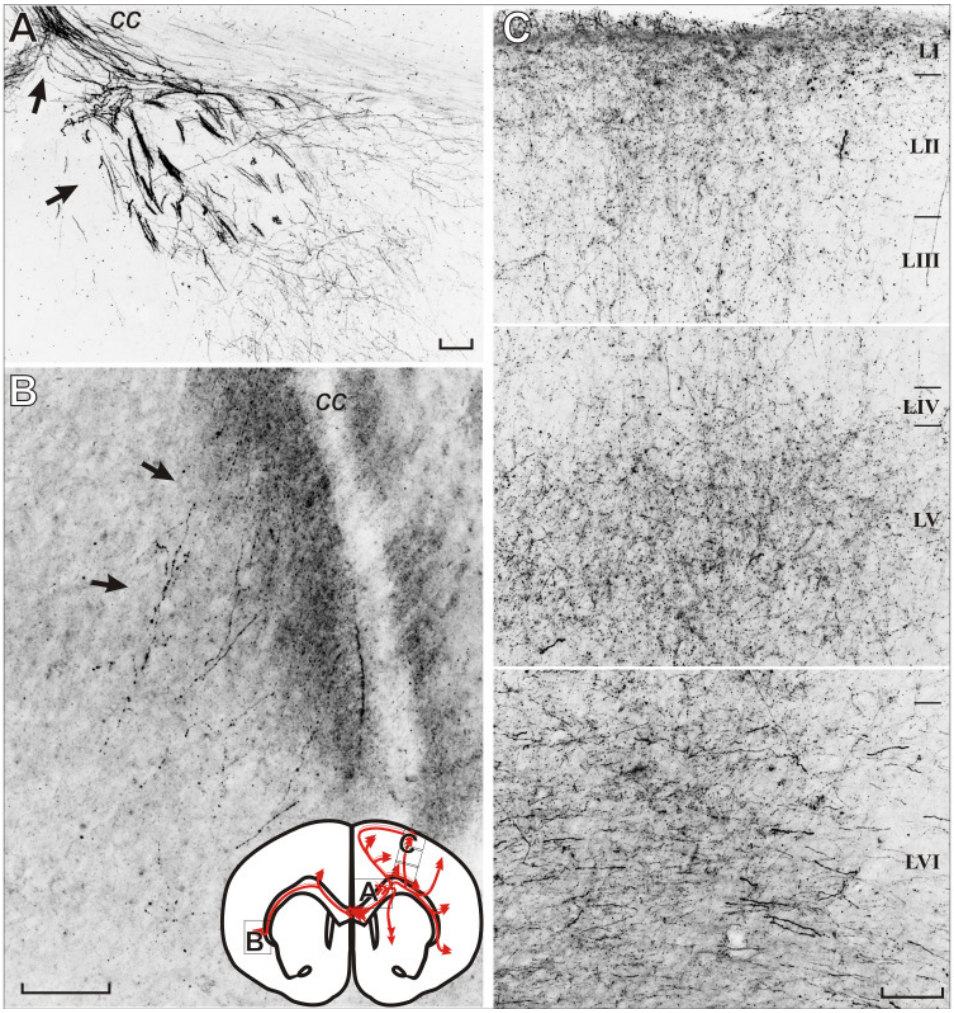

Figure 7: At the level of the aperture of the corpus callosum, one main prefrontal fibre bundle broke through the corpus callosum (cc) to project via the striatum to subcortical targets (**A**). Commissural fibres crossed to the opposite hemisphere and run along the cc until few fibres target in layer VI of the contralateral insular cortex (**B**). Other ipsilateral collaterals travelled horizontally from the layers of origin, layer I and deeper layers to innervate in a columnar manner frontal areas (**C**). Scale bar = 100 µm.

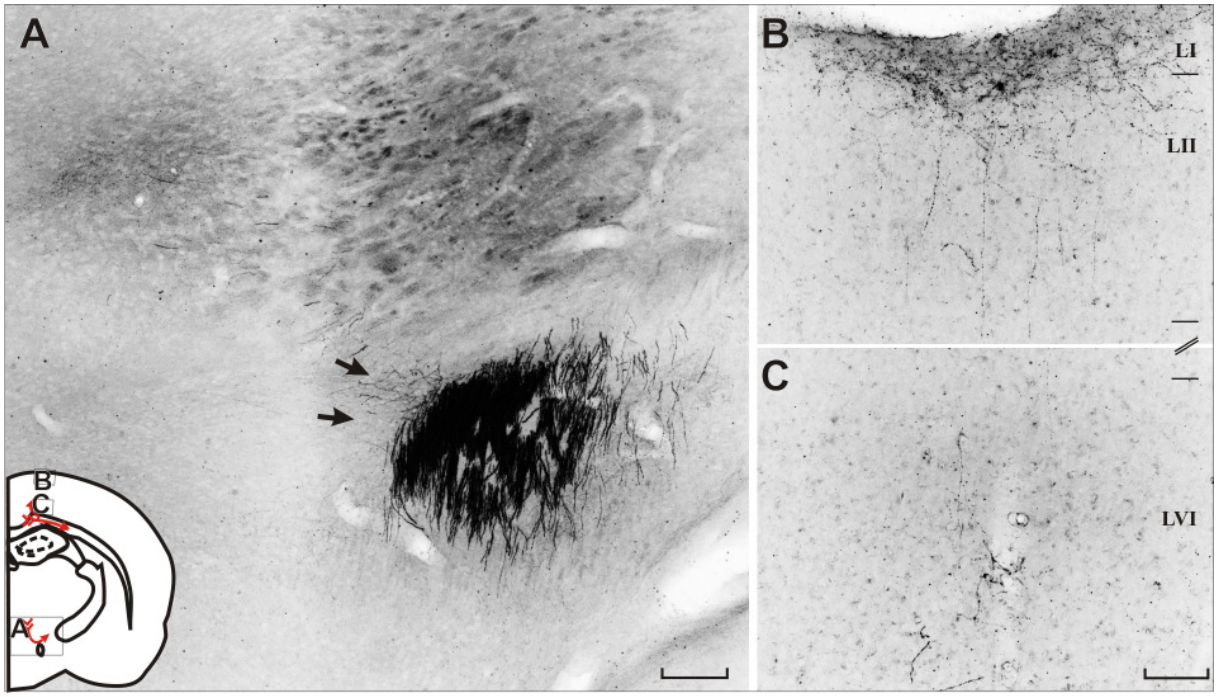

Figure 8: At the level of the hippocampus, a prominent subcortical layer V/VI-projection descended through the pallidum and surrounding fibre tracts (black arrows, **A**). Few cortical fibres and terminal fields were observed in caudal parietal areas (**B**). Scale bar = 100 µm

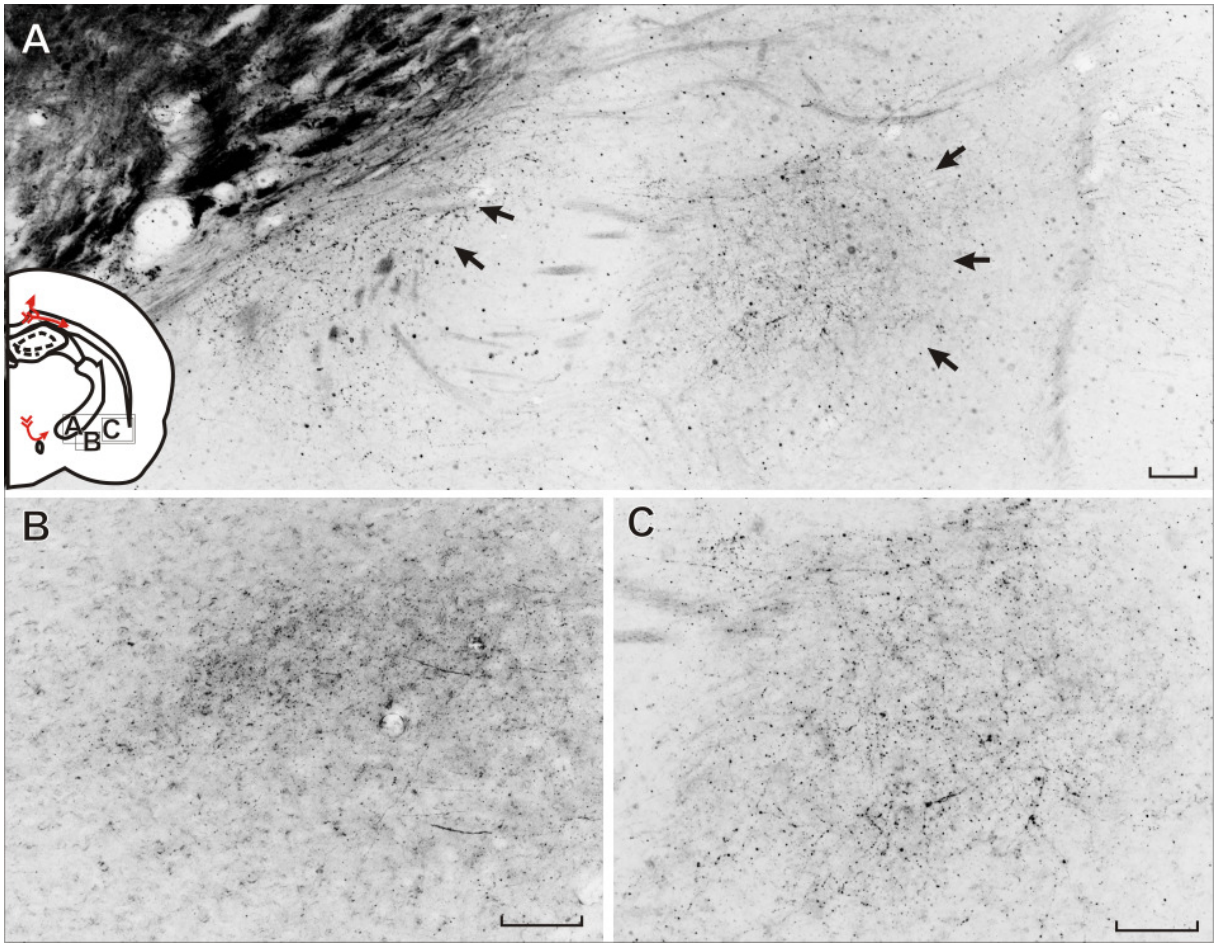

Figure 9: Incidentially, some prefrontal layer V/VI-efferents reached parts of the amygdala (black arrows, **A**). Note the fine termination jots in central (**B**) and basolateral nuclei (**C**). Scale bar = 100 µm

4.1.2 Pattern changes of juvenile fibre morphology and innervation density

Intriguingly, some impressive differences could be observed at greater magnification between age-groups following layer V/VI-injections, which were less striking after layer III-injections. Thus, prefrontal efferents in infantile animals (PD 15-19) appeared to be coarser and somewhat less ramified than axons in older animals. These early "pioneer fibres" were characterized by a thick, hardly ramified morphology and, especially in the cortical target areas, they exhibited considerable fewer ramifications and ascended somewhat orderless throughout the layers. For instance, Fig. 10 gives a detail of the innervation in target layers I-II and VI in the insular cortex of an infantile in comparison to an adolescent animal. For further visualization, please see Fig. 4-6 in Witte et al. (2007b).

Yet, these initial pioneer fibres were exclusively observed in infantile animals. In contrast, patterns of prefrontal efferents in the older age-groups (PD 20-69) resembled already the target layer-specific adult morphology. Here, well-defined terminal fields, constisting of numerous fine and densly ramified collaterals as well as multiple termination jots in layers I, V, and upper VI were separated from fibres of passage within layers II, III, and lower VI (for comparison see Fig. 10). Notably, the structural definition and precision of the adult-like pattern increased within steps of development, namely that the ramification and occurance of termination jots accumulated within age. Please see also Fig. 4-6 in Witte et al. (2007b).

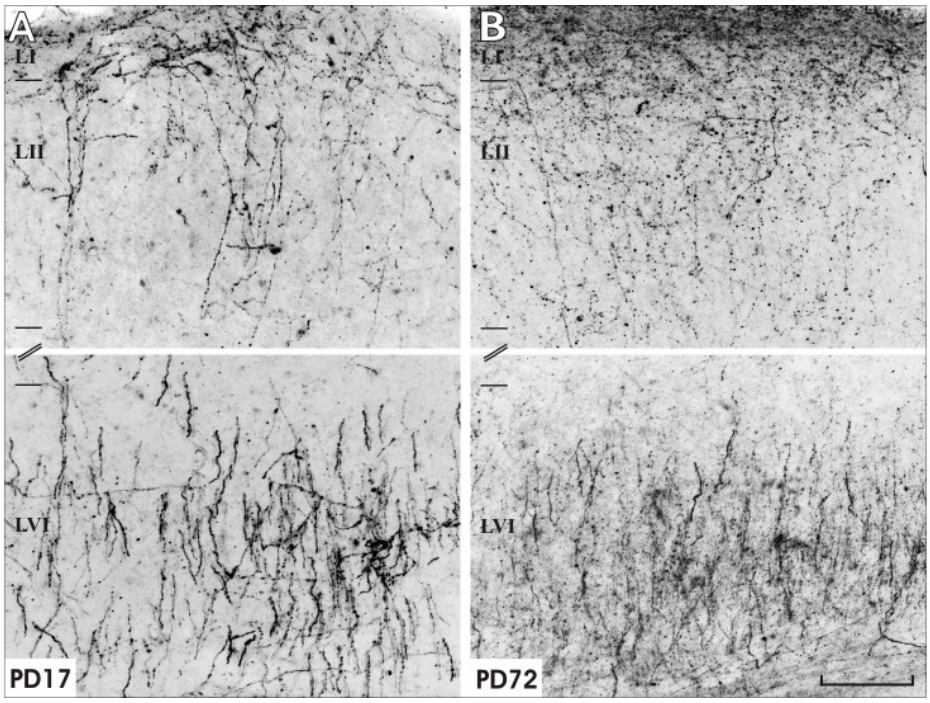

Figure 10: Pattern changes of prefrontal efferent fibres during development seen examplarily in layers I, II, and VI of the dysgranular insular cortex. Note the thick initial fibre morphology in the infantile animal (**A**) in contrast to finer, ramified fibres and multiple termination jots in the adolescent animal (**B**). Scale bar = 100 µm

4.1.3 Dynamics of fibre densities during juvenile stages

To detect further, quantitative differences between age-groups, statistical analyses of efferent fibres and terminal fields were performed in five cortical target areas, which are briefly summarized here. Therefore, the relative fibre densities in different layers of the AIC, FC, PC, DIC, and CON were measured by a computer-aided evaluation of three neighbouring brain sections for each case and target area. Layer III- and layer V/VI-efferents were checked separately for significant differences between age-groups or traget layers using adequate post-hoc-tests after significant results of a repeated measures analysis of covariance (ANCOVA) with age-group as main factor, target layer as repeated measures factor, and cortex extent as co-factor. For further details of the software procedures and statistical analyses, please see Neddens et al. (2002) and Witte et al. (2007b).

According to quantitative analyses, efferent fibre densities after prefrontal layer III-labelling appeared to remain at low levels somewhat stable until a considerable increase occured between adolescent (PD 60-79) and adult (PD 90) animals (Fig 11, grey lines). This late increase in fibre densities was statistically significant in the FC and DIC ($p < 0,01$; Fig. 11 A, E) but failed to reach significance in the remainder of the target areas (AIC, CON, PC; Fig. 11 B-D). Regarding prefrontal layer V/VI-labelling, however, fibre densities were found to indicate a remarkably different course of development. Thus, Fig. 11 (black lines) depicts an early decrease of density values as observed between the infantile (PD 15-19) and the early juvenile (PD 20-24) age-groups, followed by a steady increase to a local maximum or adult values around adolescence (PD 60-79). Thus, overall fibre densities decreased significantly between infantile and early juvenile animals in the AIC ($p < 0,05$; Fig. 11D), whereas an increase to adult values ($p < 0,05$; AIC, Fig. 11D) or to a local maximum ($p < 0,05$; FC, Fig. 11A; DIC, Fig. 11E) was found in adolescent animals. According to raw data, these dynamic differences between age-groups became likewise visible, but not statistically significant, in the other target areas (Fig. 11H-K). For further results, please see Witte et al. (2007b).

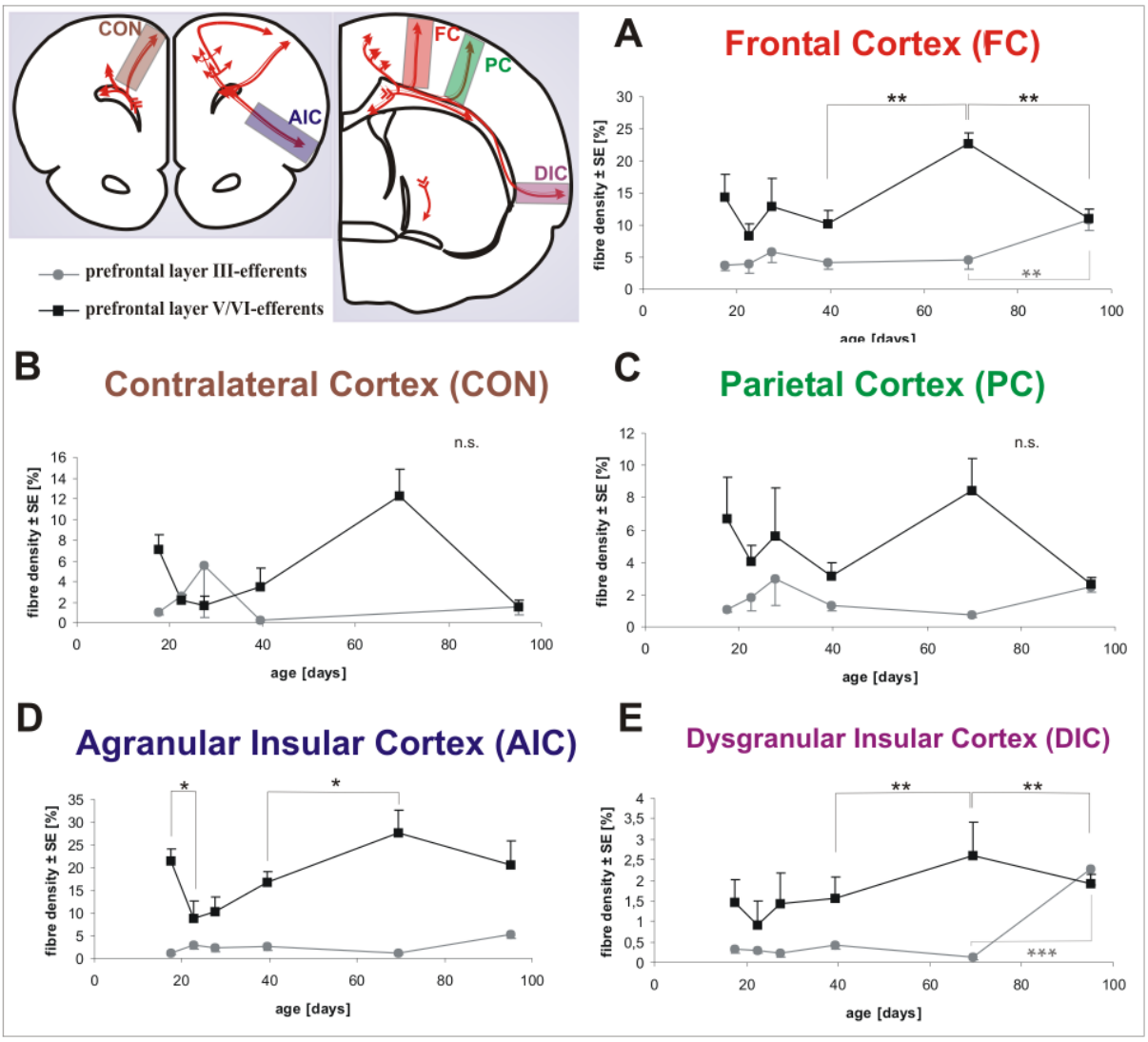

Figure 11: Overview of quantitative changes in fibre densities of prefrontal layer III- (grey line) and layer V/VI-efferents (black line) during development in different cortical target areas. Note a significant increase in layer III-fibre densities between adolescence and adulthood in the frontal (FC, **A**) and dysgranular insular cortex (DIC, **E**). In the AIC, layer V/VI-efferent fibre densities decreased significantly between infantile and early juvenile animals, while an increase to adult values set in between post-weaning and adolescent animals (**D**). The latter increase was observed also in the FC and DIC (**A, E**). As an illustration, fibre densities are given as means of the sum densities of target layers I, II, V, VI (FC, PC, CON), target layers I, II, V (AIC), and target layer VI (DIC), respectively ± standard error of the mean (SE). Asteriks indicate levels of significance as revealed by post-hoc-testing after repeated measures analysis of covarinace (ANCOVA) incorporating all target layers.

4.1.4 Coherences and backgrounds of fibre dynamics

We here described for the first time a long-lasting, considerable different postnatal development even until adulthood of distinct prefrontal layer III- and layer V/VI-efferents. Thus, our findings provide first anatomical evidence to the assumption, that the two different output subsystems should undergo highly dynamic developmental changes to lately exhibit hierarchically distinct neuronal functions (Price et al., 2006). As an add-on to the discussion part of the publication (Witte et al., 2007a), I first refer to the cytoarchitectonic development of pyramidal cells in the PFC, which might coincide with the described alterations in axon morphology and density.

The transformation of the cortical plate into the laminated adult PFC is known to start prenatally but to continue until late in postnatal life (see introduction). In rats, three different phases can be distinguished (Van Eden and Uylings, 1985a). These early findings are nicely in line with our results in gerbils and indicate a possibly similar, concurrent development of pyramidal lamination and distant axon differentiation. Although the time of gestation and weaning takes a few days longer in gerbils than in rats, eye-opening occurs around PD 14 in both species (Clancy et al., 2001; Eilam, 1997). Thus, the first developmental phase should be somewhat comparable: Until PD 18 in rats, neurons differentiate within the cortical plate and form the cortical layers (Van Eden and Uylings, 1985a). In addition, other cortical areas mature simultaneously, thereby generating specific targets for prefrontal axons. As these processes cause dramatic cytoarchitectonic changes, it seems very likely that a critical stage in the maturation of pyramidal output neurons occurs during this particular time window in rats, which is in fact corresponding to our first age-group in gerbils (PD 15-19). Prefrontal efferents may re-arrange dramatically at this developmental stage in order to innervate latterly distinctive target layers and particular neurons. Related to our quantitative data in gerbils, fibre densities following layer V/VI-injections decreased significantly in the AIC from PD 15-19 to PD 20-24, which may be due to dramatic refinements of axonal fibres. Indication of dynamic maturation during this period comes also from qualitative impressions, as only PD 15-19 animals exhibit the coarse, undefined initial fibre morphology.

As discussed in Witte et al. (2007b), further evidence for pyramidal cells of the PFC to undergo structural rebuilding at this time arises from volumetric studies, that described a transient overgrowth exclusively in prefrontal areas during the first weeks of postnatal life in rats (Van Eden and Uylings, 1985b). In addition, magnetic resonance imaging studies indicated that PFC-specific volumetric changes should also occur in infantile primates and in children (Giedd et al., 1999; Jernigan et al., 1991; Sowell et al., 2002). According to van Eden

et al. (1990), changes in interneuronal connectivity might relate to these volumetric changes. Now, our recent findings describing an early reduction in prefrontal efferent fibre densities provide first evidence to these assumptions. Thus it seems likely, that the dynamics in fibre densities should reflect an intense phase of axonal refinement during this time window.

Regarding further maturation until PD 30 in rats, little changes were observed in the cytoarchitectonic characteristics of the PFC (van Eden and Uylings, 1985a). Consistently, axonal fibre densities and their morphological image remain somewhat stable in gerbils, even until PD 49. Here, the delayed post-weaning time course of gerbils in comparison to rats may contribute to the consistency in our relatively wide ranged age-group after weaning from PD 30 until PD 49, thereby reflecting a prolonged phase-two period in gerbils.

The third phase in rats (PD 30 until PD 90) is characterized by the appearance of greater individual cytoarchitectonic differences and less defined boundaries of the cortical layers (Van Eden and Uylings, 1985a). We observed in gerbils that a remarkable increase in fibre densities to adult values sets in considerably earlier regarding layer V/VI-efferents in contrast to layer III-efferents. Thus, layer V/VI-fibre densities exhibited adult values (AIC) or even an overshoot (FC, DIC) already around adolescence (PD 60-79). In contrast, layer III-fibre densities started to outgrow first between adolescence and adulthood (PD 90) as seen in the FC and DIC. Interestingly, the peripubertal phase was also found in monkeys to be especially critical for synaptic reorganisation processes (Anderson et al., 1995; Woo et al., 1997).

In summary, our findings clearly demonstrate layer III- and layer V/VI-pyramidal efferent fibres to follow considerably separate phases of axonal refinement and growth. Moreover, it could be supposed that these distinct developmental time courses might lead to the establishment of hierarchically different, layer-dependent functions in adulthood (see e.g. Price et al., 2006). Thus, the two distinct higher-order prefrontal output functions might crucially depend on dynamic structural reorganisation processes, occurring during the infantile and a prolonged adolescent time-window.

4.1.5 Critical events during development

Referring to Witte et al. (2007b), we discussed in detail that the early decrease in layer V/VI-efferent fibre densities (and also the observed changes in axon morphology) might result from a retraction or shrinking of transiently oversprouted layer V/VI-axons. According to various studies in rodents, cats, monkeys, and even humans, it has since long been claimed that the refinement of axons e.g. by means of transient synapses and collaterals should play a specific functional role in the organization of proper cortical connectivity (Dehay et al., 1984;

Innocenti, 1981; Ivy et al., 1984; Jeffery et al., 1984; Kennedy et al., 1989; Killackey and Chalupa, 1986; Lent et al., 1990; Olavarria and Van Sluyters, 1985; Price and Blakemore, 1985; Provis et al., 1985). The dynamic changes in fibre densities found in our material might further reveal a delayed, somewhat similar organization and refinement of prefrontal efferents in comparison to sensoric efferents. For example, early studies in the visual system of kittens found that initial lower layer V–corticocortical axons are pruned in the third postnatal week, whereas new axons sprout from layer III (Price and Blakemore, 1985). Likewise in our gerbils, prefrontal layer V/VI-fibre densities decreased during early juvenile ages and increased again between post-weaning and adolescence. In contrast, layer III-fibre densities exhibited no fibre decline in early life but increased between adolescence and adulthood. This is also in line with more recent studies reporting only feedback pathways (originating in layer V/VI) to be remodelled during maturation (Batardiere et al., 2002).

Yet, the underlying mechanisms that control axonal refinement are still unclear. Some authors suggested that apoptosis might account for axonal withdrawal (Ivy and Killackey, 1981; Kim and Juraska, 1997; Nunez et al., 2001). In contrast, we did not qualitatively observe a decline in biocytin-filled pyramidal cells at the prefrontal injection site within our investigated age-group. Cell counting in the PFC of rats (van Eden and Uylings, 1986) further support these findings, thus, apoptosis should not explain the early decrease in fibre densities observed in our material. Hence, the supposed shrinking of the terminal arbour might rather depend on activity-guided processes within the prefrontal pyramidal cells. A particular focus in this context lies on the extended growth of morphogenetic DAergic fibres in the PFC, which intriguingly undergo a dramatic augmentation during adolescence (Dawirs et al., 1994; Kalsbeek et al., 1988). Supposingly, alterations in the DAergic innervation might crucially shape neuronal properties of PFC-pyramids, since DA exert both direct and indirect presynaptic influence on pyramidal cells (Tseng and O'Donnell, 2004). The extended, prolonged maturation of the DAergic projection might thus contribute to the observed pattern changes in prefrontal efferents.

Notably, how exactly the DAergic system might account for axonal changes in pyramidal cells remains to be speculative. Both pyramidal output systems certainly receive functionally different input activities in the PFC (Lewis et al., 2002), which might indeed control some aspects of neuronal connectivity e.g. via complex molecular guiding cues and second messenger cascades. The functional diversity of pyramidal cells not only between layer III and layer V/VI, but also within the same layer, is for example supported by electrophysiological studies by Thomson and Bannister (1998), who found layer V-pyramidal

neurons to comprise two distinct subclasses of output neurons. Thus, functionally diverse pyramids should be affected by considerably different (pre- and/or postsynaptic) neuronal systems, therefore experiencing distinct stimuli and firing patterns during development. As a consequence, it could be supposed that separate activity-dependent processes should in turn lead to separate developmental time courses as found in our study for functionally distinct pyramidal cells in the PFC.

Underlining this rather complex development, one should consider that communicating cells also influence each other to a great extent. Thus, the pyramidal cells should also generate activity-dependent cues to their pre- or postsynaptic cells. Since both layer III- and layer V/VI-efferents (see Fig. 3 on p.17) compete for the same postsynaptic targets (Bannister, 2005), one could hypothesize on the basis of our results that the later ingrowing layer III-efferents could act as a regulating factor for the growth of layer V/VI-collaterals. We found at least in some target areas layer III-fibre densities to increase simultaneously to stagnancy or even decrease of layer V/VI-fibre densities. Conceivably, it could be assumed that the ingrowing layer III-fibres might suppress a further spread of layer V/VI-fibres, since they might occupy potential dendritic postsynaptic targets of layer V/VI-terminals by means of activity-dependent stabilization processes.

In summary, this long-time anterograde tracing study in juvenile gerbils points to highly interrelated and self-stabilizing reorganisation processes during development, promoting once again the compensatory theory of brain organization as described above (Wolff and Wagner, 1983). Notably, a prolonged structural development as prominent feature of the PFC might stand not only for the ability to enable context-generated, higher-order cognitive functions, but also for a specific vulnerability of prefrontal networks to epigenetic challenges.

To reveal possible impacts of a disturbed development on the two prefrontal output systems, I investigated the effects of early adverse interventions in another tracer-study in our animal model of psychosis (Witte et al., 2007b). As a result, we described for the first time dramatic rearing-dependent modifications of prefrontal pyramidal efferent innervation in the contralateral prefrontal target areas.

4.2 Mal-adaptive pattern changes of prefrontal efferents (Witte et al., 2007b)

Adverse epigenetic influences have been shown to substantially affect brain organization as well as behaviour in rodents (for review, see Hall, 1998). Various studies revealed that the DAergic system, which plays a crucial role in modulating cortical processes particularly in the

limbic-prefrontal system (for review, see Le Moal and Simon, 1991), is substantially affected by epigenetic factors (Jones et al., 1992). Moreover, it seems very likely that in humans, cognitive and affective disorders such as schizophrenia are often linked to disturbances during childhood and adolescence (for review, see Sanchez et al., 2001). Thus, our group has extensively studied in a "two-hit" animal model of psychosis (Bagorda et al., 2006; Dawirs and Teuchert-Noodt, 2001; Teuchert-Noodt, 2000) the impacts of early non-invasive acute and/or chronic interventions (for details see below). These non-invasive traumatisations mimic a degeneration of DAergic terminals primarily in the PFC, partly via autotoxic occupancy of DAergic reuptake-transporters on the presynapses (Jones et al., 1992; Neddens et al., 2002; Seiden and Sabol, 1996; Teuchert-Noodt and Dawirs, 1991; Winterfeld et al., 1998). Moreover, we found evidence that this severly suppressive maturation of DA causes further changes in diverse other transmitter systems at a structural level and also dysfunctional behaviours in adulthood (for review, see Brummelte et al., in prep.). As to glutamate, Bagorda et al. (2006) recently described a dramatic, presumably dysfunctional wiring of ipsilateral cortico-cortical efferents originating in the PFC of methamphetamine-intoxicated (MA) and isolated reared (IR) animals in adulthood compared to enriched reared (ER) controls: Using the above mentioned anterograde tracing methods, it was found that under either IR- or MA-condition, animals exhibited fewer efferent fibre densities in the FC, PC, and DIC originating from both layer III- and layer V/VI-prefrontal pyramids compared to ER-controls. In contrast to this somewhat equal down-regulation of prefrontal fibre densities after a single treatment, the combined, two-hit IR- and MA-treatment led to a considerable, presumably pathologic imbalance between the two output layers. Thus, rather stable layer III-fibre densities but highly oversprouted layer V/VI-fibre densities were observed with most signifance in the PC and DIC of IR-MA-animals. These findings (for details, see Bagorda et al., 2006) provide first evidence for the hypothesis of a prefrontal "*dys*connection" underlying the pathology of schizophrenia (Weinberger and Lipska, 1995).

Yet, since especially in psychotic patients a prefrontal mal-functioning has been suggested to depend also on a disturbed interhemispheric exchange (Innocenti et al., 2003), I studied the potential effect of early epigenetic challenges in contralateral target areas of prefrontal efferents and completed therewith the previous results (Witte et al., 2007b). In this study, prefrontal layer III- and layer V/VI-efferents were labelled with biocytin in a total of 47 male gerbils reared under either ER, ER-MA, IR, or IR-MA conditions (for details, see Bagorda et al., 2006). On PD 14 (eye-opening), animals received either as sham a saline-injection or as acute neuro-intoxication a single high dose of methamphetamine in saline (50 mg/kg *i.p.*).

ER-conditions included an enriched environment (expanse = 1m², Fig. 12A) with many possibilities to hide and play as well as social contacts to mother (until weaning) and siblings (whole period). IR animals were bred and fostered in standard laboratory cages (Makrolon® type IV; expanse = 0.12 m²) until weaning (PD 30), whereupon they were kept separately in standard home cages (Makrolon® type III; expanse = 0.06 m²; Fig. 12B). After biocytin-injection and standard DAB-reaction, fibre densities were assessed in the target area CON. Statistical analyses were computed using post-hoc-tests after a two-way repeated measures ANOVA with main factors of treatment-group and rearing-group, and a repeated measures-factor of target layer (6 levels). For further details, please see Bagorda et al. (2006) and Witte et al. (2007a).

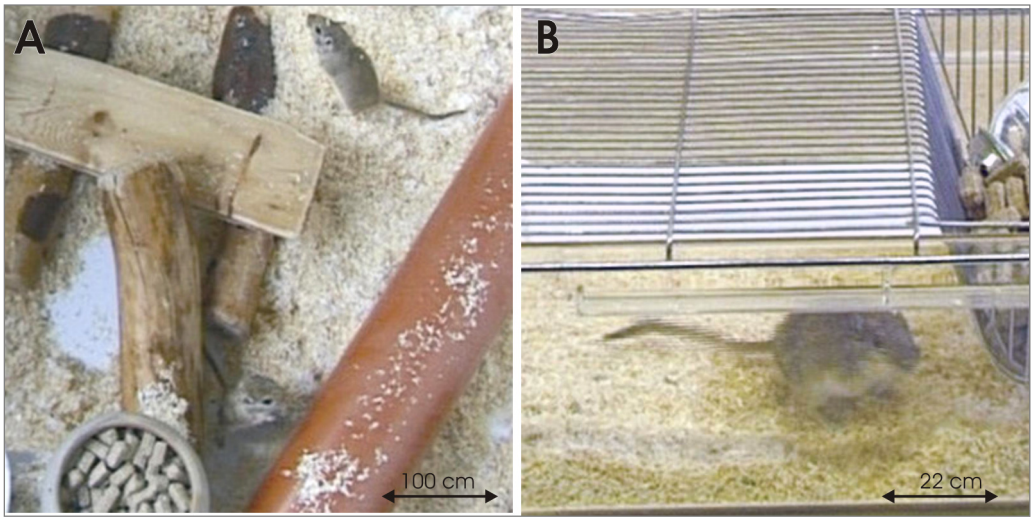

Figure 12: Different rearing conditions: Animals were either kept with their siblings in an enriched environment (ER, **A**) or socially isolated in standard laboratory cages under impoverished conditions (IR, **B**).

4.2.1 Effects of adverse epigenetic interventions

Biocytin-labelling led to considerably lower axonal fibre densities in the adult CON after layer III-injections in comparison to layer V/VI-injections (Fig. 13). Quantitative analysis showed no differences between groups after layer III-injections. Conversely, after layer V/VI-injections, both IR- and ER-MA conditions led to significant lower fibre densities in target layers V and VI in comparison to ER-controls ($p < 0.01$), whereas IR-MA animals exhibited significantly enhanced fibre densities in all target layers ($p < 0.001$). For further results, please see Witte et al. (2007a).

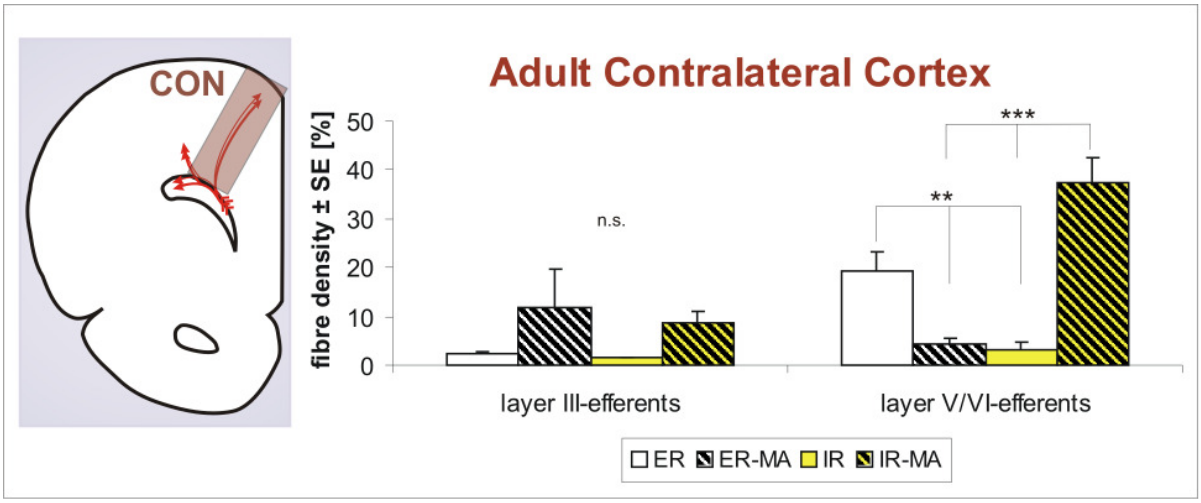

Figure 13: Axonal fibre densities in the contralateral prefrontal cortex (CON) of adult animals after layer III- and layer V/VI-injections with biocytin. Animals were either kept under enriched (white, ER) or impoverished (yellow, IR) rearing conditions and received on postnatal day 14 either a saline injection (plain columns) or a methamphetamine (MA) injection (striped columns). As indicated by asteriks, significant lower layer V/VI-axonal fibre densities occurred in ER-MA and IR animals compared to ER controls, whereas IR-MA treatment lead to significantly enhanced fibre densities according to post-hoc testing after repeated measures analysis of variance (ANOVA). As an illustration, fibre densities are given as means of the sum densities of target layers I, II, V, VI ± standard error of the mean (SE). Asterisks give levels of significance. Sample sizes: layer III-efferents: ER: $n = 9$, ER-MA: $n = 3$, IR: $n = 6$, IR-MA: $n = 9$; layer V/VI-efferents: ER: $n = 4$, ER-MA: $n = 5$,IR: $n = 6$, IR-MA: $n = 5$.

Possible methodical aspects were discussed in Witte et al. (2007a) that could explain why the environmental challenges in postnatal life did not alter commissural layer III-efferents in our study. However, Batardiere et al. (2002) found in the visual system of the monkey, that callosal feed-forward projections (i.e. layer III-efferents) are not subjected to remodelling processes during development, such as e.g. the elimination of inappropriate connections (Barone et al., 1995). Moreover, Batardiere et al. (1998) already suggested that the maturation of layer III-axonal fibres might depend somewhat to a lesser extent on activity-dependent epigenetic influences but rather on intrinsic signals. This might provide insights why the environmental interventions did not substantially modulate adult layer III-fibre densities in our material, however, this has to remain unclear.

Regarding layer V/VI-efferents, both IR- and MA-treatments led to dramatic changes in adult CON fibre densities. This crucially underlines our findings of considerably separate and different rules of maturation regarding the two prefrontal output systems (Witte et al., 2007a), which was previously assumed by Price et al. (2006). Conceivably, the adverse IR- and MA-treatments might have led to a distortion of normally regressive events occurring only in the layer V/VI-efferent path. Thus, it is assumed that in IR- and ER-MA-animals, an excessive pruning of connections, hypothetically due to a lack of DAergic input to these pyramidal cells, might have led to the observed decrease in fibre densities compared to ER-controls. Yet

in IR-MA-animals, it could be speculated that the combined treatment could have induced further, even more extensive manipulations of neuronal signalling. This might have affected the activity-dependent processes of layer V/VI-axonal refinement in a turn-over fashion.: The remarkably suppressed DAergic innervation after IR-MA treatment might result in a large-scale imbalance and putative compensatory mal-adaptation of prefrontal networks, for instance in a significant serotonergic distortion (Neddens et al., 2003; Neddens et al., 2004), and manifested not by a further reduction but in fact by a boost of layer V/VI-efferent fibre densities.

4.2.2 Relevance to human pathologies

At least layer V/VI-prefronto-callosal connections were found to be affected by epigenetic interventions in the same way like previously investigated ipsilateral prefrontal connections (Bagorda et al., 2006). Under normal conditions, Barbas et al. (1989) reported a parallel organization of contralateral and ipsilateral prefronto-cortical efferents in the monkey. Thus, the recently found dramatic imbalance of contralateral fibre densities after the combined IR-MA-treatment provides further evidence for a pathologic miswiring of prefrontal efferents after early adverse interventions in rodents, which might also occurr in primates, and even in humans. Consistently, a prefrontal miswiring is considered as an anatomic correlate for severe cortical dysfunctions that could, intriguingly, also contribute to human psychosis (Stephan et al., 2006; Weinberger and Lipska, 1995), which first-time structural evidence was previously discussed by Bagorda et al. (2006).

Regarding in particular callosal connections, it is known that contralateral projection neurons are critical for the functional integration of the hemispheres in rodents and in humans (Gazzaniga, 2000). Moreover, abnormalities of cortical lateralisation have consistently been linked to schizophrenia (Crow, 1997; Sommer et al., 2001). Not only a shift of cortical lateralisation as found in anatomical and physiological parameters (Falkai et al., 1995a; Falkai et al., 1995b; Mitchell et al., 2004; Sauer et al., 1999), but also a change of cortical hierarchies between different areas, involving in particular the PFC as described by Schröder et al. (1995). and Heckers et al. (2002). This can now be supported by our results revealing a change of layer-III associated dominance to layer V/VI-dominance after the combined treatment due to extremely high layer V/VI-fibre densities in the CON. Regarding these callosal fibre densities, our findings might further indicate that the prefrontal lateralisation is substantially suspended: Contralateral layer III-efferents might not be able to compensate an assumably dysfunctional over-activation provided by higher densities of layer V/VI-efferents.

As a consequence, the whole prefrontal networks are severely "dysconnected" by the combined epigenetic challenge, which might account for a highly disturbed neuronal functioning and, regarding schizophrenia, even for psychotic symptoms in humans. Whether these assumptions find their direct analogy in schizophrenic patients, remains to be shown in future research, e.g. via imaging methods and post-mortem analyses.

4.3 Maturation of the GABAergic system (Brummelte, Witte et al., 2007)

Beside the glutamatergic system, diverse other transmitters are thought to be structurally altered in the brains of schizophrenic patients, including DA, serotonin, and also GABA (Jones, 1992; Lewis et al., 1999; Lewis and Anderson, 1995). For instance, the number of GABAergic synapses on pyramidal cells in the frontal cortex is thought to be considerably reduced (Blum and Mann, 2003), accompanied by an enhanced, probably compensatory, $GABA_A$-receptor density at the pyramidal somata (Benes et al., 1996; Lewis and Anderson, 1995). Notably, GABA is carried by numerous, functionally distinct subtypes of local inhibitory interneurons in the cortex (Baimbridge et al., 1992; Celio, 1990). Regarding its broad inhibitory potential, GABA is also known to play a substantial role in re-arranging, shaping, and modulating neuronal circuits (Chen et al., 2002; Jacobs and Donoghue, 1991; Tamas et al., 2000; Teuchert-Noodt, 2000). In addition, the GABAergic system is subjected to various changes in its metabolism and acts as a morphogenetic factor during normal development (Chronwall and Wolff, 1980; Nguyen et al., 2001). Thus, the detailed study of its postnatal development on a further structural level might reveal promising insights into how and when potential interactions between GABA and glutamate might contribute to a functional or even mal-adaptive prefrontal maturation.

Therefore, I participated in a study dealing with the postnatal maturation of the GABAergic system in the PFC and in the basolateral part of the amygdala (BLA) of gerbils at different ages, ranging from infantile to old adult stages. The animals were divided into the following age-groups covering convincing periods of the whole lifespan: PD 14 (infantile, $n = 11$), PD 20 (early juvenile, $n = 6$), PD 30 (weaning, $n = 12$), PD 70 (adolescent, $n = 11$), PD 180 (adult, $n = 8$), PD 540 (middle adult, $n = 8$), and PD 720 (aging, $n = 4$). GABAergic cells and fibres were stained using wide established immunohistochemical methods and standard DAB-reaction (for a detailed description of methods, please see Brummelte, Witte et al. 2007). A specific GABAergic subpopulation was additionally investigated using selective staining of the calcium-binding protein Calbindin (CB), which is often used to discriminate different GABAergic subpopulations (Baimbridge et al., 1992). Statistical analyses of cells and fibres included our standardized computerized measurements and post-hoc-tests after a two-way

ANCOVA with main factors of age (7 levels) and area (PFC: three levels, BLA: one level), two co-factors of area size and GABA or CB cell number, and the dependent variable GABA or CB fibre density. For further details, please see Brummelte, Witte et al. (2007).

In general, the overall GABAergic and CB fibre distribution pattern appeared to be somewhat similar in all investigated age-groups (for a visualization, please see illustrations in Brummelte, Witte et al. 2007). With the exception of layer I (which is consistent with other species, Hof et al., 1999), GABAergic and CB cells were found to be distributed equally throughout the medial PFC, and throughout the BLA. Notably, we observed a population of lightly stained CB-pyramidal-like neurons, similar to earlier studies (Celio, 1990; Kemppainen and Pitkanen, 2000). Figure 14 shows a summary of the quantitative results in the PFC. Here, we observed a considerable increase in GABAergic fibre densities between infantile and early juvenile age-groups ($p < 0{,}001$), followed by a further enhancement until weaning ($p < 0{,}05$). Interestingly, fibre densities continued to grow until late in life, significantly in comparison of the adolescent and old adult age-groups ($p < 0{,}001$). GABAergic cell densities also showed dynamics during postnatal life: While from infantile to early juvenile stages, cell numbers substantially decreased ($p < 0{,}001$) corresponding to the increase in fibre densities, cell numbers increased again until weaning ($p < 0{,}01$), and dropped afterwards until a stable level became obvious in adolescent animals ($p < 0{,}05$). Likewise to the GABAergic values, CB fibre densities also increased from infantile to early juvenile age-groups ($p < 0{,}01$). Conversely, both CB fibre densities ($p < 0{,}05$) and cell numbers ($p < 0{,}001$) decreased between weaning and adolescent age-groups, followed by a further decrease in fibre densities from adult to old adult stages ($p < 0{,}01$).

Statistical analysis further revealed significant contributions of the co-factors cell number and area size to fibre density values. However, since fibre densities increased although cell numbers decreased, possible interactions should be negligible. Consistently, a volume expansion of the mPFC of rats around PD 20 as shown by van Eden and Uylings (1985f) was suggested already earlier to account for a decrease in prefrontal cell numbers (Vincent et al., 1995). Thus, we here described for the first time long-lasting structural changes in GABAergic prefrontal interneurons from early until considerably late in postnatal life. For further results, please see Brummelte, Witte et al. (2007).

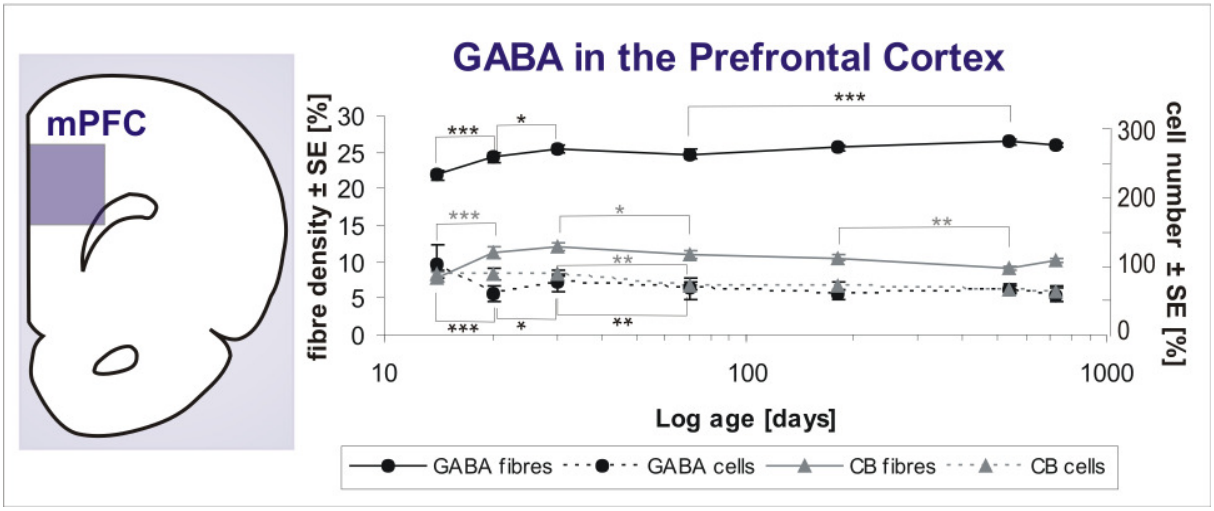

Figure 13: Development of GABAergic and Calbindin (CB)-containing cells and fibres in the medial prefrontal cortex (mPFC) of gerbils. Note the early considerable increase in GABAergic and CB fibres until weaning on postnatal day (PD) 30. Another steady increase of GABAergic fibres occurred until old age (PD 520), whereas CB fibres slightly decreased. Asterisks indicate levels of significance according to post-hoc testing after repeated measures analysis of covariance (ANCOVA).

4.3.1 Long-time alterations in GABAergic fibre densities

The present findings clearly point to a substantial involvement of GABA in the development and in particular even maintenance of prefrontal network function. The increase in GABAergic and CB fibre densities from infantile to early juvenile ages underlines the above identified first time-window of dynamic structural reorganization processes during this period.

The second postnatal week in rats is characterized by a phenotypic shift of some subgroups of CB- to Parvalbumin (PV)-containing interneurons in the cortex (Alcantara et al., 1996). PV is another calcium-binding protein, which is carried to a great extent in GABAergic basket-like cells in the adult cortex (Celio, 1990) and was found to occur considerably later than CB in development (Alcantara et al., 1993). Basket-like cells are known to exert a highly potent influence on the firing activity and synchronization of pyramidal output cells via axon-somatic contacts and basket-like boutons (Freund, 2003; Gibson et al., 1999; Miles et al., 1996; Tamas et al., 2000). These characteristic boutons likewise appear considerably late in development (Bahr and Wolff, 1995). Therefore, one could assume that the maturing GABAergic basket-like subtypes in particular around PD 20 in gerbils (reflected by the increase in GABAergic but also CB fibre densities observed in our material) might directly influence the pyramidal differentiation via strong inhibitory inputs to their somata at this time window. As a consequence, the presumably reduced pyramidal excitation might lead to a loss of efferent axonal contacts, which would be strongly in line with our findings regarding a decrease in prefrontal efferent fibre densities (see above, and Witte et al., 2007a). Moreover, the DAergic prefrontal innervation modulates pyramidal output activity besides direct inputs

also indirectly via GABAergic interneurons (Penit-Soria et al., 1987; Sesack et al., 1995; Vincent et al., 1995). Thus, an increased GABAergic activation via substantially more DAergic fibre densities in the course of development (Benes et al., 2000) might additionally support this hypothesis.

Regarding further development, it is known that synaptogenesis of inhibitory GABAergic boutons seems to continue well into adulthood (Bahr and Wolff, 1995; Lewis et al., 2005). This was found for example in postsynaptic GABAergic contacts with DAergic afferents (see Benes et al., 2000). Notably, Miller (1988) has since long presumed that the late development of local circuit neurons (i.e. GABAergic cells) and the subsequent remodelling of networks may provide a morphological basis for functional plasticity in mature pyramidal neurons. Thus, long-lasting dynamics in GABAergic circuit reorganisation might even contribute to associative processes such as long-term learning and memory in relation to the external environment. This is in line with the enduring augmentation of GABAergic fibres found in the present study, and might also point to a complex involvement of GABAergic cells in maintaining the striking plastic capabilities of the cortex even in adulthood.

4.3.2 Plasticity and aging – implications for human cognition (Flöel, Witte et al., submitted)

The present results indicate CB fibre densities in the PFC of gerbils to decrease in the ongoing course of time, whereas the overall GABAergic fibre densities persist until old adult ages (see Brummelte, Witte et al. 2007). In mammals, CB-containing interneurons are known to provide rather modulatory influence via axo-dendritic contacts on pyramidal cells (Baimbridge et al., 1992). Thus, one might now be able to speculate that a steady loss of this modulatory influence during aging might somewhat account on a structural level for aging-related cognitive deficits.

In both rodents and humans, aging goes along with a somewhat slowly progress of functional decline, such as e.g. impaired short-term memory performance (Hedden and Gabrieli, 2004). Moreover, cognition includes at least in man the striking capacity to adapt to new situations and the ability of learning during the whole span of lifetime, which is nevertheless affected by aging. Considering the permanent elongation of age in Western Countries, novel mechanisms to obtain these cognitive reserve even during a prolonged phase of aging is of great importance to the public (Dwyer, 2006; Infeld and Whitelaw, 2002). As a promising approach, several studies suggested a neuroprotective action of environmental stimulation on mental aging, provided e.g. by enhanced regular exercise, a specific diet, moderate weight and alcohol consumption, as well as non-smoking (for review, see Flicker et al., 2006).

Yet, a direct association has not been described, however, a “healthy lifestyle” was found in various studies to reduce cardiovascular risk factors (Stampfer et al., 2000), diabetes (Hu et al., 2001), and the risk of stroke (Kurth et al., 2006). Underlying mechanisms of these potential protective effects on CNS functions might be due in part to benefits of the vascular system and an increased resistance against oxidative stress at the cellular level (Mattson et al., 2004). Now, whether these epigenetic factors under a combined, holistic concept most fitting to the cardiovascular domain are indeed capable to exert preventive influence also against cognitive decline is a matter of current research. In a first epidemiology study, we examined the relation between healthy lifestyle and memory performance in healthy elderly subjects (Flöel, Witte et al., submitted).

Therefore, a total of 420 healthy participants (mean age 63 ± 6.6 years) of the cohort study “Systemic Evaluation and Alleviation of Risk factors for Cognitive Health (SEARCH)” in Münster underwent a comprehensive neuropsychological test battery. In addition, they answered a detailed questionnaire about lifestyle habits including physical activity, eating and smoking habits, as well as their height and weight. Out of the five categories exercise, dietary habits, body-mass-index (BMI), smoking, and alcohol consumption, we calculated a combined lifestyle-score according to previous studies (for details, see Flöel, Witte et al., submitted; and also Kurth et al., 2006). This score provided valuable information about the overall lifestyle habits comparable among subjects, with a higher score indicating healthier behaviour. Healthiest behaviour was defined as normal weight, never smoking, intense physical activity, moderate alcohol consumption, and a dietary pattern rich in fruits, vegetables, whole grain products and unsaturated fatty acids. To assess memory performance, we used the validated German version of the Auditory Verbal Learning Test (AVLT, Helmstaedter and Durwen, 1990) in the neuropsychological test battery, in which a list of 15 words has to be remembered and recognized after 30 min. Since the cardinal symptom of cognitive decline is an impaired episodic memory with less ability of learning (Blacker et al., 2007; Peters, 2006), we chose the delayed recall and recognition task out of the AVLT (i.e. items 6, 7, and 8). To check whether the composite lifestyle-score would be associated with better memory performance, we conducted a linear regression analysis (forward inclusion model) with the factor lifestyle score, and the dependent variable “memory score” (AVLT 6, 7, and 8). Confounders (age, sex, education, and systolic blood pressure) were entered successively into the model, to test if the effects of lifestyle factors on memory performance would be predicted by the confounders only. For further details, please see Flöel, Witte et al. (submitted).

For the first time, this cross-sectional study revealed a significant positive association between lifestyle and memory performance even after accounting for possible confounders like age, sex, years of education, and blood pressure. Thus, a healthier lifestyle was able to predict better delayed recall and recognition memory ($p < 0.05$; multivariate linear regression). These findings crucially underline the importance of looking beyond isolated aspects of a healthy lifestyle in favour of discriminating a holistic approach to enhance cognitive functions in the elderly, which is already claimed for cardiovascular risk factors (Ganne et al., 2007; Singh et al., 2006). As a consequence, future strategies for the prevention of cognitive decline and further deterioration should take these comprehensive interrelations into account. Conceivably, a novel public education targeting for instance on combined exercise- and diet-interventions might improve - in a rather simple, cost-efficient way - healthy cognition in the aging population. In a current study, we elaborate by means of a prospective interventional design the direct effects of a change to more healthy dietary habits on cognition and memory performance in healthy elderly subjects (Witte et al., 2007c).

5 Epilogue

Our recent studies revealed for the first time highly dynamics in the postnatal development of the PFC due to the very prolonged maturation of transmitters, neurons, and fibre systems. Regarding the excitatory glutamatergic system, both prefrontal layer III- and layer V/VI-pyramdial efferent paths were found to follow separate developmental time courses: A substantial growth from initially low to high adult values of layer III-efferent fibre densities first set in between adolescence and adulthood (PD 90) in ipsilateral frontal, parietal, insular, as well as contralateral prefrontal target areas. Conversely, layer V/VI-efferents exhibited an early decline of initially higher fibre densities in the third postnatal week, followed by a substantial (re-)growth from weaning to adolescence. As to the inhibitory GABAergic system, our studies described an early strong increase in prefrontal overall fibre densities during infantile ages until weaning (PD 30), followed by a rather gradual increase until aging (PD 520). The GABAergic subclass of CB-containing interneurons underwent a similar early increase in prefrontal fibre densities, but exhibited afterwards a slightly reduction of fibre densities until old age.

Based on these findings, some valuable conclusions can be drawn with regard to the functional establishment of higher-order prefrontal networks in the course of postnatal development. Thus, quite selective and dynamic maturation patterns of morphogenetic transmitters such as glutamate, DA, serotonin as well as GABA are considered to play the essential role in the successive organization of the prefrontal cytoarchitecture. During juvenile time windows, dynamic structural changes in related systems might determinate each other and presumably induce those reorganisation processes, which lead to appropriate functional outcome by taking both activity-driven extrinisic and intrinsic stimuli into account. Therefore, it seems very insightful that especially this highest brain centre, the PFC, requires a very prolonged, long-lasting postnatal development to enable the generation of exceedingly complex, highly integrative functions. Obviously, the glutamatergic pyramidal maturation attracts special attention in this dynamic, functional organization of the PFC during juvenile development.

Figure 14 gives an impression how prefrontal networks are functionally interconnected: The main excitatory glutamatergic pyramidal cells in layers II, III, V, and VI are in the centre of functional columnar modules that generate higher-order control via intrinsic and associational connections (see also Fig. 3 on p. 17). They integrate various inputs provided by other pyramidal cells, local GABAergic interneurons as well as input from glutamatergic thalamic and DAergic mesencephalic afferents, which give rise to considerable influence of these

subcortical systems during prefrontal development. Our results now indicate two different time-windows of considerable reorganisation processes, revealing a dynamic period after eye-opening during the third postnatal week and another critical phase during adolescence around PD 60 in gerbils.

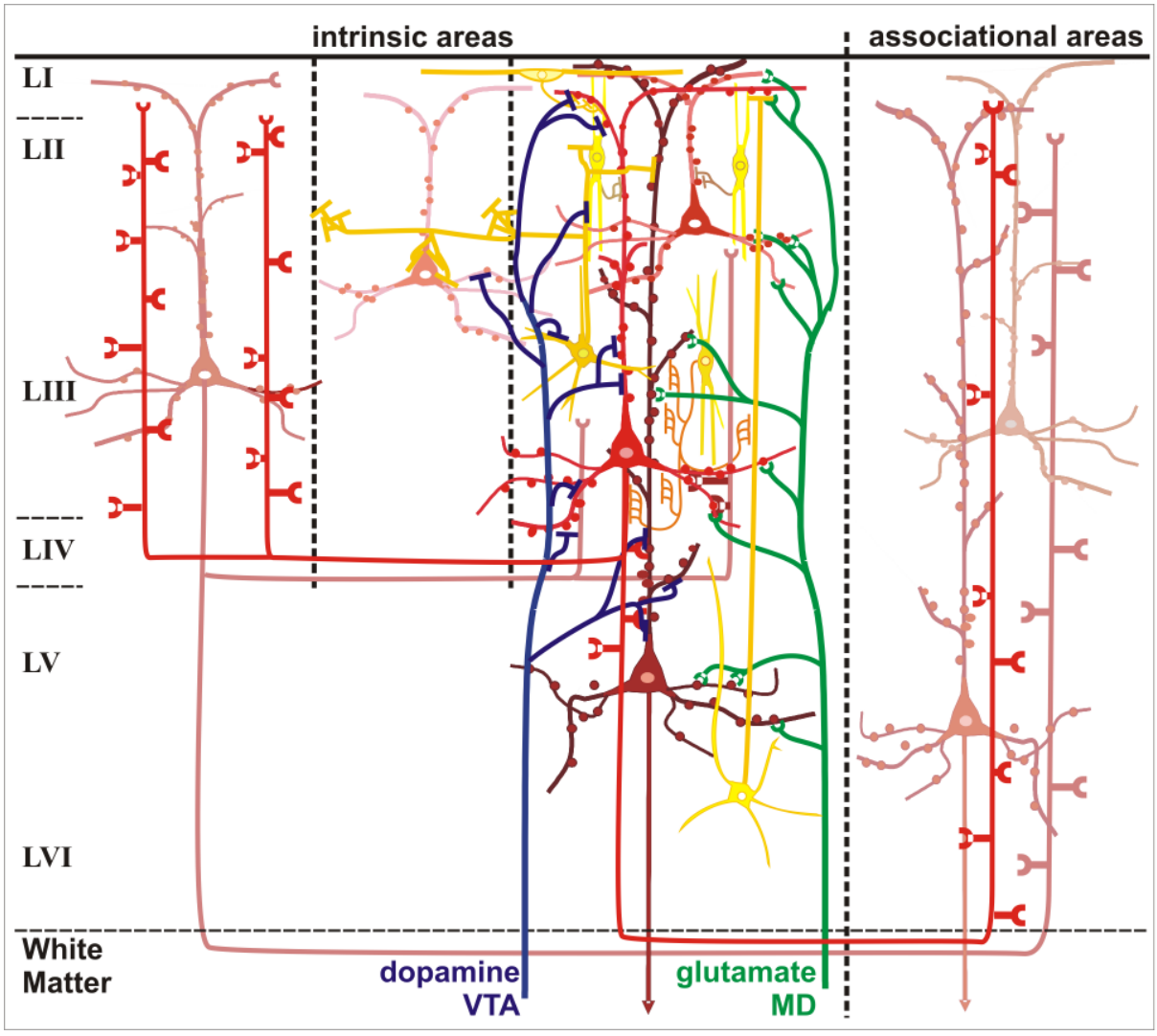

Figure 14: Cytoarchitecture of the prefrontal cortex. Glutamatergic pyramidal cells (red) situated in layers II and III exert excitatory input (open circle) to other pyramidal cells either in layers V and VI of the same column (middle), via intrinsic fibres to adjacent stripes (left) or via associational connections to more distant areas (right). Within the same column and between adjacent stripes, different types of GABAergic cells (yellow) provide modulatory dendritic or rythmic somatic inhibitory inputs (orthogonal lines) onto pyramidal output cells. Glutamatergic afferents (green) arising from the mediodorsal thalamus (MD) mature at early stages and innervate pyramidal cells. Dopaminergic afferents (blue) project from the ventral tegmental area (VTA), targeting both pyramidal cells and GABAergic interneurons. Notably, these fibres substantially grow until young adulthood. Adapted from Lewis et al. (2002).

In the early postnatal phase, thalamic afferents are thought to substantially influence pyramidal cell differentiation, pruning processes, and also the formation of cortical connections (Dehay et al., 1996; Kingsbury et al., 2002; van Eden et al., 1990; Windrem and Finlay, 1991). For example, Kingsbury et al. (2002) observed an increase in cortico-cortical connections after early visual thalamic ablation in the hamster. Considering the PFC, afferent fibres of the mediodorsal thalamus densely innervate prefrontal layers I, III, and V (van Eden, 1986) and are supposed to terminate directly via dendritic contacts in layer I on postsynaptic layer V pyramidal dendrites (Bannister, 2005). Concurrently to the establishment of thalamic afferent fibres (i.e. in rats during the second postnatal week; van Eden, 1986), we found a reduction of prefrontal layer V/VI-efferent fibre densities from initial higher values. Thus it seems likely that the refinement of prefrontal layer V/VI-efferents during the early phase might be linked to and even controlled by the mediodorsal thalamic system. Moreover, prefrontal DAergic fibre densities were also found to rapidly increase around PD 14 in gerbils (Dawirs et al., 1993), which could also be associated with a functional pruning of prefrontal layer V/VI-efferent contacts. However, the exact underlying molecular processes as well as how and to which extent in detail the different morphogenetic afferent inputs contribute or modulate pyramidal fibres remain to be widely unclear (see also Kingsbury et al., 2002). Theoretically, the changes in thalamic and DAergic inputs might shape farer axon differentiation of pyramidal cells by inducing intrinsic activity-dependent molecular signalling pathways, which are thought to be involved in pruning processes (Luo and O'Leary, 2005). For instance, activation of the small GTP-binding protein RhoA in maturing neurons is suggested to act as an important factor in the retraction process of neuronal fibres (Li et al., 2000; Nakayama et al., 2000; Song et al., 2000). Again, the exact mechanisms that remove e.g. the axon cytoskeleton are still unknown (Low and Cheng, 2006).

During the following adolescent time window, cortical fibre densities of prefrontal layer III-efferents were found to substantially increase right after those of layer V/VI-efferents have reached their maximal values. The remarkable maturation of DAergic afferents precisely during this peripubertal time window (Dawirs et al., 1993; Kalsbeek et al., 1988) might contribute to the described changes in the glutamatergic output system in gerbils. Moreover, the DAergic control is suggested to be crucially different between layer II/III- and layer V-pyramidal cells: Layer V-cells receive primarily inhibitory DAergic input (Gulledge and Jaffe, 1998; Kalsbeek et al., 1989), whereas layer II/III-pyramidal cell excitation is thought to be rather fascilitated by DA via interneuron-mediated disinhibition (Gonzalez-Islas and Hablitz, 2003). Now, it might be assumed that during adolescence, particularly layer III-

pyramdial efferents should “benefit” from the improvement in DAergic control due to the maturation of DAergic fibres. After reaching a specific innervation threshold at this adolescent period, the pyramidal layer III-cells might now form considerably stronger activity-dependent synapses in the different frontal, parietal, and insular target areas. This might further imply that layer III-efferents exhibit more axonal ramifications and thus sprout in the target areas and replace or detain layer V/VI-efferent contacts and axonal fibres. This time-shifted, layer-dependent scenario would be strongly in line with our findings indicating that layer III-fibre densities establish adult axonal patterns considerably later than layer V/VI-efferents. In addition, a suppressive mal-adaptive DAergic maturation following the IR-condition (Dawirs et al., 1994; Winterfeld et al., 1998) might produce less intense activation of layer III-efferents. Thus, a reduction in fibre densities of both efferent paths in adulthood compared to ER-controls might be the result, which fit well to our previous results (Bagorda et al., 2006).

Considering the distinct developmental time courses of the two prefrontal output layers, their reciprocal interdependencies should be seriously taken into account. Prefrontal layer III-pyramids form selective excitatory intrinsic and associational connections to cortical layers II, III, and also V (Melchitzky et al., 1998, DeLane et al., 2005), and exhibit directly input to pyramidal layer V-cells (Thomson et al., 2003; Bannister et al., 2005). Thus, by means of increasing layer III-fibre densities during adolescence (as found in the present work), layer III-cells should also provide an increasing control onto layer V/VI-pyramids. This could somewhat lead to a completion or cessation of fibre outgrowth in the latter layer V/VI-output system. It can be thereby supposed that layer III-efferents somehow organize adult-like patterns in layer V/VI-efferent fibres increasingly during later adolescent periods via their now maturing direct input to layer V/VI-pyramdial cells in the same cortical module. Without this arranging control served by layer III-efferents, layer V/VI pyramidal cells might conserve early, initial connection patterns. On the other hand, layer V-pyramids target via feedback-projections inhibitory layer III-GABAergic interneurons that inhibit and somewhat organize layer III-pyramidal excitation (Thomson and Bannister, 1998). Thus, one could further speculate that layer III- and layer V/VI-pyramids might increasingly influence each other and thus more and more contribute to a balance in neuronal activity during later juvenile and asolescence development, which is crucially for functional integrated networks (Lehmann et al., 2005).

One should also consider that especially the prefrontal network is truely affected by multiple neuromodulatory systems and various converging developmentally long-lasting processes, not

only regarding the maturation of DAergic but also GABAergic and serotoninergic fibres and other transmitters (Benes et al., 2000). The local GABAergic system, for example, should act very efficiently as a rythmic organizator during the whole lifespan and especially also during specific early postnatal time windows (Cruz et al., 2003). For a detailed discussion, please see p. 37-39. Thus under normal conditions, the whole juvenile phase is presumed to be critical for fibre organization during the early juvenile and adolescent time windows, while on the other hand plastic, life-long adaptations even beyond the level of synapses seem to play a crucial role in a well-functioning prefrontal network in relation to the environment even until old age. The latter will now be further elucidated in a recent study dealing with synaptic plasticity and lysosomal degradations in relevant cortical areas in adult animals after different rearing conditions (Neufeld, Witte et al., in prep.).

As consequence of highly selective and dynamic maturation processes, the PFC is supposed to be exceptional vulnerable to epigenetic challenge. By means of adverse juvenile interventions, the findings of our "two-hit animal model" (Bagorda et al., 2006; Mednick et al., 1998) has now since long detected a dramatic, even pathologic alteration of brain organisation due to mal-adaptive structural changes. In more detail, the neuromodulator DA is strongly suggested to exhibit a supressed fibre growth in the PFC after early interventions (Dawirs et al., 1993; Winterfeld et al., 1998), which should in turn induce further, presumably dysfunctional modifications in related systems (see e.g. Bagorda et al., 2006).

My findings now indicate the glutamatergic intra- as well as intercortical output system to re-act to the altered DAergic input with a disrupted target innervation especially due to missing steps of structural (and thus even functional) reorganization processes during the two selective developmental time windows. With regard to the different epigenetic conditions, it seems that a single challenge (either MA or IR alone) induces fewer, somewhat less dramatic alterations than the combined challenge (MA-IR). Moreover, this combined condition led to a critical imbalance in the hierarchically different efferent systems: It might, instead of a down-regulated prefrontal control, rather reflect an imbalanced, pathologic mis-wiring of prefrontal efferents, also termed as "dysconnection" (Weinberger et al., 1995; see Bagorda et al., 2006 for first anatomical evidence). It might now be suggested that the extreme loss of DAergic innervation due to the combined epigenetic traumatisations might affect the pyramdial efferent systems in such a way that their reciprocal organization is severly suspended: Layer V/VI-efferents of IR-MA animals might grow exuberantly compared to IR-animals due to a the lack of pruning during the third postnatal week. Apallingly, pyramidal layer III-neurons fail to compensate for or down-regulate those abnormal layer V/VI-fibre densities in the

course of development, since their DAergic organizational input does not mature appropriately in the adolescent time window, and they do not receive sufficient well-defined DAergic control. Thus, the required control of layer III-efferents on layer V/VI-efferents might be extremely vanished. Moreover, it can be assumed that during the adolescent phase, when normally layer III-efferents reach high adult fibre density values in distant cortical target areas, their reduced activity (due to the lack of ingrowing DAergig afferent input) might be thus deficient to not overcome the oversprouted layer V/VI-efferent activities. Therefore layer III-connections might fail to stabilizise and thus stay at their initial low density-values even in adulthood. Thus, the affected, high-regulated layer V/VI-fibre densities in contrast to decreased layer III-densities in the animal model can now be interpreted not only as a dramatic imbalanced miswiring, but also as a presumably pathologic preservation of early postnatal, initial prefrontal connection patterns, which should clearly represent a loss of crucial developmental steps and the absence of functional reorganisation processes during ontogeny to establish well-functioning higher-order prefrontal cytoarchitecture.

As previously discussed for ipsilateral connections, the trauma-induced changes reveal valuable insights into the underlying neuroanatomical causes of human schizophrenia. Thus theoretically also in humans, early traumatic experiences might affect the DAergic prefrontal system, which in turn leads to severe alterations not only in the serotonergic and GABAergic system, but also in the glutamatergic output pathways. Therefore, a miss of integrated prefrontal higher-order functions might give rise to severe psychotic symptoms. As a consequence, in future research one should now bear in mind these neuroanatomical structural findings and further engage to find possible treatment mechanisms that help to ameliorate pathologic brain changes. In this context, the findings that a healthy lifestyle-behaviour in elderly people is associated with better cognitive performance (Flöel, Witte et al., submitted) gain in importance, since it is assumed that positive stimuli e.g. provided by exercise and a healthy diet might indeed reflect the positive effects of an enriched environment as described in the animal model. In a current prospective clinical study, these coherences will be further elucidated by different dietary interventions and related cognitive outcome in healthy elderly subjects (Witte et al., in prep.).

In summary, enduring plastic capacities on a structural level are a vulnerable characteristic of particularly the PFC in rodents and also in humans, both during juvenile developement but also throughout life. As revealed by the present findings, the maturation of transmitter systems and their life-long morphogenetic potential is crucially dependent on epigenetic factors. This clearly points to the importance of a healthy environment for brain maturation

and functional maintenance even during aging, in accordance to functionally integrated structural rebuilding processes. Therefore, the important feature of persisting cortical re-organization should be regarded as a fascinating phenomenon to ensure the establishment and preservation of environment-adaptive, well-functioning neuronal processes during childhood and throughout life.

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Short Communication

Contralateral prefrontal projections in gerbils mature abnormally after early methamphetamine trauma and isolated rearing

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Summary As previously shown, a miswiring of ipsilateral prefrontal projections after methamphetamine (MA) intoxication and/or isolated rearing (IR) may serve as a model of so-called "dysconnection" in human schizophrenia. We here find that deep prefrontal projections to contralateral targets were drastically reduced by both MA and IR alone, but remained equally dense if both impairments cumulated. Projections from superficial layers were not altered by MA and/or IR. These findings confirm that the normal intercortical integration of information is compromised in this animal model of schizophrenia.

Keywords: Methamphetamine, prefrontal cortex, dysconnection, laterality, cortico-cortical projections

A miswiring of efferents from the prefrontal cortex (PFC), dubbed "dysconnection", has been hypothesised to represent the neural substrate of schizophrenia's positive symptoms (Weinberger and Lipska, 1995). In recent years, data from post-mortem human brains and diffusion tensor imaging have lent growing support to this concept (Kubicki et al., 2005). Direct histological confirmation of the dysconnection hypothesis, however, has still been impossible to obtain. We therefore used an animal model of trauma-induced aberrant brain maturation, the methamphetamine (MA)-intoxicated gerbil, which bears resemblance with human schizophrenia in several aetiological, structural and behavioural aspects, i.e. causation by severe trauma in infancy and second hit in adolescence (cp. Read et al., 2001), alterations in prefrontal and striatal dopamine and serotonin innervations (Dawirs et al., 1994; Winterfeld et al., 1998; Neddens et al., 2002, 2003; Lehmann et al., 2003), disturbances in cortical lateralisation (Neddens et al., 2004; Lehmann et al., 2004) and impairments in PFC-based learning abilities (Dawirs et al., 1996; Polascheck, 2004). In this model, we could recently demonstrate an abnormal development of PFC projections to ipsilateral frontal, parietal and insular cortices (Bagorda et al., 2006). This miswiring involved the efferents from both superficial and deep prefrontal laminae. In general, projections from both laminar origins were reduced by each an early traumatic event (intoxication with a single high dose of MA on postnatal day 14 in contrast to a saline injection) and chronic environmental deprivation, i.e. isolated rearing (IR) after weaning (PD30), alone. The combination of both strains, in contrast, caused an imbalance of superficially originating fibres, which were attenuated, and efferents from deep layers, which were kept. Apart from ipsilateral cortico-cortical connections, the balance and communication between the two hemispheres is also known to be compromised in schizophrenia (Innocenti et al., 2003). We therefore expected prefrontal projections into the contralateral PFC to be similarly disrupted in our animal model.

To test this assumption, fibres marked in 50 µm sections of the left PFC (Fr2 region) of gerbils by an injection of biocytin (0.5%, 3 µl) into the right PFC's deep or superficial laminae were visualised by standard avidin-peroxydase and DAB staining methods (see Bagorda et al., 2006 for details), detected by image analysis, represented as lines of one pixel width, and their density calculated as

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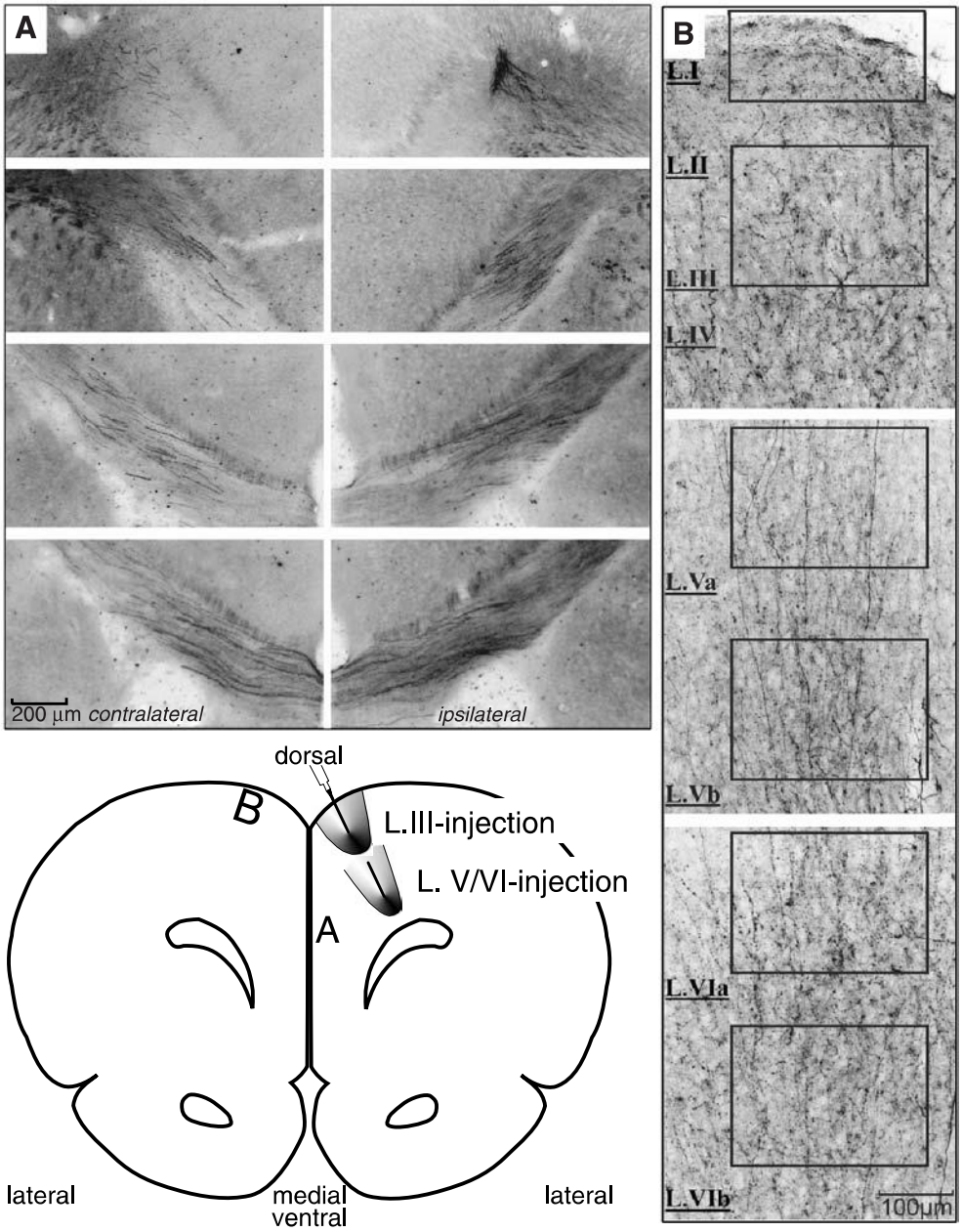

Fig. 1. Efferent biocytin-stained fibres pass from ipsilateral medial prefrontal cortex (mPFC, Cg1/Fr2-region) to contralateral medial prefrontal targets. Biocytin injections into lamina III- and lamina V/VI, respectively, are indicated by greyscaling in the schematic drawing. (A) The descending axoncollaterals enter the corpus callosum, cross to the opposite hemisphere, and rise contralaterally to the injection area. Densities of passing fibres and axon terminals in the contralateral cortical projection area were assessed by digital image analysis in six separate measurement windows (B). Pictures A and B taken of enriched reared control animals

the area of the line in relation to the region of interest, in percent (Fig. 1). For image analysis, six mutually adjacent windows throughout the depth of the cortex were defined in Fr2 cortex at a magnification of 200×, using a Polivar microscope (Reichert-Jung) with a digital video camera (ProgRes 3008 mF, Jenoptik) feeding images at a resolution of 2048×1450 square pixels into the programme KS300 (Carl Zeiss Jena). Data were statistically analysed by two-way ANOVA using two main factors of rearing and MA treatment. Subsequent post-hoc testing and contrast analysis were done as appropriate.

As a result, fibres labelled by injections into superficial layers of the Fr2 cortex were markedly fewer than those from deeper layers in all groups, but there were no detectable differences among groups (data not shown). In contrast, the densities of fibres marked by deep injections were changed by rearing ($F(1,16) = 7.63$, $p = 0.014$), MA treatment ($F(1,16) = 9.15$, $p = 0.008$), and the interaction of the

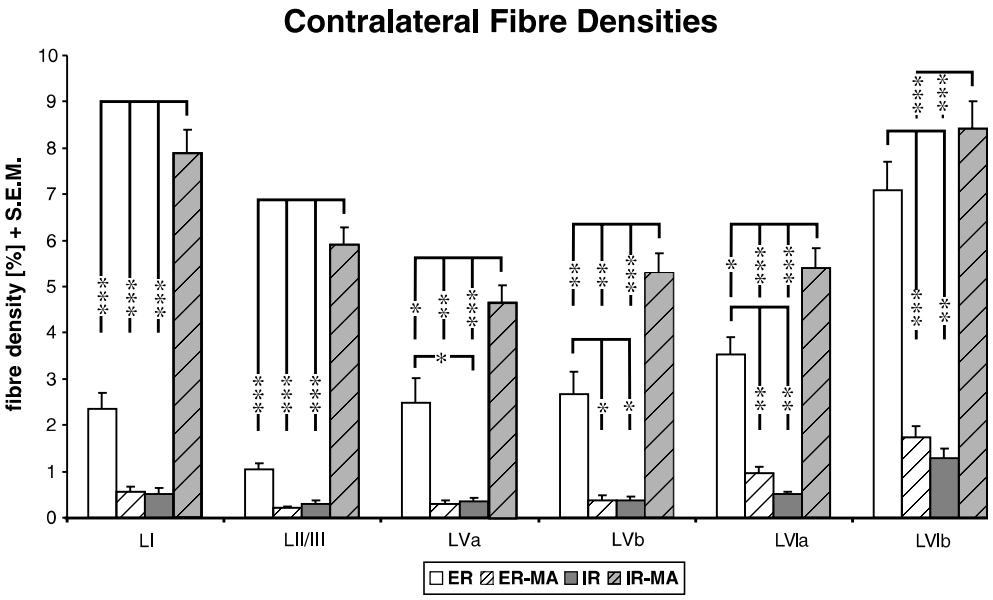

Fig. 2. Densities of axon terminals and passing fibres in the contralateral prefrontal cortex stained by biocytin injections into deep layers (lamina V/VI-injections) of the right mPFC. Data are given as means + standard error of the mean (S.E.M.). Sample sizes: Enriched reared (ER): n = 4; MA-injected ER (ER-MA): n = 5; isolation reared (IR): n = 6; MA-injected IR (IR-MA): n = 5. Asterisks indicate differences among groups within a lamina. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

two factors ($F(1, 16) = 60.04$, $p = 0.000$), according to ANOVA (Fig. 2). Specifically, as detailed by contrast analysis, IR had opposing effects, depending on whether the animals had previously been traumatised by MA or received a control injection of saline: Whereas IR reduced the fibre densities in infragranular layers ($p < 0.05$) of control animals, it increased them in MA injected gerbils throughout all layers ($p < 0.01$). Accordingly, a MA injection on P14 had opposing outcomes in the adult animal, depending on the subsequent rearing conditions: MA injected enriched-reared (ER) animals had lower fibre densities in the infragranular layers than their respective controls ($p < 0.05$). In contrast, after IR, stained contralateral fibres were denser throughout the cortex in MA injected animals, compared to saline injected IR controls ($p < 0.001$). These findings correspond closely to our previously reported results in ipsilateral cortical target areas of prefrontal efferents (Bagorda et al., 2006).

Mitchell and Macklis (2005) recently showed that in mice, approximately 40% of neurons in premotor cortex with projections into the ipsilateral sensorimotor cortex also project into the contralateral premotor cortex. Since their tracer injections were only slightly lateral to ours, a similar collateralisation of prefrontal efferents in gerbils may be supposed and explain the similarity of results in ipsi- and contralateral target areas. That study (Mitchell and Macklis, 2005) also demonstrated that markedly fewer neurons make long range connections in layer III than in layer V, and that a much smaller proportion makes dual projections into the two hemispheres. That we did not detect significant effects of MA nor IR on contralateral projections from outer layers may therefore reflect different developmental timecourses of layer III versus layer V pyramidal cells, as reported for interhemispheric visual connections (Ivy and Killackey, 1981), or it may be due to the very low fibre densities.

In both ER controls and MA injected ER animals, fibres originating from deep injections terminate with higher densities in deeper than superficial laminae. This relationship is weakened in IR controls and completely lost in MA-pretreated IR gerbils. Thus, in unimpaired animals, most pyramidal-collaterals from deep layers project symmetrically again into deep layers (Gerfen, 1989). Since projections in MA-pretreated IR animals, in contrast, are dense and widespread throughout all layers, it becomes likely that the interhemispheric exchange of information is severely impaired in these animals.

Abnormalities of cortical lateralisation have consistently been linked to human schizophrenia (Crow, 1997; Falkai and Bogerts, 1992; Harrison, 1999; Sommer et al., 2001). Apart from a shift in hemispheric dominance that is reflected by both anatomical and physiological parameters (Falkai et al., 1995a,b; Highley et al., 1999; Mitchell et al., 2004; Rasser et al., 2005; Richardson et al., 1999; Sauer et al., 1999), there is also an altered degree of intercortical organisation in schizophrenics: Artiges and colleagues (2000) described lower activations of left frontal regions in combination with weaker deactivation of right inferior parietal areas during

word production tasks, whereas other studies detected a reversed lateralisation effect in frontal, sensorimotor and visual cortices in schizophrenic patients compared to healthy subjects (Heckers et al., 2002; Schroder et al., 1995). Thus, our findings presented here might contribute to the anatomical background of abnormalities in interhemispheric information processing. The enhancement of callosal collaterals and Fr2-terminals in MA-intoxicated IR animals might lead to an over-activation of contralateral prefrontal networks, providing smaller effects of lateralised functions. Further studies may help to better understand how pathologic prefrontal lateralisation could emerge from early traumatisation and lead to abnormal behavioural effects.

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Developmental pattern changes of prefrontal efferents in the juvenile gerbil (*Meriones unguiculatus*)

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Summary Previous findings of our group showed that early traumatisation leads to a dysfunctional organisation of prefrontocortical efferents in adulthood. To identify vulnerable time windows during maturation, we labelled either layer III- or layer V/VI-pyramidal cells with biocytin in the prefrontal cortex of gerbils (*Meriones unguiculatus*) from the age of postnatal day (PD) 15 up to adulthood (PD 90). The density of passing fibres and axonal terminals in distinct cortical columns in specific prefrontal projection areas was assessed by digital image analysis. Following layer III injections, fibre densities reached adult values between adolescence (PD 60) and adulthood (PD 90). However, layer V/VI-fibre densities decreased after eye-opening (PD 15), followed by an increase to adult values after weaning (PD 30). These findings are the first to describe dynamic structural changes even beyond adolescence of functionally diverse prefrontal output systems. External interventions might exert adverse influences on the establishment of integrated prefrontal networks especially during the early phase of re-arranging.

Keywords: Glutamate; prefrontal cortex; biocytin; cortico-cortical connections

Introduction

Cortical projection systems originate from excitatory pyramidal cells, which are distributed throughout specific layers of the cerebral cortex. In general, pyramidal cells positioned in laminae V and VI project to both subcortical and distant cortical areas, while pyramidal cells situated in laminae II/III are known to form mainly, but not exclusively, intrinsic connections between adjacent cortical areas (Bannister 2005). Indeed, early studies suggested that the distinctive laminar distribution of the pyramidal cells may correspond to different output functions (Jones 1984). Thus, the pyramidal efferents of the evolutionary youngest part of the brain, the prefrontal cortex (PFC), target multiple cortical and subcortical fields and play a crucial role in the processing of complex functions such as generation and revision of planned behaviours or purposive movements. These higher-order functions are realized not only in humans, but also in other mammals such as primates and rodents (Kolb 1984; Uylings and van Eden 1990). Since various dysfunctions of PFC-related behaviours are presumably caused by cell disturbances during cortex development (Weinberger and Lipska 1995), the present experiments focus on the establishment of the prefrontal output system at structural level in adolescence, which may be reflected in aberrant formation of PFC-projections. The present experiments focus on the time course of the physiological establishment of the prefrontal output systems in adolescence on a structural level.

In the period of embryonic differentiation, the axonal growth cones of pyramidal cells are conducted by multiple cues in their intrinsic environment (reviewed in Dodd and Jessell 1988; Tessier-Lavigne and Goodman 1996). Since the neuronal differentiation and the formation and refinement of synaptic connections cause continuing changes in intrinsic properties and synaptic transmission of pyramidal cells, these factors are known to play a considerable role in the axon guidance (Katz and Shatz 1996; Spitzer 1991). In addition, the myelination process contributes to the axonal growth in the proceedings of neuronal development (Hu and Strittmatter 2004; Sherman and Brophy 2005).

In early postnatal stages, the impacts of extrinsic cues on the formation of area-specific patterns of connectivity gain

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in importance. For instance, it is shown that neonatal ablation of the prominent visual thalamocortical projection leads to a drastic disturbance of the pyramidal cell innervation in the visual cortex in hamsters (Kingsbury et al. 2000, 2002). In contrast, non-sensoric cortex areas, especially the PFC, exhibit a late and prolonged postnatal development beyond those of sensoric areas (Fuster 2002; Goldman-Rakic 1987; Levitt 2003; van Eden et al. 1990). Dopamine (DA) fibres, for example, do not reach adult levels until early adulthood in the PFC, where they target mainly on pyramidal cells (Dawirs et al. 1993b; Kalsbeek et al. 1988). Moreover, the myelination process, which triggers the cessation of fibre outgrowth, occurs last in the PFC in postnatal life (de Graaf-Peters and Hadders-Algra 2006). Thus, the very slow maturation and the ongoing augmentation of fibres cause a high structural plasticity of this area. As a result, the PFC is supposed to be especially vulnerable to disturbing influences during an extended juvenile development.

Indeed, previous findings of our group described early adverse interventions to induce highly interrupted transmitter maturation patterns especially in the PFC: Both impoverished rearing after weaning on postnatal day (PD) 30 and a single early methamphetamine (MA)-intoxication on PD 14 affected primarily the maturation of the DAergic innervation (Dawirs et al. 1994; Teuchert-Noodt and Dawirs 1991; Winterfeld et al. 1998). In addition, the suppressed DA maturation in the PFC is supposed to cause further extensive alterations not only in other mesolimbic and mesocortical DAergic projections (Busche et al. 2004), but also in the serotonin (Neddens et al. 2003, 2004) and the GABA system (Nossoll et al. 1997). As another and even more direct consequence, these early non-invasive interventions led to a miswiring of pyramidal PFC-efferents to specific ipsilateral and contralateral target areas in adulthood (Bagorda et al. 2006; Witte et al. 2007).

Now the question arose, whether a direct derangement of regressive events, which are known to play a key role in modifying neuronal connectivity (Low and Cheng 2006), or an abnormal prolonged progressive development of pyramidal efferents may cause this pathology. Therefore, the goal of this study was (1) to determine the precise maturation of the prefrontocortical projection system, depending on cortical target areas and layers of origin; and (2) to identify possible time windows during juvenile development where critical refinements of axonal branching may occur, vulnerable to epigenetic abnormalities. This may help to better understand how and when environmental challenges in postnatal development could crucially affect the establishment of prefrontal networks.

Material and methods

Animals

In total, 148 male gerbils were bred in standard laboratory cages (Makrolon® type IV). Seventy-eight pups received biocytin injections in three age-groups on PD 15–19 (infantile, $n = 31$), 20–24 (early juvenile, $n = 29$) and 25–29 (pre-weaning, $n = 18$), respectively. The remainder of the animals were weaned on PD 30 and kept separately in standard home cages (Makrolon® type III). Fifty-eight animals were injected with biocytin either after weaning on PD 30–49 (post-weaning, $n = 45$) or around puberty on PD 60–79 (adolescent, $n = 13$). Twelve gerbils were allowed to reach adulthood and received biocytin labelling on PD 90. Data of the 12 adult animals have been published before partially (for details see Bagorda et al. 2006; Witte et al. 2007). Food and water were provided ad libitum. All gerbils were on natural day/night cycles. The experimental procedures were approved by the appropriate committee of animal care in accordance with the guidelines of the European Communities Council Directive.

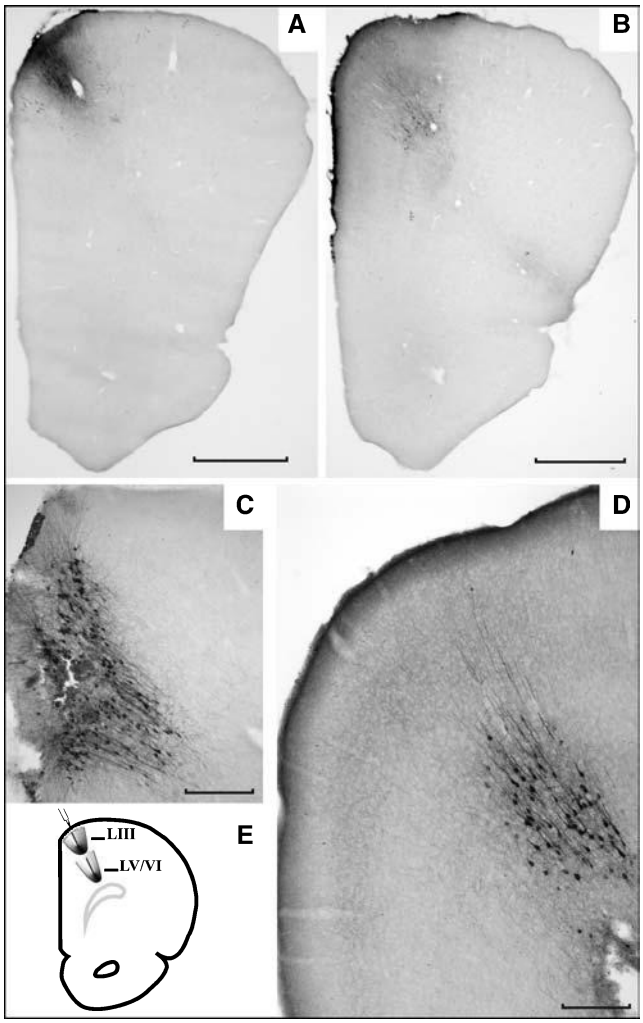

Fig. 1. Prefrontal pyramidal cells filled by an injection of biocytin into superficial layers (LIII, **A** and **C** for details) or deep layers (LV/VI, **B** and **D** for details) of the medial prefrontal cortex (Cg1/Fr2-region) in front of the corpus callosum (**E**). Scale bar: A, C = 1000 µm; B, D = 200 µm

Biocytin fibre tracing

The animals were fixed into a stereotaxic frame after anaesthesia with diethylether. At the level of the prefrontal cortex a hole was drilled into the skull to apply the anterograde tracer biocytin. Three µl of 0.5% biocytin solution in 0.1 M phosphate buffer (PB, pH 7.4) were injected at midline into the shoulder subfield which encompasses the medial precentral (Fr2) and the dorsal-anterior cingulate (Cg1) cortex at 4.5 mm anterior to bregma. Based on the frequently used definition of the PFC by Rose and Woolsey, that includes all cortex with reciprocal connections from the mediodorsal thalamus (Rose and Woolsey 1948), we considered Fr2 and Cg1 as part of the PFC. Notably, PFC-classification in rodents still remains controversial

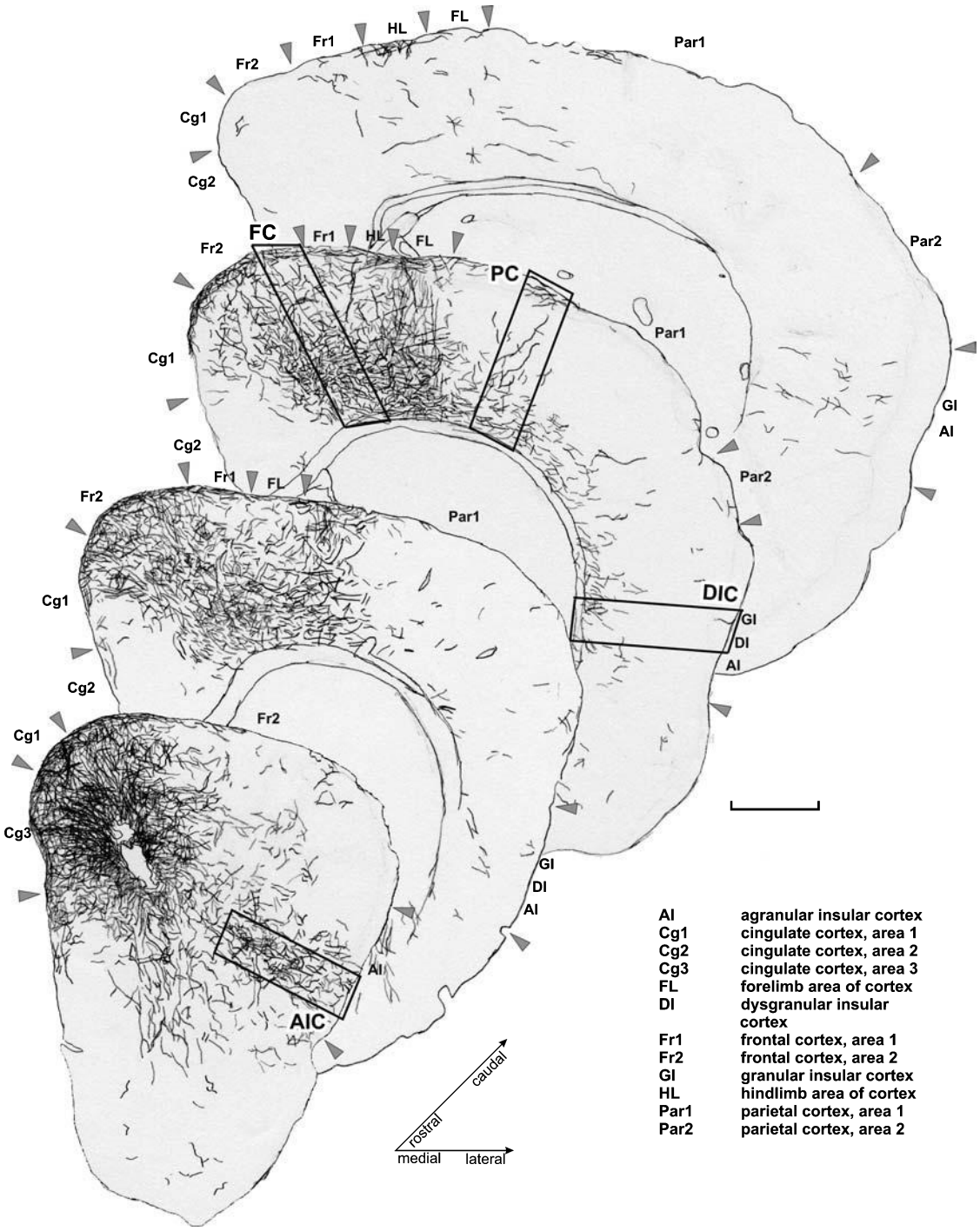

Fig. 2. Ipsilateral camera lucida drawings of selected frontal slices through an exemplary 17 days old gerbil brain with layer V/VI-efferents of the medial prefrontal cortex (Cg1/Fr2-region, see Fig. 1), labelled by a deep biocytin-injection. The boxes in the first and third section depict the columns in which fibre densities were assessed; i.e. in the agranular insular cortex (*AIC*), frontal cortex (*FC*), parietal cortex (*PC*), and dysgranular insular cortex (*DIC*). Scale bar = 1000 µm

(Brown and Bowman 2002; Dalley et al. 2004). Either superficial injections ($n = 77$), aiming at pyramidal cells in layer III, and deep injections ($n = 71$), targeting pyramidal cells in layer V/VI (Fig. 1), were performed. The skull was closed with hystoacryl. After 24 hours, the animals were transcardially perfused under deep chloralhydrate anesthesia (1.7 g/kg, i.p.) with 200 ml PB, followed by 4% paraformaldehyde and 0.5% glutardialdehyde in 0.1 M PB. The brains were removed and kept in fixative to increase background staining. After one week, frontal brain sections of 60 µm thickness were cut on a frigomobile (Reichert-Jung) and every other section was collected in 0.01 M PB. Subsequently, slices were incubated with 1% sodium borohydride (Sigma) for 20 min and treated overnight after washing in PB with ExtrAvidin-Peroxidase (Sigma) diluted 1:125 in 0.01 M PB containing 1% bovine serum albumin and 0.5% triton X-100 (Sigma). On the following day, sections were washed twice in PB, twice in 0.05 M Tris-buffer (pH 7.6) and stained by DAB reaction. After washing in PB, the slices were mounted on coated glass slides, dried overnight, and coverslipped in DePeX (Sigma). Biocytin is a sensitive tracer capable of detailing the morphological features of axonal fibres and terminal fields within the neocortex. It is efficiently transported to the cell soma and axon by neuronal dendrites, which provides valuable information about the location of the cell within the prefrontal area: superficial injections labelled primarily layer III-pyramids, whereas deep injections labelled primarily layer V/VI-pyramids.

Qualitative evaluation of projection patterns

To delineate the different injection sites and the staining in general, assembly drawings were made on every animal before assigning to quantitative evaluation. Those who showed either injections at wrong stereotactic positions at the rostro-caudal level or those with irregular distribution of biocytin-filled cells were sorted out. Further, only cases with comparable numbers of biocytin-filled cells were used for statistical analysis. In order to gather an overview of the projection pattern and get a general impression of a deep injection of a young animal (PD19), camera lucida drawings were made of four ipsilateral and one contralateral section (Figs. 2 and 3). The rostrocaudal positions of these sections with distances to bregma transferred to the rat brain (Paxinos and Watson 2005) were: a) the appearance of the forceps minor of the corpus callosum (ipsilateral and contralateral, 4.2 mm anterior to bregma), b) the commissura anterior approaches the lateral ventricle (ipsilateral, 0.84 mm anterior to bregma), c) the commissura anterior crosses to the lateral stripe of the striatum (ipsilateral, 0.12 mm posterior to bregma), and d) at the most rostral appearance of the hippocampus (ipsilateral, 1.72 mm posterior to bregma).

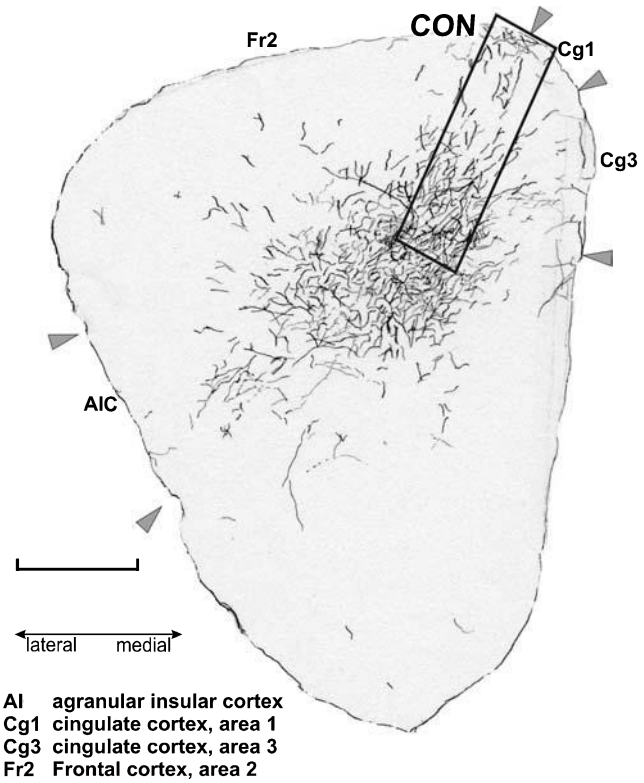

Fig. 3. Contralateral camera lucida drawing of a selected frontal slice of an exemplary 17 days old gerbil brain with layer V/VI-efferents of the medial prefrontal cortex (Cg1/Fr2-region; see Fig. 1), labelled by a deep biocytin-injection. The box depicts the column in which fibre densities were assessed; i.e. in the contralateral prefrontal cortex (*CON*). Scale bar = 1000 µm

Computerised assessment of terminal field densities

Prefrontal projections enter their receptive fields in distinct columns spanning the whole depth of the cortex. For evaluation, we chose five characteristic columns (Figs. 2 and 3), which reliably appear at the rostrocaudal level of either the forceps minor of the corpus callosum (4.2 mm anterior to bregma) or the commissura anterior crossing to the lateral stripe of the striatum (0.12 mm posterior to bregma). According to the brain atlas of the rat (Paxinos and Watson 2005), columns are located in the agranular insular (AIC), frontal (FC), parietal (PC), and dysgranular insular (DIC) cortices (ipsilateral) and in the contralateral PFC (CON). The AIC is part of the lateral PFC (van Eden et al. 1990), whereas in the FC prefrontal and premotor aspects intermingle (Kolb 1984). The PC contains somatosensory features like the barrel field (Frostig 2006), whilst the DIC act as a somatic 'viscerosensory' area (Gabbott et al. 2003). Three neighbouring sections at these levels were evaluated. The sections were viewed in dark field at 125× magnification on a microscope (Polyvar, Reichert-Jung). Pictures were taken by a digital camera (ProgRes 3008mF, Jenoptik, Jena) and processed by software for image analysis (KS300, Zeiss, Jenoptik, Jena). Either two (AIC) or three (FC, PC, DIC, CON) separate pictures were taken of superficial, middle and deep target laminae, converted to black-and-white pictures, and inverted. Background was suppressed by high-pass filtering. DAB-stained fibres of different diameter were standardised to identical thickness and visualised using a combination of Gauss filter and Gerig operator ("valleys operator") that depicts differences of grey values of adjacent pixels and transforms the result into binary images. The fibre density was calculated as percentage of the measurement window, which was the same size for all target laminae. In the AIC, laminae I, II and V were evaluated separately. In the FC, PC, DIC, and CON laminae I, II, V, and VI were examined. In addition, the extent of each cortex area was measured in every animal to adjust the measurement windows for individual brain sizes. The analysis was performed under blinded conditions with regard to animal age and injection site.

Statistical analysis

In total, 90 animals of comparable biocytin injection and fibre staining were selected for quantitative evaluation (PD 15–19: L III $n = 5$, L V/VI $n = 8$; PD 20–24: L III $n = 11$, L V/VI n = 8; PD 25–29: L III $n = 5$, L V/VI $n = 4$; PD 30–49: L III $n = 14$, L V/VI $n = 14$; PD 60–79: L III $n = 4$, L V/VI $n = 5$; PD 90: L III $n = 6$, L V/VI $n = 6$). In each projection area, fibre densities were cecked for statistical differences between age-groups after either layer III-injections or layer V/VI-injections. Comparisons between groups were done by a repeated measures analysis of covariance (ANCOVA, general linear model), using one main factor of age-group, a cofactor of cortex area extent, and a repeated-measurements factor of target lamina. The cofactor was included in order to adjust data for individual brain sizes, which did not correlate significantly with age-groups (Pearson; AIC: $p = 0.75$; FC: $p = 0.15$; PC: $p = 0.16$; DIC: $p = 0.61$; CON: $p = 0.93$).

Subsequently, single comparisons were performed by LSD-post hoc-testing, first to describe general differences between age-groups and, if necessary, to specify differences between age-groups at single target lamina-level, i.e. when only specific target laminae show significant changes in fibre densities. Differences were evaluated by *t*-test after F-test. Data are represented as means ± standard error of means (S.E.M.). All statistical analyses were computed with Statistica 6 (StatSoft, Tulsa, USA). The levels of significance were set at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

Results

Qualitative analysis

Light-microscopical analysis of anterograde biocytin labelling in the developing PFC showed in principle fewer projections following layer III-pyramidal cell-injections as compared to layer V/VI-injections. This proportion and the cortical distribution pattern of prefrontal efferents, independent of injection site, was somewhat similar in juvenile compared to adult gerbils and also consistent with earlier studies in the rat (Sesack et al. 1989). Camera lucida drawings (Figs. 2 and 3) give representative projection patterns (subcortical projections not shown) up from PD 15 following layer V/VI-injections (for comparison with PD 90 animals, see also Bagorda et al. 2006; Witte et al. 2007). The axonal fibres travelled from layer of origin preferentially through layers V and VI, but also ascended and ran along layer I. Fibres ramified in a columnar manner in ipsilateral target areas, i.e. the agranular insular cortex (AIC), the frontal cortex (FC), the parietal cortex (PC), and the dysgranular insular cortex (DIC). In addition, branching collaterals entered slightly caudal to the injection site the corpus callosum and crossed to the contralateral hemisphere. Here, one main fibre bundle rose directly through layer VI to form another distinctive column corresponding to the injection site in the contralateral PFC (CON).

Observation of labelled fibres in cortical target areas at higher magnification revealed some impressive differences between age-groups after layer V/VI-injections. The differences were less striking after layer III-injections. Analysis of distribution and morphology of labelled fibres in the different age-groups revealed specific steps in the maturation of prefrontal projection patterns.

Infantile animals (PD 15–19) exhibited a different state of the cortical projections compared to older animals with terminal fields not yet established. In this age-group, layer V/VI-injections led to a labelling of multiple coarse, hardly ramified axonal fibres, which travelled either horizontally from layer of origin, ascended to layer I or descended ventralwards (Fig. 4, CG left). These pioneer fibres reached via layer I or via layers V and VI various cortical areas and branched in the above described target columns apparently orderless throughout all target laminae. In addition, they exhibited terminal fields in laminae I, V, and VI, mainly consisting of multidirectional fibres rather than fine termination jots (Figs. 4–6, left columns). Following layer III-injections, a similar distribution was seen, although the pioneer fibres were slightly thinner and the fibres in the terminal fields appeared to be somewhat finer (data not shown). Early juvenile, pre- and post-weaning animals (PD 20–49) already showed characteristics of the adult termination pattern like a distinctive laminae-specific distribution of axonal fibres in the cortical columns (see Bagorda et al. 2006; Witte et al. 2007). This was even more the case in adolescent animals (PD 60–79). The early fibre pattern had developed from an unrefined, coarse state over time to an organized, laminae-specific distribution that was segregated into fibres of passage in laminae II, III and VI, and dense, intertwined terminal fields in laminae I, V, and VI (Figs. 4–6, middle columns). Taken from qualitative impression, the abundance of fibres and termination-jots increased from early juvenile to adolescent animals, most obvious in the FC and PC (Figs. 4–6, right columns). In these age-groups (PD 20–49), layer III-injections led again to similar, but somewhat finer projection patterns. This appeared to partially remain until adulthood (PD 90; data not shown).

Quantitative analysis

In the AIC, repeated measures ANCOVA did not show any effect of age-group and target lamina or their interaction on the projection densities from layer III-injections (Table 1, Fig. 7A). Conversely, after layer V/VI-injections, ANCOVA detected significant effects of either age-group ($F(5, 38) = 2.16$; $p = 0.027$) and the interaction of age-group and target lamina ($F(10, 76) = 4.84$; $p < 0.001$) on the efferent fibre densities in the AIC (Fig. 7B). Regarding general differences between age-groups, post-hoc-testing revealed a significant decrease in overall fibre densities about 40% in early juvenile (PD 20–25) compared to infantile animals (PD 15–19; $p = 0.015$, Fig. 7B). In contrast, the overall fibre densities accumulated to finally about 130% of the initial level in adolescent animals (PD 60–79), showing a significant increase in overall fibre densities to adult values between weaning and adolescence ($p = 0.044$, Fig. 7B).

In the FC, repeated measures ANCOVA described strong effects of age-group ($F(5, 38) = 2.87$; $p = 0.009$) and the interaction of age-group and target lamina ($F(15, 117) = 2.81$; $p < 0.001$) after layer III-injections. According to post-hoc-testing, overall fibre densities increased significantly first

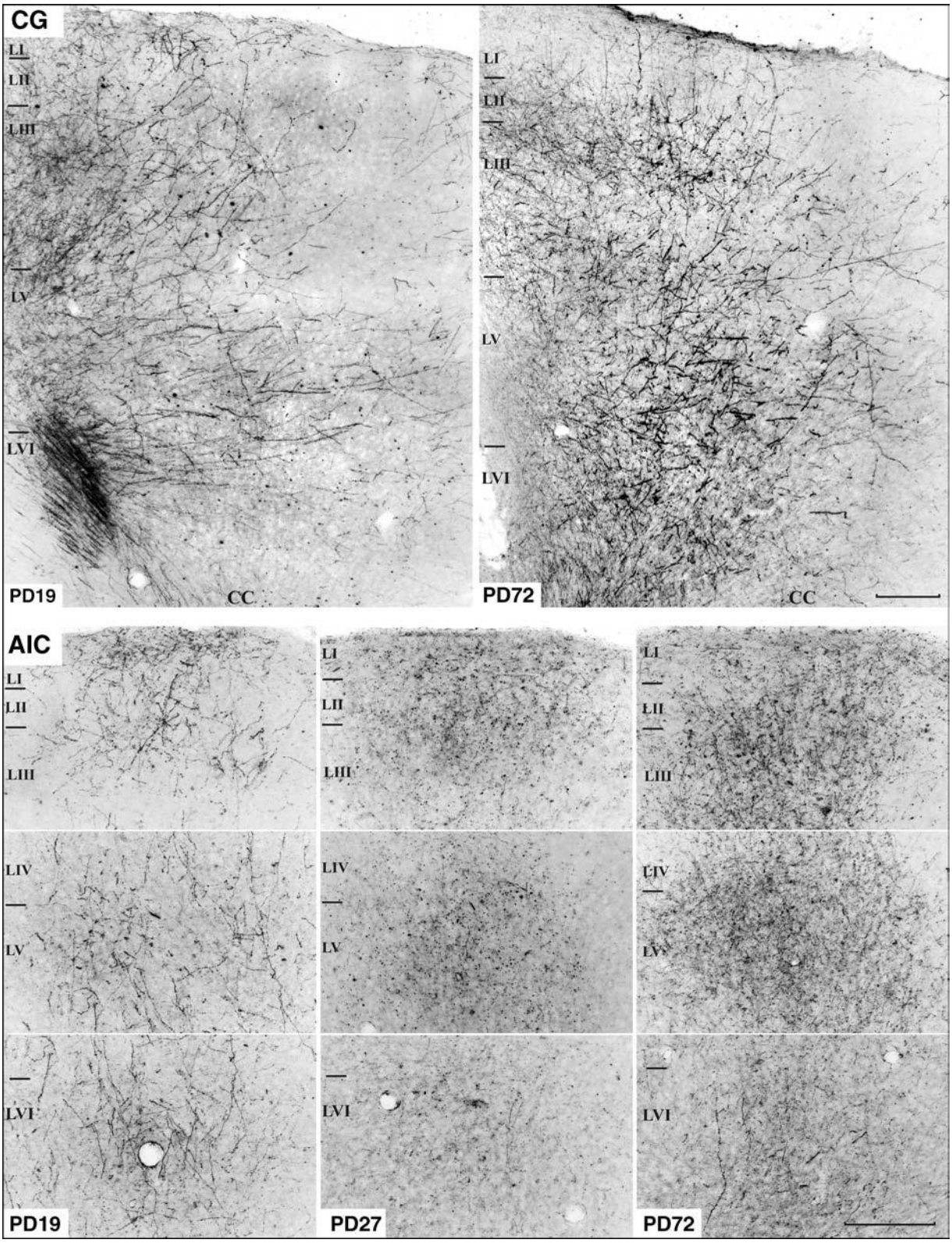

Fig. 4. Prefrontal projection fibres in the ipsilateral caudal Cg1/Fr2-region (*CG*, *top*) and agranular insular cortex (*AIC*, *bottom*) after layer V/VI-injections with biocytin in infantile (PD 19, left), pre-weaning (PD 27, middle), and adolescent (PD 72, right) animals. Note the initial unramified fibres (PD 19) in comparison to more arborized fibres in the older animals in target laminae II, III and VI and the fine terminal jots throughout the laminae. *PD* postnatal day; *CC* corpus callosum; scale bar = 200 µm

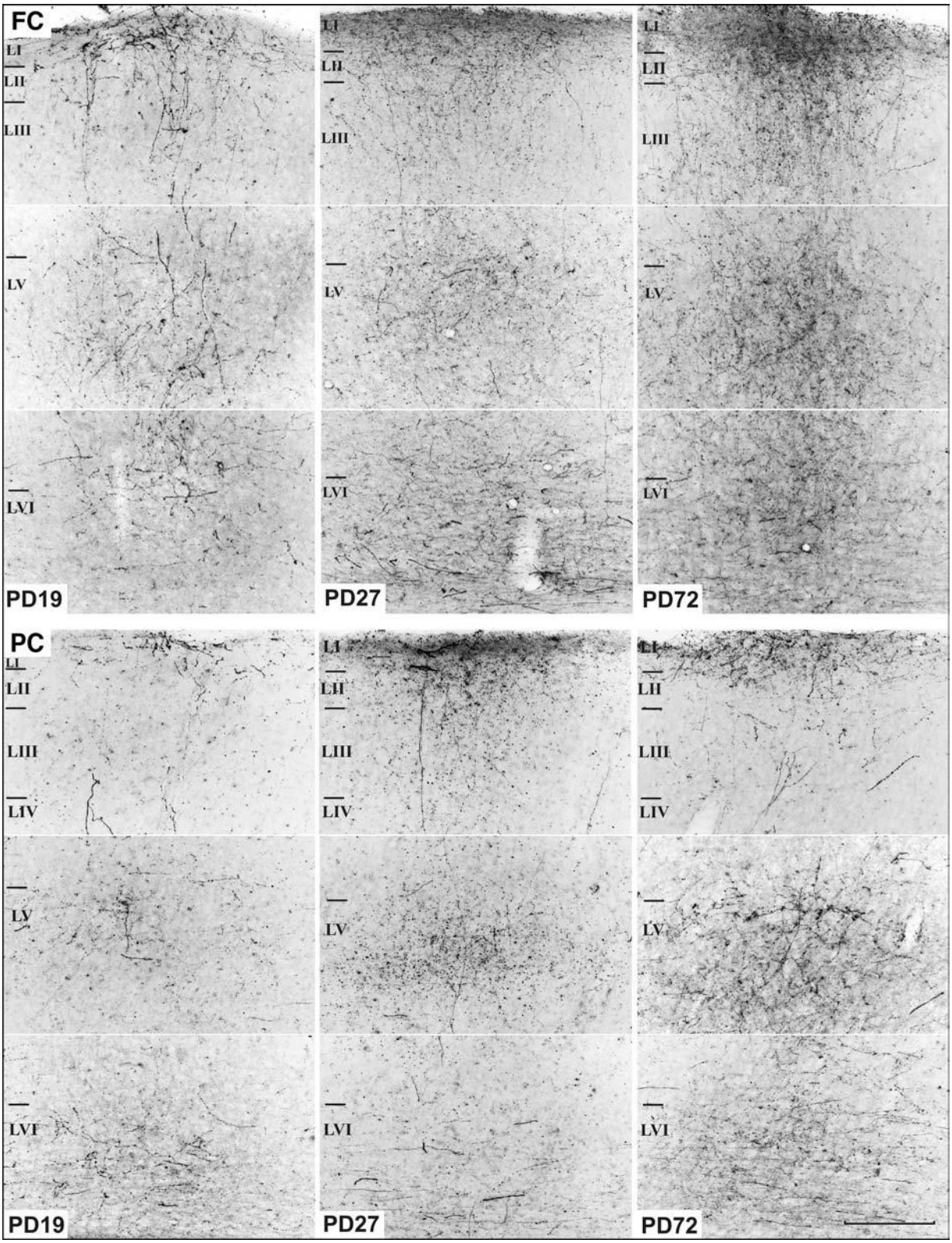

Fig. 5. Prefrontal projection fibres in the ipsilateral frontal cortex (*FC*, *top*) and parietal cortex (*PC*, *bottom*) after layer V/VI-injections with biocytin in infantile (PD 19, left), pre-weaning (PD 27, middle), and adolescent (PD 72, right) animals. Note the different projection patterns during development, manifested by more intertwined, arborized fibres and fine terminal jots in laminae I, upper V and VI, densiest in the adolescent animal. *PD* postnatal day; scale bar = 200 µm

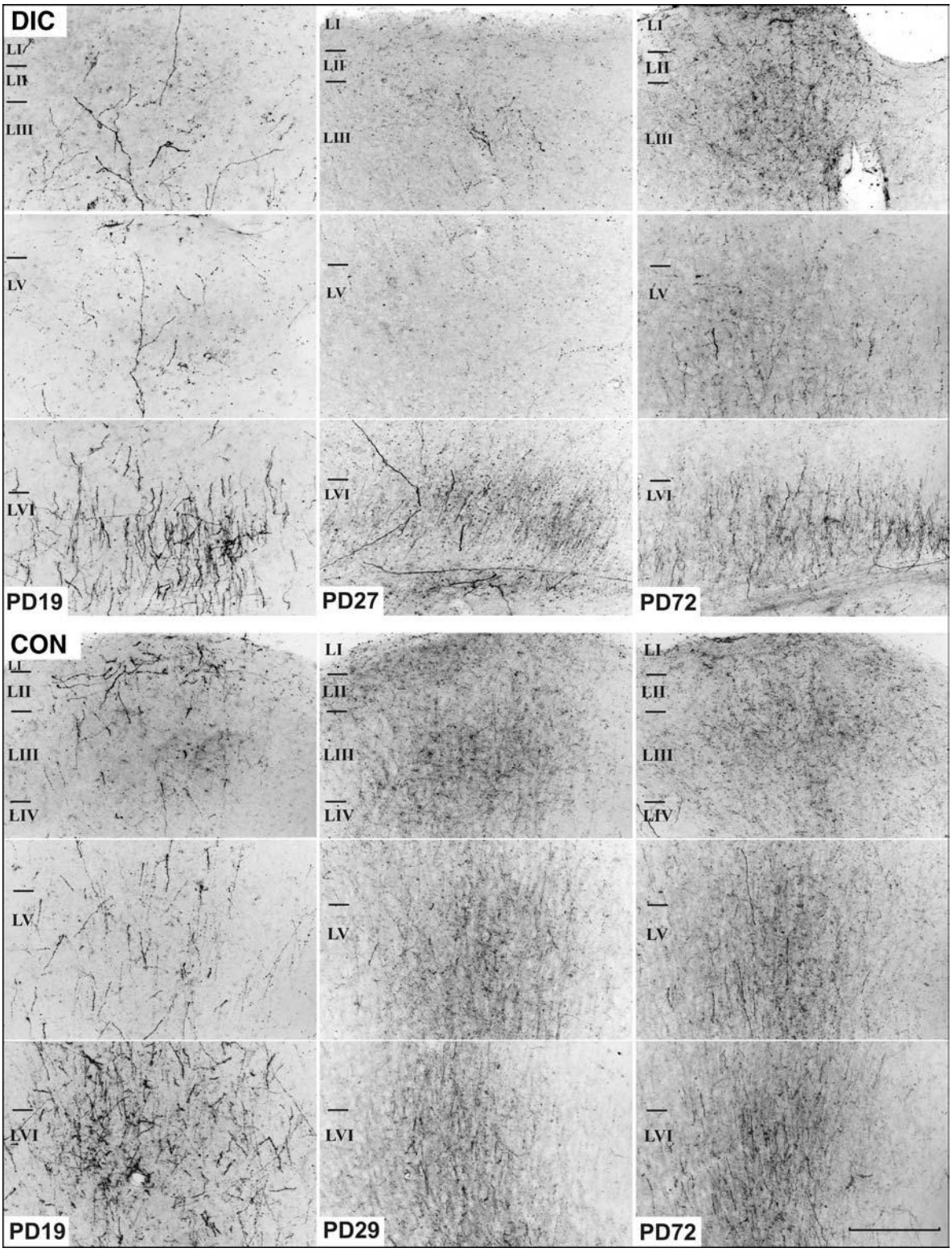

Fig. 6. Prefrontal projection fibres in the ipsilateral dysgranular insular cortex (*DIC*, *top*) and contralateral prefrontal cortex (*CON*, *bottom*) after layer V/VI-injections with biocytin in infantile (PD 19, left), pre-weaning (PD 29, middle), and adolescent (PD 72, right) animals. Note the developmental pattern changes by thinner, multiple arborized fibres and fine terminal lots throughout the target laminae in the organization of the older animals, most precise in the adolescent animal. *PD* postnatal day; scale bar = 200 µm

Table 1. *Results from repeated measures analysis of covariance (ANCOVA), using one main factor of age-group, a repeated measurement factor of lamina, and the cortex extent as a cofactor. Significant effects are indicated by italic font*

Target area	Injection site	Repeated measures analysis of covariance (ANCOVA)		
		Age-group	Target lamina	Age-group*Target lamina
AIC	L III	F (5,38) = 2.16 p = 0.08	F (2,76) = 0.86 p = 0.43	F (10,76) = 1.28 p = 0.25
	L V/VI	*F* (5,38) = 2.87 *p* = 0.02	F (2,76) = 1.3 p = 0.28	*F* (10,76) = 4.84 *p* = 0.000
FC	L III	*F* (5,39) = 3.59 *p* = 0.009	F (3,117) = 0.19 p = 0.9	*F* (15,117) = 2.81 *p* = 0.0009
	L V/VI	*F* (5,37) = 2.49 *p* = 0.048	F (3,111) = 2.02 p = 0.11	F (15,111) = 0.65 p = 0.83
PC	L III	F (5,23) = 1.58 p = 0.21	*F* (3,69) = 0.54 p = 0.66	F (15,69) = 1.63 p = 0.088
	L V/VI	F (5,33) = 1.94 p = 0.11	F (3,99) = 0.21 p = 0.89	F (15,99) = 0.84 p = 0.63
DIC	L III	*F* (5,20) = 9.28 *p* = 0.0001	F (3,60) = 0.73 p = 0.54	*F* (15,60) = 10.39 *p* = 0.000
	L V/VI	F (5,33) = 0.42 p = 0.83	F (3,99) = 0.95 p = 0.42	F (15,99) = 1.04 p = 0.43
CON	L III	F (4,24) = 1.9 p = 0.17	F (2,24) = 2.71 p = 0.087	*F* (8,24) = 3.62 *p* = 0.0067
	L V/VI	*F* (5,29) = 3.09 *p* = 0.023	*F* (2,58) = 3.97 *p* = 0.024	F (10,58) = 1.5 p = 0.16

between adolescent (PD 60–79) and adult (PD 90) animals, showing adult levels over 200% of previous adolescent values ($p = 0.006$, Fig. 7C). Regarding layer V/VI-injections, ANCOVA detected likewise significant effects of age-group on prefrontal projection fibre densities ($F(5, 37) = 2.49$; $p = 0.048$). Subsequently, post-hoc-testing revealed a significant increase of overall fibre densities over 90% between post-weaning (PD 30–49) and adolescence (PD 60–79, $p = 0.0025$), followed by an approximately 50% decrease to pre-weaning values in adult animals ($p = 0.0014$, Fig. 7D). The withdrawal of overall fibre densities as described for the AIC between infantile and early juvenile animals was not evident in the FC.

However, in the PC repeated measures ANCOVA detected no significant effect between groups after layer III- or layer V/VI-injections. Nevertheless, the projections in the PC appeared to develop in a somewhat similar fashion to that of FC projections: on the one hand, fibre densities increased again between adolescence (PD 60–79) and adulthood (PD 90) after layer III-injections (Fig. 7E). On the other hand, fibre densities accumulated to a local maximum around adolescence (PD 60–79) followed by lower adult values (PD 90) after layer V/VI-injections (Fig. 7F).

In the DIC, repeated measures ANCOVA showed highly significant effects of age-group ($F(5, 20) = 9.28$; $p < 0.001$) and the interaction of age-group and target lamina ($F(15, 60) = 10.39$; $p < 0.001$) on prefrontal efferent fibre densities after layer III-injections. According to post-hoc-testing, significant differences between age-groups were observed only at single target lamina-level, namely that fibre densities significantly increased about 100% in target laminae V and VI between adolescent (PD 60–79) and adult (PD 90) animals ($p < 0.001$, Fig. 7G). After layer V/VI-injections repeated measures ANCOVA revealed no significant effect. However, Fig. 7H indicated that prefrontal efferents in the DIC matured likewise in parallel with the FC.

Concerning the contralateral hemisphere, the number of comparable cases was lower than for ipsilateral evaluation, possibly due to the fact that remarkably fewer neurons make dual projections to the two hemispheres (also reported by Mitchell and Macklis 2005), which resulted in a reduced number of evaluable data for statistical analysis. However, repeated measures ANCOVA detected significant effects in the CON for the interaction of age-group and target lamina ($F(8, 24) = 3.62$; $p = 0.0067$) after layer III-injections. In addition, significant effects were found for both age-group ($F(5, 29) = 3.09$; $p = 0.023$) and target lamina alone ($F(2, 58) = 3.97$; $p = 0.024$) after layer V/VI-injections. According to post-hoc-testing, there was a significant increase in overall fibre densities after weaning (PD 30, $p = 0.0019$) and a strong decrease in overall fibre densities after adolescence (PD 60, $p = 0.001$, Fig. 8).

In total, results from repeated measures ANCOVA are shown in Table 1.

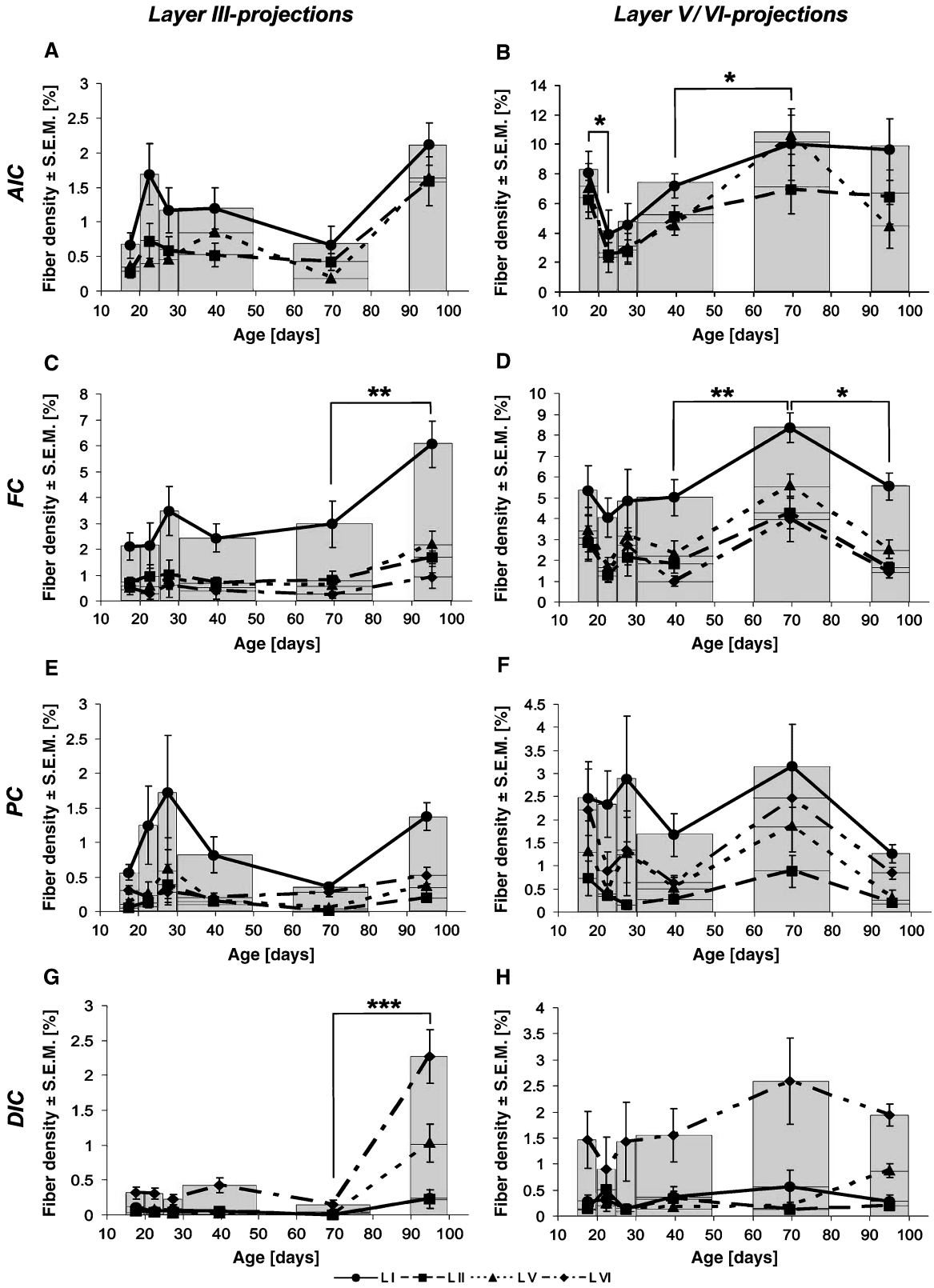

Fig. 7. Densities of terminal fields and passing fibres in the ipsilateral cortex labelled by biocytin injections into layer III (left) or layer V/VI (right) of the medial prefrontal cortex. Fibre densities were assessed in the agranular insular cortex (*AIC*; **A** and **B**), frontal cortex (*FC*; **C** and **D**), parietal cortex (*PC*; **E** and **F**), and dysgranular insular cortex (*DIC*; **G** and **H**). Data are given as means ± S.E.M. Age ranges within groups are indicated by grey columns. Asterisks represent differences between age-groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

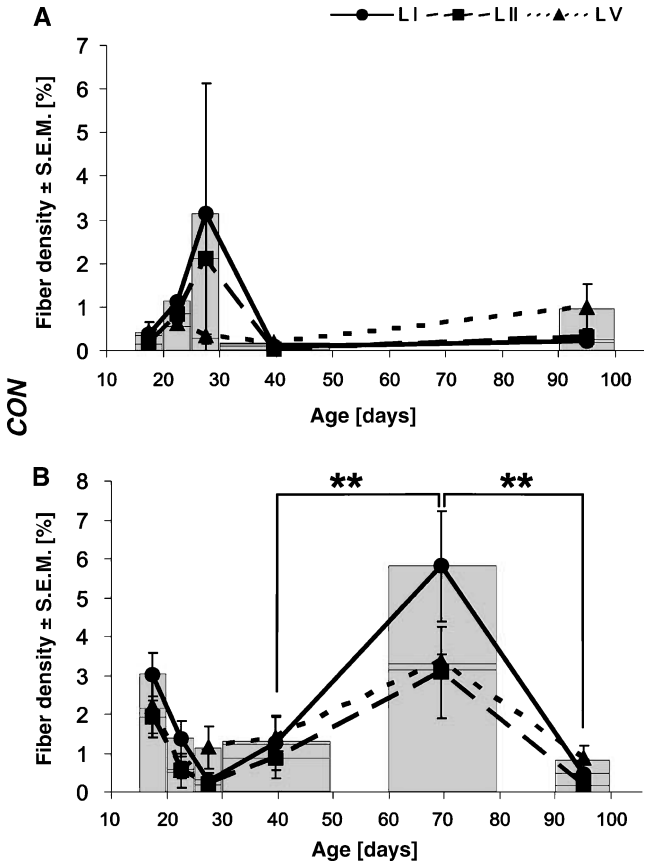

Fig. 8. Densities of terminal fields and passing fibres in the contralateral prefrontal cortex (*CON*) labelled by biocytin injections into layer III (**A**) or layer V/VI (**B**) of the medial prefrontal cortex. Data are given as means ± S.E.M. Age ranges within groups are indicated by grey columns. Asterisks represent differences between age-groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Discussion

In the current study, we present detailed evidence from anterograde tracing studies that the maturation of prefrontal cortical projection systems undergoes dynamic changes until late in postnatal life. Qualitative evaluation indicated an early axonal branching of prefrontal pyramidal efferents originating from both layer III and from layer V/VI in the infantile age-group between PD 15 and 19, which was more conspicuous in the V/VI-projections. This immature pattern was characterized by coarse, hardly ramified collaterals, which travelled primarily through deeper layers, but also ascended nearby the injection site and ran through layer I. In distinct ipsilateral and contralateral cortical areas, they distributed in a columnar manner without clearly defined termination patterns. Before weaning (PD 30), columns became more precise and axonal fibres exhibited the finer arborized adult-like (PD 90) termination pattern. In adolescent animals (PD 60–79), labelling resulted in an even more precise termination pattern.

Quantitative analysis showed impressive dynamics of the selective fibre maturation depending on layer of origin. Following layer III-injections, fibre densities remained static at low levels until a considerable (secondary) increase occurred first in adolescence (PD 60–79) and reached significantly higher values in the ipsilateral frontal cortex (FC) and the dysgranular insular cortex (DIC) in adults (PD 90). After layer V/VI-injections, however, a significant decrease in fibre densities occurred between infantile (PD 15–19) and early juvenile (PD 20–24) animals in the ipsilateral agranular insular cortex (AIC), which was indicated without significance in the other investigated areas. An increase in fibre densities followed after weaning (PD 30) and reached either adult values (PD 90: significant in the AIC) or a local maximum around adolescence (PD 60–79: significant in the FC and contralateral PFC).

In summary, prefrontal pyramidal III- and V/VI-efferents are subjected to considerably different dynamics of maturation in time and quality. Moreover, since so-called feedback pathways originate mainly from infragranular layers whereas feedforward pathways originate mainly from supragranular layers, both efferent paths are very likely to comprise hierarchically and developmentally distinctive subsystems with seperate rules of maturation (reviewed in Price et al. 2006).

Volumetric development of the PFC

During the juvenile period, plastic capabilities especially of the PFC are also reflected by volumetric studies by van Eden and Uylings (1985): all prefrontal areas exhibit a period of transient overgrowth during the first weeks of postnatal life in rats, whereas the somatosensory cortex, for example, was found to grow continuously in a greater extent than the cortex as a whole (Riddle et al. 1992). In the medial PFC (mPFC) a volume overshoot of approx. 30% occurred until PD 24 compared to adult values (van Eden and Uylings 1985), which coincides with the time window of dramatic pattern change occuring from the infantile to the pre-weaning age-group in our material. Notably, one should bear in mind that gerbils are born and weaned later than rats (birth: ED 25 and ED 21, respectively; weaning day: PD 30 and PD 21, respectively), however, their prior to weaning postnatal development should be somewhat comparable, since eye-opening occurs around PD 14 in both species (Clancy et al. 2001; Eilam 1997). In comparison with other species, more recent volumetric studies using magnetic resonance imaging (MRI) led to some controversial

findings (Pfefferbaum et al. 1994; Rathien et al. 2003; Villablanca et al. 2000). However, a volumetric decline during development seems to be PFC-specific also in primates and man (Jernigan et al. 1991; Giedd et al. 1999; Sowell et al. 2002). In addition, various histological post mortem and more recent MRI-based in vivo studies showed a protracted cycle of myelination, particularly in frontal and parietal regions continuing well into the third decade of life (Ballesteros et al. 1993; Brody et al. 1987; Huttenlocher 1979; Huttenlocher and de Court 1987; Yakovlev 1962). Since changes in axon mediated electrical activity, conceivably due to a refinement of axonal contacts, play an essential role in the myelination process (reviewed in Coman et al. 2005), axonal refinement should occur until late in postnatal life.

Van Eden et al. (1990) suggested that the reduction of PFC volume could be related to interneuronal connectivity and might be caused by a reduction or elimination of axonal branches or terminals. Indeed, exuberant productions of synapses and the following synaptic pruning seem to play a major role during maturation of the PFC at least in primates (Bourgeois et al. 1994) and man (Huttenlocher and Dabholkar 1997). Yet the decrease in AIC fibre densities after layer V/VI-injections described in the current study might reveal an insightful cause of volumetric decline in the PFC during postnatal development when considering the AIC as part of the lateral PFC (van Eden et al. 1990). Notably, the cellular and molecular determinants as well as onset and closure of these critical developmental processes in detail are still poorly understood (Woo and Crowell 2005); however, they might vary between functionally diverse systems.

Development of layer III- and layer V/VI-efferents in various cortical areas

A similar (but not simultaneous) developmental organization of both prefrontal and sensoric efferents seems likely following early studies by Price and Blakemore (1985), who investigated visual corticocortical connections in the kitten: During the third postnatal week, primary projections from lower layer V are pruned, while new axons sprout from layer III (Price and Blakemore 1985). In addition, more recent studies in the visual cortex by Batardiere et al. (2002) reported only feedback pathways (i.e. layer V/VI-projections) to undergo processes of axonal remodelling during development.

This is strongly in line with our findings, considering that projections of prefrontal layer III showed a delayed increase in fibre densities in comparison to deep projections (i.e. first after adolescence), highly significant in the FC and DIC. Moreover, this late increase in layer III-fibre densities could be rated as some kind of "fibre-release" after layer V axonal retardation and/or completion of growth. Intriguingly, the prolonged maturation of layer III-pyramidal cells in comparison to layer V-pyramids (as indicated by our data) could lead to a considerably delayed augmentation of layer III-axon collaterals. Moreover, one could even speculate that the late increase of layer III-projections might disturb or detain already existing excitatory connections rising from deeper layers, since it is known that layer III- and layer V/VI-pyramids innervate similar cortical areas and compete for the same target cells (reviewed in Bannister 2005). Consistently, fibre densities following layer V/VI-injections remained stable (or even decreased) after adolescence (PD 60). In the context of synapse forming it could be considered that the observed increase in layer III-fibre density after adolescence might be somehow interrelated negatively with axospinous synapses, which could react regressively to adjust for increasing fibre densities. This idea is supported by a study of Melchitzky et al. (1998), who found that axospinous synapses are eliminated during adolescence in the monkey, corresponding to the time where we found increasing overall fibre densities after layer III-injections in the FC. However, considering that in monkeys, the synapses are pruned to a greater degree in supragranular than in infragranular laminae (Melchitzky et al. 1998), but our observation at least in the DIC revealed a significant increase in fibre densities limited to target laminae V and VI (= infragranular laminae) after layer III-injections, the possible association between ingrowing axons and simultaneous elimination of synapses remains unclear. Yet, the adolescent elimination of axon terminals may be also selective for certain systems (Anderson et al. 1995).

According to the above mentioned pruning of layer V-efferents in the visual areas (Price and Blakemore 1985), we observed a significant decline in fibre densities arising from layer V/VI-projections in the AIC between infantile and adolescent animals, which might be caused by a retraction of axons. Early experiments have shown that developing corticocortical pathways, especially those originating from deeper layers of diverse cortical areas, are characterized by transient connections in various species (reviewed in Innocenti and Price 2005); e.g. in rats (Ivy et al. 1984; Olavarria and Van Sluyters 1985; Provis et al. 1985), hamsters (Lent et al. 1990), cats (Dehay et al. 1984; Innocenti 1981; Price and Blakemore 1985), monkeys (Jeffery et al. 1984; Kennedy et al. 1989; Killackey and Chalupa 1986), and humans (Provis et al. 1985). Ivy and Killackey (1981) presumed that these transient connections are eliminated

either by selective cell death and/or by retraction of the collaterals of the neuron's axon or by a shrinking of the terminal arborisation of the axon. On the one hand, cell death may contribute to axon withdrawal (Ivy and Killackey 1981; Kim and Juraska 1997; Nunez et al. 2001), since e.g. Parnavelas and Uylings (1980) found substantial numbers of pycnotic cells in the hamster brain between PD 5 and PD 12. In addition, Nunez (2001) observed a rapid rise in the incidence of cortical apoptosis over the first postnatal week in rats. On the other hand, Van Eden and Uylings (1986) found no developmental changes in the cell number of the shoulder subfield of the PFC (where our biocytin-injections were set). Therefore, the reduction of prefrontal efferent fibre densities found in the current study should rather be ascribed to axonal refinement than to apoptosis.

Another intriguing phenomenon, described by electrophysiological studies, could possibly contribute somewhat to the dynamic pattern change observed exclusively in layer V/VI-projections. These studies found two existing subclasses of layer V-pyramidal cells (for review see Bannister 2005), characterized by distinct spike discharge patterns (Thomson and Bannister 1998). Although the two subgroups exhibited similar axonal distributions, their input characteristics were rather selective and considerably different (Markram et al. 1997; Thomson and Bannister 1998). This could, theoretically, lead to a developmental variance between these two subclasses, characterized by temporally separated fibre maturation. Unfortunately, little is known about distinct growth rates within layer V-pyramids. However, one could speculate that one population matures earlier and exhibits the initial coarse projection pattern as seen in infantile animals. Thus, the premature fibre morphology might become in part masked or refined during the ongoing growth of axons of the other subclass (which hypothetically matures later) into the same projection area, since both layer V-subgroups compete for the same postsynaptic targets (Bannister 2005) and competition between axons for target-derived chemotrophic substances is supposed to act as a regulating factor in the remodelling process of neuronal connections (reviewed in Innocenti and Price 2005).

Related transmitter systems

Moreover, it could be assumed, that the morphological and functional maturation of related transmitter systems might underlie different processes of axonal pruning and sprouting of PFC pyramids. Then, the developmental pattern change of prefrontal projections could be interpreted as some kind of reaction caused by critical steps in the maturation of their presynaptic inputs. Indeed, certain monoaminergic systems, that exert both direct and indirect effects on the excitability of PFC pyramidal neurons (Puig et al. 2005; Seamans et al. 2001; Tseng and O'Donnell 2004), were found to mature considerably fast during early postnatal life, but to establish adult-like termination patterns not earlier than between postnatal weeks 3 and 4 (serotonin) or 8 and 9 (dopamine, DA) in rats (Berger et al. 1985; Kalsbeek et al. 1988; Lidov and Molliver 1982). Moreover, pointing to the exceptional role of the DAergic system, DA afferents start to reach cortical pyramidal cells already in the final trimester of gestation in rats (Kalsbeek et al. 1988), but continue to grow even until adulthood in gerbils (PD 90), characterized by an exponential increase between PD 14 and PD 30 (Dawirs et al. 1993a). Since DA and also serotonin are very likely to act as neurotrophic factors in the development of the PFC (Kalsbeek et al. 1987; for review Sodhi and Sanders-Bush 2004), they might exert crucial influence on pyramidal cells during the whole juvenile phase, thereby causing a dynamic, differentiated and enduring maturation of prefrontal efferents.

In addition, GABAergic neurons, which influence the pyramidal metabolism directly via their potent terminations around the pyramidal soma and axon hillock, are supposed to play a crucial role by re-arranging, shaping and modulating neuronal circuits (Jacobs and Donoghue 1991; Tamas et al. 2000). Consistent with the DAergic maturation, we recently found an early increase in GABAergic fibre densities between PD 14 and PD 20 followed by a further slow but significant enhancement beyond adolescence in gerbils (Brummelte et al. 2007). Intriguingly, the GABAergic inhibitory input intensifies (as indicated by increasing fibre densities) precisely during the same particular time window in which we detected the pyramidal fibre reduction, i.e. in the third postnatal week. Therefore, one could speculate that the increasing inhibitory signals might provoke a refinement of prefrontal pyramidal axons, since they reduce the excitation and fire abilities of pyramidal cells, thereby leading to a loss of synaptic strength on terminal target cells. Thus, as the pruning of synapses is an activity-dependent process mediated by the NMDA class of glutamate receptors via molecular cues of the presynaptic cell (Hickmott and Constantine-Paton 1997; Katz and Shatz 1996), a loss of glutamatergic excitation (provided e.g. by enhanced somatic GABAergic input) should result in a loss of synaptic contacts and thus in a retraction or elimination of pyramidal axons, which is a prominent feature in the development of many cortical axonal pathways (reviewed e.g. in Price et al. 2006).

Time schedule of crucial windows for impact of epigenetic factors

Since epigenetic factors are known to contribute heavily to the formation and sharpening of cortical networks (for review Rice and Barone 2000), and considering the dynamic postnatal maturation occurring in the prefrontal projection system, it seems very likely that environmental challenge crucially affects the prefrontal development (see also Berger-Sweeney and Hohmann 1997). Indeed, previous studies of our group reported abnormal prefrontal projection patterns in the adult gerbil after acute and/or chronic traumatisation (see introduction), which was discussed to emerge from a disturbed and therefore potentially dysfunctional branching, shrinking or refinement of these efferents during development (Bagorda et al. 2006; Witte et al. 2007). Namely, the combined challenge led dramatically to an enhancement in exclusively layer V/VI-projection fibre densities in frontal and parietal target areas, although no alterations in layer III-projections were found. These findings point on the one hand to an abnormal glutamate activity and, on the other hand, to a dramatic imbalance between the different PFC output systems. Intriguingly, an impaired glutamate transmission may act as key player in the pathology of schizophrenia (Keshavan and Hogarty 1999; Weinberger and Lipska 1995), and the so-called "dysconnection" of corticocortical connections is frequently discussed as an anatomical correlate of the disease (Feinberg 1982; Harrison 1999; Weinberger and Lipska 1995).

The present results give valuable indications regarding when and how the prefrontal dysconnection to other cortical areas might set in: conceivably, between the second and third postnatal week, the initial coarse axon collaterals may be affected by epigenetic interferences, when non-invasive methamphetamine (MA)-intoxication (PD 14) was amplified, lesioning not only ingrowing prefrontal DA terminals (Dawirs et al. 1994) but also affecting the GABA (Nossoll et al. 1997) and serotonin systems (Neddens et al. 2003, 2004). The disturbed innervation of the pyramidal cells, as a consequence, may lead to a pathologic non-pruning of layer V/VI-efferents, by impairing e.g. the cortical transmission and metabolism. Subsequently, in combination with impoverished rearing (IR) as chronic intervention, the overextension of layer V/VI-projection fibre densities might stabilize and could be described in adults as a dramatic imbalance between layer III- and layer V/VI-projections. Intriguingly, beside this assumable entire and therefore pathologic integration of excessive projections following adverse epigenetic interventions, a phenomenon that could be termed as "masking" was suggested earlier to occur as well during normal development (Murakami et al. 1992; Wall et al. 1988): The authors described a somehow subtler preservation of exuberant projections as thin, unmyelinated and poorly branched fibres beyond juvenile age. These pre-existing routes might then serve as a source of sprouting after e.g. neuronal injury (Murakami et al. 1992), thereby contributing heavily to the remarkable plasticity in the adult brain.

However, as MA-intoxication alone (i.e. without restricted rearing) rather caused an abnormal decrease than an increase in layer V/VI-projections in adult animals (Bagorda et al. 2006; Witte et al. 2007), it seems very likely, that an enriched environment could somehow reverse the MA-mediated effects. In addition, it could be assumed, that the stimulating environment by enriched rearing (ER) somehow benefits especially the disturbed maturation of the DAergic system, thereby protecting the brain from adverse influences and the further establishment of abnormal connectivity and pathologies during ongoing development.

Conclusion

The results of the current study indicate the presence of structural dynamics in the development of prefrontocortical connections even beyond adolescence. Moreover, the functionally diverse pyramidal output systems of the PFC are characterized by individual time courses of maturation: Layer III-projections reached adult fibre densities between adolescence and adulthood, whereas layer V/VI-projection-densities declined between the second and third postnatal week in gerbils and attained adult values around adolescence. Especially during the early phase of re-arranging, pruning or masking of layer V/VI-collaterals, it seems likely that disturbing factors exert crucial influence and might further lead to an impaired wiring of prefrontocortical connections. Indeed, a hindrance of the axonal pruning process caused by external interventions could explain the pathologic and potentially dysfunctional overextension of layer V/VI-projections after early traumatisation as previously observed in adult MA-IR animals (Bagorda et al. 2006; Witte et al. 2007). In addition, a stimulating surrounding might somehow attenuate the establishment of abnormal corticocortical connections: it is supposed, that an enriched environment might somehow re-animate the indispensable pruning process of layer V/VI-collaterals or reverse otherwise the imbalance between layer III- and V/VI-projections, which is consistent with the additional result of MA-ER animals that described an equable slight decrease of both projection systems in adulthood.

Eventually, developmental tracer studies of ER as well as MA-intoxicated animals would complete the present results and could provide further evidence to prove these hypotheses and underlying processes.

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Postnatal development of GABA and calbindin cells and fibers in the prefrontal cortex and basolateral amygdala of gerbils (*Meriones unguiculatus*)

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Abstract

The postnatal maturation of immunohistochemically stained gamma-amino-butyric acid (GABA) and calbindin (CB) cells and fibers were quantitatively examined in the prefrontal cortex (PFC) and the basolateral amygdala (BLA) of the Mongolian gerbil (*Meriones unguiculatus*). Animals of different ages, ranging from juvenile (postnatal day (PD)14, PD20, PD30), to adolescent (PD70), adult (PD180, PD540) and aged (PD720) were analyzed. Results reveal an increase in GABAergic fiber densities between PD14-20 in the PFC and the BLA with a concomitant decrease in cell density. After PD70 GABA fiber density slightly decreases again in the BLA, while there is a further slow but significant increase in the PFC between PD70 and PD540. Fibers immunoreactive for the calcium binding-protein CB, which is predominantly localized in particular GABAergic subpopulations, also accumulate between PD14 and PD20 in the PFC and BLA, while a concomitant decrease in cell density is only seen in the BLA. Both areas reveal a decrease of CB cells between PD30 and PD70, which parallels with a decrease of CB fibers in the PFC. However, there is no particular 'aging-effect' in the fiber or cell densities of GABA or CB in any of the investigated areas in old animals.

In conclusion, we here demonstrate long-term dynamics in cell and fiber densities of the GABAergic system until late in development which might correspond to the prolonged maturation of other neuroanatomical and functional systems.

Keywords: Gamma-amino-butyric acid; Calbindin; Calcium-binding proteins; Immunohistochemistry; Development; Limbic system

1. Introductory statement

Gamma-amino-butyric acid (GABA) is probably the most important inhibitory neurotransmitter in the mammalian nervous system. It is usually expressed in local interneurons, which can modulate and even control the neuronal activity of cortical and subcortical output neurons. Further, GABA has been shown to exert important morphogenetic influences during development (Chronwall and Wolff, 1980; Nguyen et al., 2001) and to play an essential role in reactive plasticity and reorganization processes during development and adulthood (Dawirs et al., 1997; Hensch, 2005; Merzenich et al., 1983; Zito and Svoboda, 2002). Thus, GABA has a central part in shaping and maintaining of neuronal networks.

Within the GABAergic population several classes of subpopulation can be distinguished according to their content of calcium-binding proteins (Baimbridge et al., 1992) and corresponding different maturation patterns. One of these proteins is calbindin (CB) which is, e.g. found in Marinotti, Neuroglia and Double Bouquet cells within the cortex, i.e. in cells, that primarily innervate distal parts and spines of pyramidal dendrites (Conde et al., 1994; DeFelipe et al., 1989; Gabbott and Bacon, 1996; Lund and Lewis, 1993) and appear and mature relatively early (Alcantara et al., 1993). In the amygdala, CB cells are distributed differently in the various nuclei (Kemppainen and Pitkanen, 2000), but in contrast to the prefrontal cortex (PFC), CB varicosities are found in the basolateral amygdala (BLA) to form basket-like structures around unlabelled projection neuron somata (Berdel and Morys, 2000; Kemppainen and Pitkanen, 2000; Legaz et al., 2005; Muller et al., 2003). This points to the

Abbreviations: GABA, gamma-amino-butyric acid; CB, calbindin; PFC, prefrontal cortex; mPFC, medial prefrontal cortex; BLA, basolateral amygdala; PD, postnatal day; PV, parvalbumin

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particularly interesting role of CB in this subcortical area, as it is widely known, that axo-somatic synapses have an exceptionally powerful control over target neurons compared to distal dendritic or spine contacts. In the cortex, these baskets are usually built by GABAergic cells containing parvalbumin, another calcium-binding protein, or other substances such as cholecystokinin (Conde et al., 1994; Kawaguchi and Kubota, 1998).

Despite this difference, the GABAergic innervation patterns of the PFC and the BLA bear marked resemblances (Carlsen, 1988; Muller et al., 2006), although the origin and function of the PFC and the BLA are quite diverse, which is a reason for choosing these particular two structures for investigation in the current study. Further reasons are the high interconnection and thus potential interrelation of the PFC and BLA during development and their distant positions in the brain, which imply divergent developmental patterns. In addition, both areas belong to one main circuit, characterized by the mesolimbic prefrontal dopamine projections, which originate in the ventral tegmental area and the substantia nigra (Björklund and Lindvall, 1984; Fallon and Ciofi, 1992). This dopamine fiber innervation is of particular interest as it shows a prolonged maturation until adulthood in the rodent medial PFC (mPFC) (Dawirs et al., 1993; Kalsbeek et al., 1988), while it stays relatively stable after PD20 in the gerbil amygdala or entorhinal cortex (Brummelte and Teuchert-Noodt, 2006). This is in line with the general developing pattern, with the PFC being one of the last areas to reach adult stages (Mrzliak et al., 1990; Van Eden et al., 1990), while the amygdala maturates relatively early after birth (Joseph, 1999; Morys et al., 1999).

The prenatal and early postnatal maturation of the GABAergic population in the cortex, with particular emphasis on the visual cortex, has been intensively investigated in the last two decades (Chronwall and Wolff, 1981; Del Rio et al., 1992; Parnavelas, 1992; Van Eden et al., 1989; Wolff et al., 1984). However, less research has been done concerning the late postnatal development and aging effects of GABAergic and CB fibers and concerning different cortical or subcortical areas. It is assumed that GABA exhibits a high synaptic plasticity and can help to reorganize, shape and modulate neuronal circuits not only during development (Chen et al., 2002; Teuchert-Noodt, 2000). This compensatory effect in plastic processes might be reflected in changes of the GABAergic or CB fiber densities even during adulthood and aging. As it is further supposed, that the cortex might continuously adapt to new situations and experiences by (re)arranging neuronal networks (Bagorda et al., 2006; Holtmaat et al., 2006; Trachtenberg et al., 2002), the current study was conducted to examine the life long progression of GABAergic and CB structures in two areas of the mesolimbocortical circuit, the mPFC and BLA.

2. Experimental procedures

A total of 60 male Mongolian gerbils (*Meriones unguiculatus*) were used for this study. Breeding gerbils were obtained from Harlan Winkelmann (Borchen, Germany). The animals were bred in standard cages (Macrolon type 4) and, after weaning on postnatal day (PD) 30, were reared individually in standard cages (Macrolon type 3). All gerbils were kept under natural day/night cycles with food and water being provided *ad libitum*. Seven experimental animal groups of different ages were investigated to cover convincing periods of the life span of gerbils: PD14 ($n = 11$), PD20 ($n = 6$) (juvenile), PD30 ($n = 12$) (weaning), PD70 ($n = 11$) (young adult), PD180 ($n = 8$), PD540 ($n = 8$) (adult) and PD720 ($n = 4$) (aging). Gerbils were chosen due to their very small genetic variability (Thiessen and Yahr, 1977), and their rich wild-type like behavioral repertoire (Rosenzweig and Bennett, 1969). All experimental procedures were approved by the appropriate committee for animal care in accordance with the European Communities Council Directive.

2.1. Immunohistochemistry

Animals were transcardially perfused under deep chloralhydrate anesthesia (1.7 g/kg, i.p.). The perfusion was performed with 200 ml 0.05 M phosphate buffer (pH 6.2), containing 1% sodium metabisulfite, followed by 750 ml 5% glutaraldehyde with 1% sodium metabisulfite in 0.1M phosphate buffer (pH 7.5), with appropriate amounts of solutions for younger animals. Immediately after perfusion, the brains were removed and postfixed for 30 min. Coronar sections of 50 μm were cut with a vibratome (Vibratome Series 1000, Technical Products International Inc.) of which every 3rd was used for GABA and CB immunostaining, respectively. For GABA staining sections were collected in wash buffer at 4 °C and rinsed 3 × 10 min followed by a preincubation in 10% normal goat serum and 0.4% Triton X-100 (Sigma) for 30 min. Subsequently, the sections were incubated with rabbit anti-GABA (ImmunoStar, Hudson, WI), diluted 1:5000 with 1% normal goat serum and 0.4% Triton X-100 for 48 h. Sections used for CB staining were treated in almost the same manner, but collected and rinsed in 0.05 M Tris–HCl buffered saline (pH 7.5, TBS), and were additionally incubated in 1% H_2O_2 for 10 min. The primary antibody was mouse anti-calbindin (Sigma, diluted 1:3000, for 18 h). The following rinses, all three times for 10 min, and dilutions were all done in TBS. The sections were rinsed and incubated for 30 min in biotinylated goat anti-rabbit antibody (Sigma) for GABA and biotinylated goat-anti-mouse antibody (Sigma) for CB staining, respectively, diluted 1:20 with 1% normal goat serum, rinsed again and incubated with ExtraAvidin-Peroxidase (Sigma) diluted 1:20 for 30 min. After another rinse the sections were stained in 0.05% 3.3-diaminobenzidine (Sigma) with 0.01% H_2O_2 for 4 min. Then the sections were washed, mounted on glass slides, dried overnight, dehydrated with ethanol, cleared with xylene and cover slipped with DePeX (Serva, Heidelberg, Germany). To avoid deviations due to possibly lateralized innervation densities of GABA and CB only right hemispheres were used for analyses.

For quantification of fiber densities, brain sections were chosen in areas of interest by means of anatomical characteristics according to brain atlases of the rat (Paxinos and Watson, 1986) and the mouse (Valverde, 1998). The BLA and mPFC subregions Cg1 and Cg3, with the latter being further divided into layer III and layer V, were chosen for investigation due to the clear presence of GABAergic and CB fibers and cells. The average number of analyzed sections was 5 per animal and region. In the defined region of each section all detectable fiber fragments were visualized in standard test fields using a bright field microscope (BX61, Olympus, Hamburg, Germany) and a digital camera for microscopy (ColorView II, SIS, Münster, Germany). Calbindin sections were investigated using 200-fold magnification, GABA sections at 600-fold magnification.

To account for a possible interaction of fiber density and cell density or size of the investigated area, these parameters were measured additionally for the PFC and BLA at 200-fold and 20-fold magnification, respectively. Digital images were adjusted in contrast and intensity before fibers, cells or the size of the area were quantified by software for image analysis (KS300, Jenoptik, Jena, Germany). For further details of the quantification process see (Brummelte et al., 2006; Brummelte and Teuchert-Noodt, 2006). The fiber density was calculated as a percentage of the evaluated test area, the cell density as number of cells per test area. Lightly stained cells (cf. qualitative results) were excluded in the counting by a minimum threshold of gray values for cell recognizing. All analyses were done by a person blind to the age of individual animals.

2.2. Data analysis

Measurements were computed as arithmetic means by-case and by-group ± S.E.M. The overall size of the particular area in which fiber densities were measured as well as the number of GABA or CB cells were integrated as

covariates in the statistical analysis to account for a possible interaction of an augmentation of volume or cells and fiber sprouting. For the PFC, a two-way analysis of covariance (ANCOVA) with age (7 levels) and area (3 levels) as independent variables, GABA or CB as dependent variable and area size and GABA or CB cell number as covariates were used to check for statistical significance between groups, followed by Fisher L.S.D. post hoc test for multiple comparisons if appropriate. For the BLA, the ANCOVA comprised only one area level. As the covariates revealed some significant effects on the fiber development, these parameters were also statistically analyzed using an ANCOVA (cell number, size as covariate) or ANOVA (size) and subsequent L.S.D. post hoc tests. Statistical analysis was computed with Statistica 6 (StatSoft, Tulsa, USA). The levels of significance were set at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

3. Results

3.1. Qualitative observations

The GABAergic fiber innervation is equally dense in all investigated areas, while the CB fibers are more present in the BLA compared to the mPFC. The overall distribution *pattern* of GABAergic and CB fibers, however, is similar in animals from all age stages (cf. Fig. 1). PFC and BLA contain a population of lightly stained CB pyramidal neurons, which has been previously observed in rats, too (Celio, 1990; Kemppainen and Pitkanen, 2000). In the PFC these cells are arranged in a bundle throughout lamina II (Fig. 1B.1). In concert with data from other species (Hof et al., 1999) hardly any CB or GABA cells were seen in lamina I. In the BLA, GABAergic and CB cells were distributed quite equally through the nucleus. We could not detect clear CB basket-like structures in the BLA at light microscope level, although these have been described for rats (Berdel and Morys, 2000).

3.2. Quantitative analysis

3.2.1. PFC

For the GABAergic fiber density in the PFC the two-way analysis for variance reveals a highly significant effect of age ($F(6,147) = 16.67$; $p < 0.001$), but not for area or interaction of age and area ($F(12,147) = 0.168$; $p = 0.99$). Both covariates show a significant effect on the fiber development (GABA cells: $p = 0.007$; size: $p = 0.016$). A subsequent Fisher L.S.D. test exhibits a significant increase in fiber density between PD14 and PD20 (+11%; $p < 0.001$) and between PD20 and PD30 (+5%; $p = 0.037$) and a further trend for an increase between PD70 and PD180 ($p = 0.078$), which becomes significant compared to PD540 (PD70 and PD540: +7%; $p < 0.001$) (Fig. 2A).

For the CB fibers in the PFC the ANCOVA reveals a significant effect of age ($F(6,148) = 23.94$; $p < 0.001$) and area ($F(2,148) = 6.26$; $p = 0.002$) but not for the interaction of age and area. Size as a covariate shows a significant effect ($p = 0.005$), while the CB cell number narrowly fails to reach a significant level ($p = 0.054$). The subsequent L.S.D. test for the area effect reveals a significant difference in innervation density between the Cg1 area and Cg3 lamina III and between lamina III and lamina V within the Cg3 region (both p's < 0.001). Further, a post hoc test shows a highly significant increase of fibers between PD14 and PD20 (+43%; $p < 0.001$), followed by slight decrease between PD30 and PD70 (−8%; $p = 0.017$) and PD180 and PD540 (−13%; $p = 0.003$) (Fig. 2B).

Table 1
Circumferences of the two analyzed areas: the prefrontal cortex (PFC) and the basolateral amygdala (BLA) + S.E.M.

Age (days)	PFC (μm)	BLA (μm)
14	8241.93 ± 349.47	3949.28 ± 36.90
20	8367.58 ± 561.35***	4045.24 ± 98.24
30	8248.79 ± 365.93**	4019.70 ± 30.63
70	8226.65 ± 389.70	4106.80 ± 78.18
180	8307.00 ± 340.07	4193.11 ± 112.2
540	8262.83 ± 382.16	4325.24 ± 60.86
720	8225.94 ± 491.41	4218.94 ± 85.76

The PFC reveals a peak in volume on postnatal day 20, while there is no age-dependent effect in the BLA. Levels of significance, compared to the age stage before: $^{**}p < 0.01$, $^{***}p < 0.001$.

Due to the significant effect of the covariates, the cell numbers were additionally analyzed and a two-way analysis of covariance reveals a significant effect of area ($F(4,294) = 85.38$; $p < 0.001$), with all areas being significantly different from each other concerning GABA and CB cells (all p's < 0.05). Age also shows a significant effect ($F(12,294) = 17.0$; $p < 0.001$) with GABA cells exhibiting a decrease between PD14 and PD20 (−41%; $p < 0.001$), followed by an increase between PD20 and PD30 (+26%; $p = 0.001$) and another decrease after PD30 (−11%; $p = 0.035$). The last effect could also be seen in the CB cell density (−21%; $p < 0.001$) (Fig. 2). Size as a covariate has again a significant influence ($p = 0.013$) and when therefore analyzed separately it reveals a reverse peak with an increase between PD14 and PD20 (+8%; $p < 0.001$) and a subsequent decline until PD30 (−7%; $p = 0.003$) (Table 1). As there was no interaction effect for age and area in any of the analyses, the line plots (Figs. 2 and 3) show the overall values for the PFC. The separate data for the different investigated areas of the PFC are presented in Tables 2 and 3.

3.2.2. BLA

GABAergic fibers in the BLA show a significant effect of age ($F(6,49) = 8.84$; $p < 0.001$) with none of the covariates showing a significant contribution. The L.S.D. post hoc test reveals a highly significant increase between PD14 and PD20 (+15%; $p < 0.001$) and a tendency for a decline after PD70 (PD70–PD180: $p = 0.073$), showing a significance compared to PD540 (−7%; $p = 0.012$). CB fibers in the BLA reveal no significant age-effect ($p = 0.21$), however, the CB cell number as a covariate exhibits a significance ($p = 0.031$).

An analysis of covariance of the cell numbers in the BLA shows a significant effect of age ($F(12,98) = 8.85$; $p < 0.001$) with no effect of size. A following L.S.D. test further reveals a decrease of GABAergic cells (−14%; $p = 0.025$) and CB cells (−18%; $p = 0.001$) between PD14 and PD20 with a further slow decrease until PD70 in the GABAergic population (PD20–PD70 −21%; $p = 0.007$) and a more steep decline in the CB cell density (PD30–PD70: −23%; $p < 0.001$). In addition, the CB cell number decreased between PD180 and PD540 (−14%; $p = 0.036$) (Fig. 3).

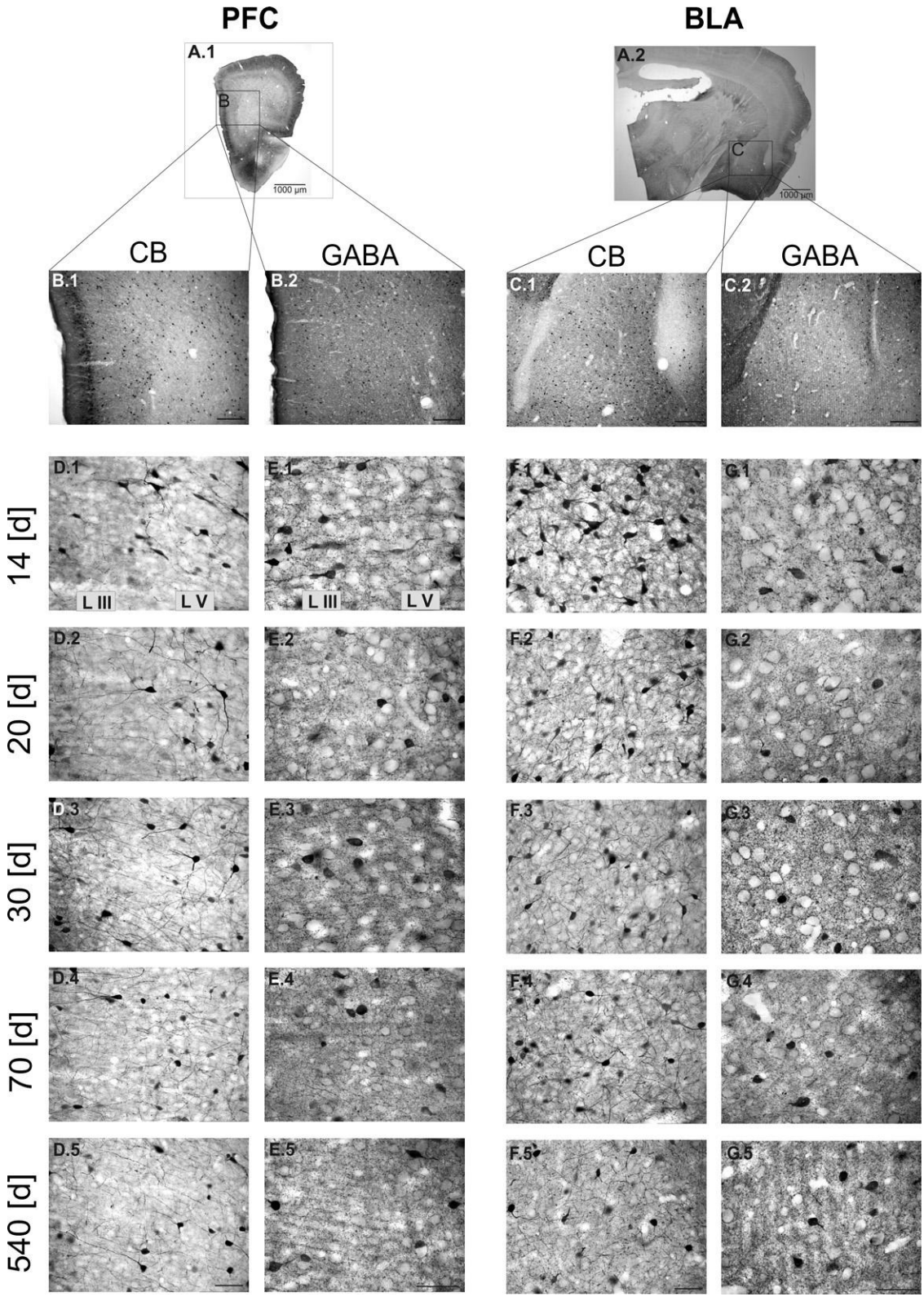

Fig. 1. Representative photomicrographs of the GABAergic and Calbindin (CB) distribution of the medial prefrontal cortex (mPFC) and the basolateral amygdala (BLA) at different age stages. A.1 and A.2 are photomicrographs of representative coronal sections at the level of the PFC and amygdala, respectively. The areas of the rectangles are magnified in panels B.1–C.2 for CB and GABA. Pictures (D1)–(G5) show examples of GABA and CB structures at juvenile age stages (PD14–PD30), adolescence (PD70) and a comparative section from an adult animal (PD540) in lamina III and V of the Cg3 region and in the BLA. Scale bars: 200 μm (B.1–C.2), 50 μm (D1–G5).

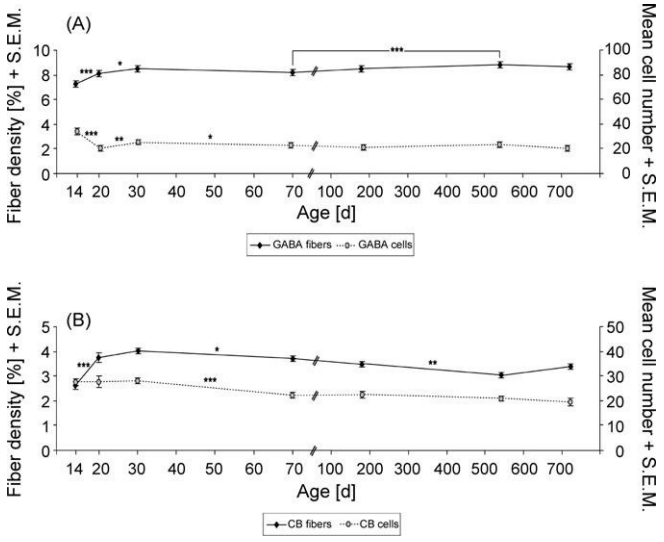

Fig. 2. Postnatal development of GABA (A) and Calbindin (CB) (B) cells and fibers in the prefrontal cortex (PFC). Shown are the fibers density means as percentage [%] and the cell number means, respectively, of the reference area + S.E.M. at postnatal day 14, 20, 30, 70, 180, 540 and 720. The double bar in the middle marks a break in the scaling of the *x*-axis. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

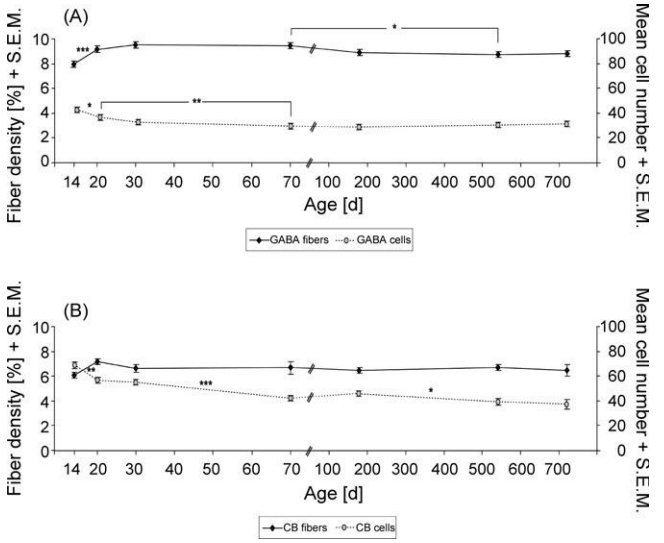

Fig. 3. Postnatal development of GABA (A) and Calbindin (CB) (B) cells and fibers in the basolateral amygdala (BLA). Shown are the fibers density means as percentage [%] and the cell number means, respectively, of the reference area + S.E.M. at postnatal day 14, 20, 30, 70, 180, 540 and 720. The double bar in the middle marks a break in the scaling of the *x*-axis. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Table 2
GABA and CB fiber densities in the various areas within the prefrontal cortex, namely the Cg1 region and lamina (L) III and V of the Cg3 region + S.E.M.

Age (days)	GABA			CB		
	Cg1	Cg3 L III	Cg3 L V	Cg1	Cg3 L III	Cg3 L V
14	7.31 ± 0.19	7.04 ± 0.27	7.52 ± 0.21	2.76 ± 0.26	2.02 ± 0.16	3.07 ± 0.33
20	8.16 ± 0.25	7.84 ± 0.26	8.26 ± 0.14	3.75 ± 0.24	3.29 ± 0.21	4.22 ± 0.38
30	8.52 ± 0.25	8.35 ± 0.23	8.57 ± 0.17	3.95 ± 0.15	3.80 ± 0.10	4.29 ± 0.19
70	8.22 ± 0.24	8.15 ± 0.28	8.34 ± 0.19	3.84 ± 0.19	3.50 ± 0.16	3.76 ± 0.17
180	8.59 ± 0.15	8.29 ± 0.15	8.71 ± 0.11	3.62 ± 0.11	3.57 ± 0.13	3.30 ± 0.12
540	8.88 ± 0.16	8.75 ± 0.09	8.83 ± 0.19	3.35 ± 0.09	3.02 ± 0.09	2.79 ± 0.05
720	8.82 ± 0.07	8.58 ± 0.04	8.63 ± 0.12	3.59 ± 0.09	3.29 ± 0.11	3.34 ± 0.07
Mean	8.31 ± 0.11	8.09 ± 0.11	8.38 ± 0.09	3.55 ± 0.09	3.21 ± 0.10*	3.56 ± 0.11

There was a difference in the mean innervation density concerning the CB fibers in lamina III of the Cg3 region compared to the other two areas ($^{*}p < 0.001$).

Table 3
GABA and CB cell densities in the various areas within the prefrontal cortex

Age (days)	GABA			CB		
	Cg1	Cg3 L III	Cg3 L V	Cg1	Cg3 L III	Cg3 L V
14	26.42 ± 6.97	23.54 ± 8.54	47.03 ± 10.2	23.86 ± 1.57	22.14 ± 1.12	36.72 ± 1.69
20	15.90 ± 2.65	10.90 ± 2.11	29.65 ± 5.99	23.38 ± 1.56	21.18 ± 2.11	38.65 ± 3.27
30	18.99 ± 3.69	16.21 ± 3.27	36.23 ± 5.97	24.42 ± 0.62	22.86 ± 1.09	37.09 ± 0.99
70	16.21 ± 4.05	14.52 ± 4.98	32.86 ± 5.02	19.16 ± 1.16	18.11 ± 1.37	29.27 ± 2.06
180	16.26 ± 1.50	10.68 ± 1.95	29.21 ± 3.23	20.35 ± 1.42	18.35 ± 1.76	28.76 ± 1.49
540	16.66 ± 1.83	16.29 ± 2.89	29.91 ± 3.14	21.57 ± 1.09	16.42 ± 0.76	24.83 ± 1.70
720	14.78 ± 1.85	12.50 ± 1.58	26.81 ± 6.04	18.54 ± 2.10	16.60 ± 2.24	23.77 ± 3.22
Mean	18.53 ± 5.43*	15.70 ± 6.17*	34.42 ± 8.80*	21.93 ± 0.54*	19.78 ± 0.60*	31.96 ± 0.97*

Lamina V of the Cg3 region revealed the highest cell density and lamina III the lowest. All areas were significantly different from each other ($^{*}p < 0.01$).

4. Discussion

The current study provides first data for GABAergic and Calbindin (CB) cell and fiber densities in two prominent structures of the mesolimbocortical circuit from the juvenile period to aging in the Mongolian gerbil. The fluctuations in fiber densities might in part be due to variances in the cell numbers or expansions of the reference area as these parameters

reveal significant contributions in the analyses of covariance. For instance, the PFC exhibits a peak in volume around PD20, which has been reported for rats before (Van Eden and Uylings, 1985), and which is accompanied by a low level of GABAergic cells. Interestingly, fiber densities in general tend to increase, although the according cell densities decrease during development. We additionally report long-term dynamic variations of the GABAergic fiber system in the gerbil brain, which are probably independent of the early changes in cell number or volume.

4.1. Species- and area-specific maturation of GABAergic cell and fiber densities

Previous prenatal, and early postnatal investigations have shown, that GABAergic cells appear in the rodent visual or somato-sensory cortex as early as embryonic day (ED) 14–16 (Chronwall and Wolff, 1980; Del Rio et al., 1992) and that there is no apparent change in cell density after the third postnatal week (Chronwall and Wolff, 1980). CB cells have also been observed to appear prenatally in the rat cortex and increase until PD8–11, but their number seems to decrease notably between PD11–15 while reaching adult levels around the end of the third postnatal week (Alcantara et al., 1993). In the amygdala first CB cells appeared around ED13 in the mouse (Legaz et al., 2005), and were observed on ED 20 in the rat, where they reached adult levels around PD20 (Berdel and Morys, 2000).

These previous observations are in part at variance with our present results from the Mongolian gerbil. One first explanation for this might be the different developmental pattern of gerbils compared to rats or mice. The gerbil is known to develop its auditory and visual capacity later than the rat (Seto-Ohshima et al., 1990) and to lag behind about 2 weeks in reaching its sexual maturity. In addition, the dopaminergic innervation of the mPFC shows a prolonged maturation until PD60 in the rat (Kalsbeek et al., 1988), while dopaminergic afferents continue to grow until PD90 in the gerbil (Dawirs et al., 1993). Thus, the partially highly significant increase in GABAergic and CB fiber densities until PD30 and the fluctuations in the cell densities we observed in the mPFC and the BLA in the present study might indicate the postponed maturation of the gerbil nervous system compared to other rodents and the later onset of functional systems.

The apparent discrepancy of former and our present results might further be due to the developmental differences of particular areas. Thus, calcium-binding proteins occur several days later in the associative cortices compared to the primary visual cortex (Alcantara et al., 1993). In addition, Wolff et al. (1984) found no notable difference in GABA cell proportion after P3 in the layers II–VI in the visual cortex, while Vincent et al. (1995) found a decrease of GABA cell density until PD15 in the mPFC of rats. They further suggest that this decrease is associated with an expansion of the cortex. The severe decrease of GABA cells between PD14–PD20 in the gerbil might therefore be due to the peak in mPFC volume around PD20. Intriguingly, we did not observe such a decline in CB cell density during this particular time. This might depend on a variation of the amount of the protein within the cells, so that despite the decreasing number of GABAergic cells more of the remaining cells expressed enough CB to reach the minimum gray value for cell counting. The inverse effect might also account for the later decline in the CB number (PD30–PD70), which would be in line with the hypotheses that one population of CB cells only expresses the protein transitorily, while the other neurons are permanently immunoreactive for CB (Alcantara et al., 1993).

The maturation of GABAergic fibers in the BLA seems to differ slightly from the cortex. One reason for this could be the different targets and functions of the subpopulations in the cortex and the amygdala, as for instance, CB cells have been shown to build basket like structures in the BLA (Berdel and Morys, 2000) but not in the cortex. Further, CB interneurons in the cortex rather present a minor subpopulation (Celio, 1990), while they constitute almost 60% of the GABA-containing population in the BLA (McDonald and Mascagni, 2001) which would be in line with our observation of a higher CB fiber density in the BLA compared to the cortex. In addition, another 60% of these CB neurons have been shown to also coexpress another calcium-binding protein, parvalbumin (PV), permanently (McDonald and Betette, 2001), while such a coexpression is only transiently observed in the cortex (Alcantara et al., 1996). This might explain the existence of CB baskets around unlabelled pyramidal neurons in the BLA and further hints to the particular role of the various calcium-binding proteins in different subpopulations of the brain.

4.2. GABA plasticity from adolescence to aging

Regardless of fluctuations in cell densities, there are prolonged variations in fiber densities and thus in the inhibitory networks of the particular areas during adolescence and even adulthood. Generally it is thought that local circuit neuron connections mature relatively late compared to projections from efferent neurons (Miller, 1988). In addition, GABA appears to exert direct and indirect trophic action and thus initiate the establishment of synaptic contacts such as excitatory synapses, which usually appear 1–4 days after the GABA cells (Wolff et al., 1978, 1993). The synaptogenesis of inhibitory GABAergic boutons seems to be even further delayed and continues well into adulthood (Bahr and Wolff, 1985; Lewis et al., 2005), which would be in line with a continuing augmentation of fibers.

GABA is known to undergo a shift from an excitatory transmitter before birth into an inhibitory transmitter after birth (Ben-Ari, 2002; Cherubini et al., 1991; Ganguly et al., 2001). Further, there is a potential postnatal shift between the different GABAergic subpopulations with a decrease in CB immunoreactivity, which is usually accompanied by the appearance of PV-positive structures in various areas of the brain (Cruz et al., 2003; Davila et al., 2005; Erickson and Lewis, 2002; Legaz et al., 2005). PV cells mature considerably late during development (Alcantara et al., 1993), e.g. in the gerbil, first PV neurons appear around PD14 in the mPFC, though their number is very small (unpublished data), and are known to

build axo-somatic contacts and basket like boutons around pyramidal somata, which likewise appear considerably late (Bahr and Wolff, 1985). These types of connections have a particularly powerful influence on the firing activity and synchronization of target neurons (Freund, 2003; Gibson et al., 1999; Klausberger et al., 2003; Miles et al., 1996; Tamas et al., 1997; Tamas et al., 2000). Such oscillatory (rhythmic) synchronization is for instance generated by a BLA PV network during emotional arousal (Muller et al., 2005) and is further believed to create the necessary temporal and spatial frame for functions such as working memory in the PFC (Constantinidis et al., 2002; Lewis et al., 2005) or consolidation of emotional memories in the amygdala (McDonald and Mascagni, 2004). In addition, it has been assumed that morphological changes in response to learning stimuli may include a shift of synapses nearer to neuronal somata (Murakami et al., 1988). Taken together, these evidences underline the importance of somatic and axonic inhibitory synapses, although the majority of GABAergic contacts terminate on dendrites or spines of the postsynaptic cells (Beaulieu et al., 1992; Beaulieu and Somogyi, 1990; Nitsch and Riesenberg, 1995), which in turn emphasizes the general importance of understanding the involvement of the GABAergic system and its different subpopulations in neuronal circuits and plasticity.

Different transmitter systems have been shown to exhibit high plastic potentials during adolescence and adulthood and thus contribute to the shaping or remodeling of neuronal circuits. For instance, the dopaminergic innervation modulates neuronal out-put activity by directly terminating on glutamatergic projection neurons in the PFC and amygdala and indirectly via GABAergic interneurons (Asan, 1998; Brinley-Reed and McDonald, 1999; Sesack et al., 1995) and thus may have a particularly important part in shaping neuronal connectivity. A similar innervation pattern was revealed for the BLA input to the PFC, which connects to pyramidal spines as well as to GABAergic local circuit neurons (Bacon et al., 1996; Gabbott et al., 2006) and thus may also be essential for the establishment of neuronal circuits. It becomes apparent that irrespective of the art of input, the GABAergic transmitter system seems to be generally perfectly positioned to mediate between the various incoming projections and the efferents. As the dopaminergic innervation continues to grow into the PFC during adolescence (Dawirs et al., 1993; Kalsbeek et al., 1988) and the connections from the BLA to the PFC also mature relatively late compared to other connections arising from the amygdala (Diergaarde et al., 2005), it seems likely that local interneurons might continue to adapt to the changing input by enlarging or rearranging their fiber densities. It has already been assumed, that the late development of the local circuit neurons and the subsequent remodeling of networks may provide a morphological basis for functional plasticity in mature cortical neurons (Miller, 1988) and thus it might even contribute to complex processes as long-term learning and memory.

As we could recently show in our laboratory using an animal model of early traumatized gerbils, epigenetic disturbances during development can cause a shift within the GABAergic system, with a loss of GABAergic boutons around pyramidal somata and an increase in lamina I/II GABAergic fibers in the mPFC of adult animals (Brummelte et al., in press). The lessening of somatic inhibition and the potential subsequent interference of the synchronization of whole pyramidal populations might contribute to the observed deficits in PFC-related behaviors and functions such as working memory after this early developmental disturbance (Dawirs et al., 1996). In addition, GABA has also been shown to exhibit a high plasticity when challenged in adult animals (Dawirs et al., 1997). Therefore the question arose, if GABA keeps its natural neuroplastic potential even up to adulthood, especially as it is believed that disturbances in the GABAergic inhibitory regulation of cortical networks contribute considerably to cognitive impairments as seen in schizophrenia (Benes and Berretta, 2001), which's onset is usually in young adulthood. We here demonstrated that there are indeed long-term variations in the GABAergic system during adolescence.

Intriguingly, there was no aging-related change in the fiber densities of GABA or CB in neither the PFC nor the BLA. Several studies have reported about an age-related decrease in CB immunoreactivity in basal forebrain cholinergic cells (Geula et al., 2003; Wu et al., 1997, 2003) and also about age-related changes of CB structures in some cortical and subcortical areas (Bu et al., 2003; Hwang et al., 2002; Kishimoto et al., 1998). Further, several studies have shown alteration in the CB immunoreactivity in Alzheimer patients compared to controls (Ichimiya et al., 1988; Lally et al., 1997; McLachlan et al., 1987). However, further investigations suggest, that it might rather be a decrease in the expression of the protein than a decline of whole cells or branches (Kishimoto et al., 1998). Nevertheless, a decline in CB within the cell might cause a diminished capacity to buffer high levels of calcium, thus leading to a higher vulnerability towards pathological processes that might cause the degeneration of the cell in the end (Bu et al., 2003). However, to our knowledge, there is no study revealing a significant age-related effect in CB immunoreactivity for the PFC or BLA. But there are other hints for alterations within the GABAergic system as, e.g. differences in GABA activity in specific hypothalamic areas (Jarry et al., 1999) or age-related changes in GABA receptor compositions (Caspary et al., 1999). Such changes can not be excluded in the old gerbil referring to our data, but there is apparently no alteration in fiber densities. However, we have to admit, that 110 weeks is the mean survival of a male gerbil, meaning that in the individual case the maximum age lies higher (Troup et al., 1969), thus our results of stable GABAergic and CB fiber densities up to PD720 do not exclude variations in still older animals.

Nevertheless, GABA seems to appear relatively consistent against the deleterious effects of age compared to other transmitters as, e.g. dopamine, which is believed to play a role in various age-related diseases such as Alzheimer, Parkinson's or Huntington disease (reviewed in: Backman and Farde, 2001; Morgan et al., 1987; Ossowska, 1993) and shows a decline of fibers in 720 days old gerbils in the PFC (unpublished data) but

not in the amygdala (Brummelte and Teuchert-Noodt, 2006). On a highly speculative level it might be assumed that neurodegenerative diseases are likely to appear when the GABAergic plasticity finally vanishes during aging.

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Lifestyle and memory

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Abstract:

Objective: Healthy lifestyle has been associated with a decreased risk for cardiovascular disease, but the relationship with memory functions has been inconclusive so far. Previous studies tried to correlate isolated aspects of a healthy lifestyle with memory functions, while the effect may involve complex interactions. Here, we tested the hypothesis that a composite lifestyle score can reliably predict memory performance.

Design: Survey

Setting: Department of Neurology, University of Muenster.

Participants: 420 healthy individuals (mean: 63 years; population-based sample)

Main outcome measure: All participants took tests of verbal episodic memory. The following lifestyle dimensions were considered: Physical exercise, dietary habits, body mass index (BMI), smoking, and alcohol consumption. From these factors, a composite lifestyle score was calculated. Healthiest behavior was defined as a BMI < 22, a diet high in fruit, vegetables, low-fat dairy products, wholemeal foods, and unsaturated fatty acids, energy expenditure through physical activity > 13000 kcal/week, a history of never smoking, and an alcohol consumption of 4 -10 drinks per week.

Results: Linear regression analysis revealed that a higher lifestyle score was associated with a better memory score, after adjusting for age, sex, years of education, and blood pressure. None of the 5 dimensions of the lifestyle score alone predicted memory scores.

Conclusions: This cross-sectional study of elderly individuals revealed that a composite account of lifestyle factors predicts memory performance, while isolated lifestyle factors do not. Our findings point to the importance of a comprehensive modulation of lifestyle in order to preserve memory functions in the elderly.

Introduction

Lifestyle involves our conscious choice to engage in behaviour that can remarkably influence the fitness level of our body and brain.[1] Given the prospected rapid growth of the aged population and related staggering costs it is becoming an imperative public health goal to identify effective mechanisms for warding off structural and functional declines in the aging brain.[2;3] A healthy lifestyle, incorporating factors like eating habits, exercise, alcohol consumption, and smoking, has been associated with decreased risk for cardiovascular disease,[4] stroke,[5] and diabetes.[6]

For memory impairment and dementia, the association is as of yet unclear. Isolated aspects of a healthy lifestyle have been linked to preserved memory functions in some studies, while other studies could not confirm these findings: Epidemiological and interventional studies have suggested benefits of exercise on memory functions, particularly in aging populations (see ref. 7 for review) but negative findings have been reported as well.[8-11] Diets high in folate, Vitamin B12, C, and E, polyunsaturated fats, and omega-3 fatty acids (seafish) have been linked to better cognition and memory,[12-14] but other studies could not confirm these findings.[15-17] Epidemiological studies have mostly suggested an inverse association of BMI with cognitive parameters including memory from middle age to very old age,[18-21] but negative reports can be found as well.[22] Alcohol consumption may be beneficial in low[23;24] and deleterious in high quantities,[25] whereas other studies could merely reveal a positive effect on vascular parameters.[26;27] Smoking is held to be detrimental for cognitive functions including memory by some,[28;29] but beneficial or even neuroprotective by others.[30;31]

These contradictory findings may have arisen due to the fact that lifestyle factors are probably complexly interrelated, and may compensate for one another.[28;32] A composite lifestyle assessment has recently been recognized as a more suitable concept for the development of cardiovascular disease and its prevention.[4;5] This concept has not been translated into the domain of cognition so far.

In the present study, we tested the hypothesis that a composite lifestyle score, including exercise, diet, BMI, alcohol, and smoking, would predict memory performance, rather than individual lifestyle factors.

Methods

Study sample

Study subjects were all participants of the Münster project on healthy aging in community-dwelling individuals (Systemic Evaluation and Alleviation of Risk factors for Cognitive Health = SEARCH-Health). Participants were identified from a city registry of Muenster, Germany, based on their date of birth. They were contacted by letter and screened when agreeing to participate and signing a written informed consent. Criteria for exclusion were history of stroke or dementia based on scores below 25 on the Mini-Mental State Examination (MMSE). Participants underwent a structured interview (previous medical history), a medical examination including blood pressure, heart rate, electrocardiogram, routine laboratory testing, and a standardized neurological examination. Territorial brain infarction was excluded by brain imaging. Neuropsychological assessment excluded any individual with pre-existing cognitive impairment. Three out of 436 individuals were excluded because of MMSE < 25. Thirteen additional subjects had to be excluded due to missing data, leaving 420 for this analysis. These subjects (37-84, mean 63 ± 6.6 years; see Table 1 for details) were recruited over the course of two years. They answered a detailed questionnaire about lifestyle habits as well as their height and weight (see below, and Table 2, for details). The research protocol was approved by the local ethics committee.

Psychometry

The cardinal feature of dementia of the Alzheimer type (AD) and its precursor, “mild cognitive impairment”, type is an impairment of episodic memory and new learning.[36;37] We therefore considered the delayed phase (recall and recognition) of a word learning task as our primary outcome measure ("memory score", items 6-8 of the German version of the Auditory Verbal Learning Test, AVLT).[38] Participants were asked by clinical neuropsychologists to remember and recall as many words as possible after hearing a list of 15 words, which were consecutively presented for 5 times. After the fifth recall, subjects were distracted by a different task. They then had to recall the word list again after a short delay (AVLT 6) and a 30-minutes delay (AVLT 7). Subsequently, subjects had to recognise these words out of a total of 45 mixed words (AVLT 8).

Lifestyle Score

To create a lifestyle score as previously described,[4-6] we considered self-reported lifestyle information from the baseline questionnaires, incorporating information about BMI, dietary habits, exercise, smoking, and alcohol consumption. The following definitions and cut-off values were used:

Exercise: A validated questionnaire was used that encompasses questions on daily-life physical activities (e.g., cycling, stair-walking), and on "sport" exercise (e.g., nordic walking, swimming, tennis.[33] A specific metabolic equivalent task (MET)-score was assigned to each activity.[34] The hours spent on different activities in a week, multiplied with their specific MET score, were summed and then divided by the square of body weight. Finally, individual values, measured as kcal per week, were divided into quintiles (13000 kcal per week or more, 7000 to 12999 kcal, 3000 to 6999 kcal, 1000 to 2999 kcal, and less than 1000 kcal). The most intense physical activity (≥ 13000 kcal) was categorized as healthiest behavior, the least physical activity (< 1000 kcal) as unhealthiest behavior.

Dietary habits: An index was created that incorporated 37 questions on frequency of different food intake and dietary habits. The items included different forms of meat, poultry, fish, fruits, vegetables, corn products, dairy products, eggs, sweets, salty snacks, margarines, oils, other sources of fats, as well as salting habits (adapted from a short, qualitative food frequency list used in several German large scale surveys).[35] Subjects were asked to recall their "average intake" of the respective food item in the following six frequency categories: "Almost daily", "several times per week", "about once a week", "several times per month", "once a month or less", or "never". From this information, a diet score was calculated that ranged from 127 points (a diet rich in raw fruits and vegetables, low-fat-, whole-meal- and low-sugar-products, fish, oils and margarines high in unsaturated fatty acids, moderate sheer meat and salting habits) to 20 points (a diet rich in fatty products, sausages, soft drinks, sweets, butter, salt and low in fruits and vegetables). The diet score was then divided into quintiles, with the highest score (≥ 117) indicating the most healthy, the lowest score (< 90) most unhealthy behavior.

BMI: Calculated as weight in kilograms divided by the square of height in meters, BMI was divided into five strata: 22.0, 22.-24.9, 25-29.9, 30.0-34.9, and 35.0 or more.[5]

Smoking: We categorized participants as never smokers (most healthy behavior), past smokers who smoked less than 20 pack-years, past smokers who smoked 20 or more pack-years, current smokers who smoke fewer than 15 cigarettes per day, and current smokers who smoke 15 or more cigarettes per day (most unhealthy behavior).

Alcohol: Alcohol intake was categorized as never or less than 0.1, 0.1 to 1, 1.1 to 3.9, 4 to 10.4, and 10.5 or more drinks per week. We assigned the healthiest behavior to moderate drinking habits (4 to 10.4 drinks per week), followed by an alcohol consumption of ≥ 10.5, a consumption of 1.1 to 3.9, alcohol consumption of 0.1 to 1, and an alcohol consumption of less than 0.1 drinks per week. In an exploratory analysis, we assigned alcohol consumption to a "modified J-shaped curve", where highest alcohol consumption (10.5 or more drinks per week) is now classified as the most unhealthy behavior, and no alcohol as the second unhealthiest behavior.

The overall "lifestyle score" was calculated by assigning scores of 1 to 5 to each individual variable category, for which a higher point value indicates a healthier behavior.[5] The lifestyle score in our study population ranged from 4 to 19. Healthiest behavior was defined as normal weight, never smoking, intense physical activity, moderate alcohol consumption, and a dietary pattern rich in fruits, vegetables, whole grain products and unsaturated fatty acids (Tab. 2). We a priori divided the lifestyle score into five categories: ≤ 8, 9-11, 12-14, 15-17, and ≥ 18 (see also ref. 5).

Statistical analysis

Normal or near-normal distribution for all lifestyle categories and the memory scores was standardized ascertained first.

We then asked if either the individual lifestyle habits or the composite lifestyle score would be associated with better memory performance. Linear regression analysis (forward inclusion model) with predictor exercise or dietary habits or BMI or smoking or alcohol or total lifestyle score, and the dependent variable "memory score" (sum of scores AVLT 6, 7, and 8) was conducted. Confounders (age, sex, education, and systolic blood pressure) were entered successively into the model, to test if the effects of lifestyle factors on memory performance would be predicted by the confounders only.

Univariate analysis of variance with factor age or education or sex or blood pressure and the dependent variable lifestyle score was used to check for co-linearity.

Results:

In total, 190 healthy elderly men and 230 healthy elderly women, aged 65 ± 6.2 and 62 ± 6.8, respectively, were enclosed for statistical analysis. Details on baseline characteristics in the five groups of the lifestyle score are shown in Table 1.
The distribution of lifestyle characteristics within each group of the overall lifestyle score can be seen in Table 2. As expected, participants in the most healthy category were more likely to exhibit intense regular physical activity, to follow a healthy diet, to be of normal weight, to be current and past non-smokers, and to exhibit moderate alcohol consumption (see also ref. 5).

Neuropsychological testing showed a total memory score of 34 words ± 8.2 SD for delayed verbal memory (AVLT 6-8).

Linear regression analysis with the predictor “lifestyle score” and the dependent variable “memory score” and age, sex, and education as confounders revealed that lifestyle was associated with a significantly higher memory score (AVLT 6-8: standard coefficient beta = 0.09, T = 2.02, $p < 0.05$, see Fig. 1).

Additional adjustment for the potential consequences of an unhealthy lifestyle (systolic hypertension) did not attenuate these associations. According to the model, we found a 3% better memory score for every 5 points improvement in lifestyle score (score max. = 25, see Tab. 2).

In an exploratory analysis, re-assigning the highest alcohol consumption to the most unhealthy behavior (possible neurotoxic effect) showed a similar result to the J-shaped curve but failed to reach significane (AVLT 6-8: $p = 0.098$).

None of the five individual factors showed a significant predictive value for memory score (all T’s < |1.93|; all p’s > 0.05).

Univariate analysis of variance revealed that confounders alone did not account for the lifestyle score (age: $F_{(1,36)} = 1.31$, $p = 0.12$; education: $F_{(1,11)} = 1.27$, $p = 0.24$; sex: $F_{(1,1)} = 2.59$, $p = 0.18$; blood pressure: $F_{(1,137)} = 1.04$, $p = 0.4$).

Discussion:

In this cross-sectional study of elderly individuals, we found that a healthier lifestyle that incorporated BMI, physical and dietary habits, smoking, and alcohol consumption was significantly associated with memory performance, even after accounting for possible confounders. In isolation, none of the lifestyle factors showed a significant association with memory.

Individual lifestyle factors and cognition

The influence of individual lifestyle factors on brain health and memory is as of yet controversial:

A number of epidemiological and interventional studies have suggested benefits of physical activity on brain health and memory, particularly in aging populations[39-45] but negative findings have been reported as well.[8-10;46] The present study did not show a positive association of physical activity alone on memory, but history of physical activity contributed to the significant association of lifestyle score with memory.

The evidence for a "brain–protective" diet (other than meeting the basic requirements for micro- and macronutrients, which are necessary for cognitive health in the elderly)[47;48] is scarce. For Vitamin B12, compelling evidence for a positive effect of supplementation on cognition only derives from data on individuals with deficiencies in Vitamin B12.[49] Definite effects of folate are still out, but four small randomized trials did not find an effect.[50] For Vitamin C and E, some initial promising results[51;52] could not be supported in a systematic review.[53] For seefish/omega-3-fatty acids, potentially protective effects for dementia have been assumed, however, no definite recommendation may be issued here either.[54;55] For high intake in polyunsaturated fats, the evidence is not conclusive yet.[54;56-58] Here, higher intake of unsaturated fatty acids supposedly decreases the incidence of dementia,[59] whereas higher intake of saturated or so-called trans-fatty acids may increase it.[60-63] These reports concur with our non-significant findings of a brain healthy diet. Note that a study on the incidence of stroke in women found a higher risk for stroke in those with a diet high in antioxidants and unsaturated fatty acids.[5]

The association between an individual's body mass index (BMI) might crucially depend on the age of the study population. An inverse association of BMI with global brain volume,[19] cognitive functions,[18] and risk of AD[64] was found in middle-aged healthy men and women. They may already be suffering from differentially greater brain atrophy, and may be at greater risk for future decline in memory. Studies on elderly subjects found an almost reverse effect, with poorer cognitive performance in individuals with lower BMI[22] and greater weight loss on the course of 10 years,[65] although an inverse association was also reported,[20] However, it may be important to look separately at the middle old and very old, in whom low weight may be an indicator of other disease processes like malignancy or low food-intake due to cognitive problems.[66]

Smoking was thought to be protective of AD, possibly by a neuroprotective effect of nicotine receptor agonists.[31] However, a systematic review demonstrated case-control and cohort studies to be inversely associated with AD.[28;29] Other cohort studies did not find this effect.[30;67] Negative effects of smoking may be partly mediated by the well known effects on physical illness. For example, in a cohort of 558 women aged 70 years or more, current smoking was not a significant risk factor for either cognitive decline or physical decline, but was for those experiencing a decline in both.[68] Smoking has also been associated with poorer health-related quality of life.[69] Thus, the evidence seems to indicate that smoking strongly increases the risk of unhealthy mental aging.[70]

The effect of alcohol on cognitive functions including memory is probably complex: there may be a neuroprotective effect of low levels of alcohol consumption, e.g. 4.5 to 18 drinks per week,[71] but higher levels of consumption are detrimental, as assessed by brain volume studies[25] and behavioural endpoints.[72;73] We tried to incorporate those findings in our score for alcohol, assigning the "most healthy" category to those individuals who consumed a moderate amount of alcohol per week according to previous studies.[5;26;74;75] Thus, mild to moderate alcohol consumption contributed to the positive association of lifestyle and memory. In an exploratory analysis, we evaluated alcohol intake by assigning the "most unhealthy behavior" category to individuals with the highest alcohol intake. The association between this lifestyle score and memory remained significant but weakened, suggesting that at

least in the quantities consumed by our participants, a clearly neurotoxic effect could not be established.

Combined effect of lifestyle

Comprehensive lifestyle scores have been developed to assess the risk of coronary heart disease,[4] diabetes mellitus,[5] and stroke.[6] To the best of our knowledge, we present the first study using a composite lifestyle score on cognition in an apparently healthy elderly population. The combined lifestyle score demonstrated an association of healthy behavior with memory, while failing to show a significant association with isolated lifestyle factors. These findings support our hypothesis that the effects of lifestyle factors on cognition are complexly interrelated ("pleiotropic effect"), with certain healthy behaviors "neutralizing" the effect of unhealthy behaviors. For example, lifestyle implementations like exercise or moderate alcohol consumption may activate systems that act on metabolism and plasticity, like neurotrophic factors. Thus, they may ameliorate the oxidative stress that our brains accumulate in the face of a diet low in antioxidants and high in saturated fatty acids or by means of smoking[1].

Several limitations should be considered when interpreting our findings. First, despite adjustments for potential confounding factors, residual confounding remains possible. Second, the relationship between the lifestyle score and cognition is influenced by the weight given to each component of the score. Because we did not aim to maximize the likelihood of observing a significant effect, we chose to give equal weight to each component, similar to previous studies. [5] However, because the use of equal weight is an imperfect approximation of the underlying biological relationships, further analyses should examine the issue of weighting. Third, the negative findings of individual lifestyle factors may be due to a lack of power, because we did a cross-sectional one-time assessment of factors that probably take several years or even decades to develop.

Strengths of our study included standardized neurological examination and brain imaging to rule out pre-existing territorial brain infarction, as well as detailed information on a wide array of lifestyle factors. By simultaneously examining the

effect of several lifestyle variables, we took into account the clustering of healthy behaviors within individuals.[4]

Another strength was the primary outcome measure chosen for the present study, the delayed phase of verbal memory. It mimics episodic memory encoding in the verbal domain, and can thus be considered as the most sensitive marker of cognitive changes seen in aging with regard to the risk of dementia;[76;77] (see ref. 45 for review). Furthermore, the parameter "memory performance" allowed us to assess both lifestyle and cognitive variable as a continuum with a high variability, instead of using a dichotomised variable such as "dementia". This approach is in line with the idea that lifestyle factors exert a gradual rather than a threshold effect on cognitive functions.[4;5]

Our study sample was relatively homogenous with regard to memory performance. Therefore, the significant effects of lifestyle on memory performance that emerged even in our homogenous population are a conservative approximation; a much larger effect in the general population might be expected. Also, since part of the lifestyle-associated performance is mediated through improvements in blood pressure,[78] our adjustment for blood pressure might underestimate the overall benefit of lifestyle on memory score.

Conclusion:

The present study indicates that memory function in the elderly may be predicted by a composite assessment of lifestyle rather than by studying isolated lifestyle choices. A similar phenomenon has been suggested for the so-called "metabolic syndrome" and the risk for cardiovascular disease. Here, a comprehensive assessment and subsequent therapeutic approach that takes into account all factors contributing to the metabolic syndrome significantly reduces cardiovascular disease.[79;80] Future therapeutic interventions that try to preserve and improve memory functions in the elderly may profit from similar comprehensive approaches.

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Figure titles and legends:

Figure 1: Association between verbal memory and lifestyle.

Standardized verbal memory score (AVLT 6-8) residual values adjusted for age, years of education, and sex in the five lifestyle score categories. Note that a higher lifestyle score indicates healthier behavior (forward inclusion model, $p < 0.05$). Bars give standard error of the mean (SE).

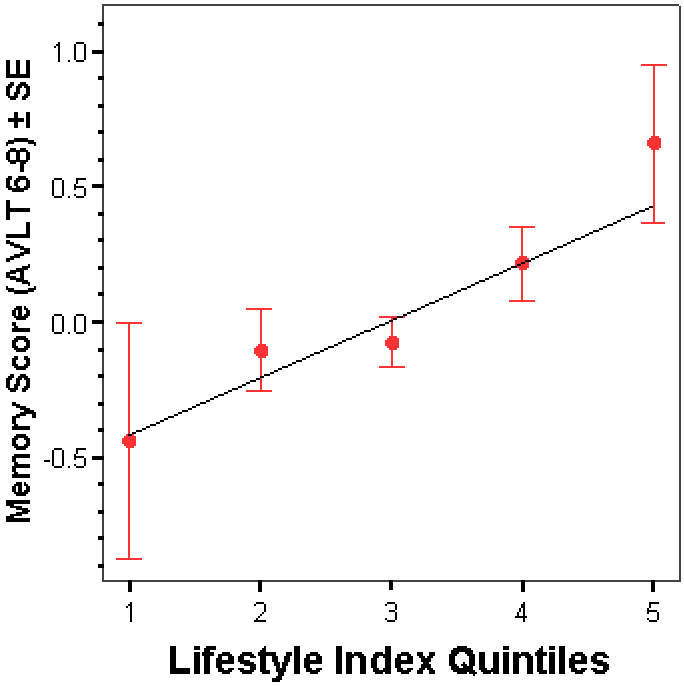

Figure 1

Table 1: **Baseline characteristics according to lifestyle score categories**[†]

Characteristics	Lifestyle score					Total
	≤ 8 n = 28 (6.7%)	**9 - 11** n = 88 (21.0%)	**12 - 14** n = 160 (38.1%)	**15 – 17** n = 125 (29.8%)	**≥ 18** n = 16 (3.8%)	n = 420
Age [mean years ± SD]	59.9 ± 7.8	63.2 ± 9.3	63.1 ± 7.5	62.3 ± 7.0	64.6 ± 5.1	62.7 ± 7.8
Female [%]	51.7	47.8	56.9	58.4	50	54.8
Years of education [mean years ± SD]	12.8 ± 3.5	13.8 ± 3.1	13.6 ± 3	13.9 ± 3.2	13.9 ± 3.3	13.7 ± 3.1
Systolic blood pressure [mean mmHg ± SD]	150.3 ± 20.4	145.2 ± 22.1	144.8 ± 20.5	140.9 ± 17.6	155.2 ± 17.4	144.5 ± 20.1
Diastolic blood pressure [mean mmHg ± SD]	86.1 ± 11.8	83.9 ± 9.7	84.7 ± 12.1	83.7 ± 10.7	91.3 ± 8.9	84.6 ± 11.1
History of hypertension* [%]	48.3	41.1	37.5	38.4	37.5	39.3
Total hypertensive [%]**	72.4	65.6	68.8	62.4	87.5	67.1
History of severe disease* [%]**	27.6	18.9	18	20	6.3	19

[†] Percentages may not add to 100 because of rounding.

*Defined as treated self-reported hypertension

**Defined as systolic blood pressure > 140 mm Hg or treated self-reported hypertension

***Myocardial infarction, coronary heart disease, cancer, diabetes

Table 2: Distribution of modifiable lifestyle factors according to lifestyle score categories†

Individual components		Points*	Lifestyle score					Total
			≤ 8	**9 - 11**	**12 - 14**	**15 – 17**	**≥ 18**	
			n = 28 (6.7%)	n = 88 (21.0%)	n = 160 (38.1%)	n = 125 (29.8%)	n = 16 (3.8%)	n = 420
Smoking [%]	Never	5	10.3	30	44.4	60	87.5	45.2
	Past < 20 pack-years**	4	6.9	36.7	36.3	32.8	12.5	32.4
	Past ≥ 20 pack-years	3	41.4	24.4	16.3	7.2	0	16.4
	Current <15 cigarettes/ day	2	3.4	3.3	2.5	0	0	1.9
	Current ≥15 cigarettes/ day	1	37.9	5.6	0.6	0	0	4.0
Body mass	< 22	5	10.3	8.9	14.4	24	43.8	16.9
index in kg/m²	22 to 24.9	4	10.3	16.7	40	52	43.8	36.7
[%]	25 to 29.9	3	62.1	61.1	38.1	22.4	12.5	39.0
	30 to 34.9	2	17.2	8.9	6.3	1.6	0	6.0
	≥ 35	1	0	4.4	1.3	0	0	1.4
Physical	≥ 13000	5	0	4.4	8.8	9.6	37.5	8.6
exercise in	7000 to 12999	4	6.9	15.6	25.6	28.8	37.5	23.6
kcal/week [%]	3000 to 6999	3	20.7	42.2	41.9	48	25	41.7
	1000 to 2999	2	44.8	25.6	20.6	12	0	20.0
	< 1000	1	27.6	12.2	3.1	1.6	0	6.2
Alcohol	Nondrinker	1	58.6	40	21.3	3.2	0	21.7
	0.1 to 1	2	34.5	47.8	26.3	4	0	23.8

consumption	1.1 to 3.9	3	3.4	7.8	14.4	9.6	0	10.2
[%]	4 to 10.4	5	3.4	1.1	15.6	24.8	6.3	14.0
	≥ 10.5	4	0	3.3	22.5	58.4	93.8	30.2
Diet score	1st	5	0	2.2	5.6	10.4	31.3	6.9
quintiles* **	2nd	4	6.9	17.8	30	40	50	29.5
[%]	3rd	3	31	40	44.4	40.8	18.8	40.5
	4th	2	41.4	36.7	20	8.8	0	21.0
	5th	1	20.7	3.3	0	0	0	2.1

† Percentages may not add to 100 because of rounding.

* assigned point value to create the lifestyle score.

** 1 pack-year = smoked 20 cigarettes per day for one year.

*** a diet high in fruits, vegetables, low-fat dairy products, wholemeal foods, unsaturated fatty acids and low in high-fat products, sweets, sugary drinks, and saturated fatty acids.