Joseph Knoll: Enhancer Sensitive Brain Regulations and Synthetic Enhancers (Selegiline, BPAP) Which Counteract the Regressive Effects of Brain Aging

Contents

Introduction

It is a horrifying fact that in Germany's history millions of average normal people who had previously lived honest simple lives and had never belonged to extremist groups, dramatically changed within a few years after 1933. Imbued with Nazi ideology, they became unbelievably complicit in the murders of innocent civilians during World War II. This phenomenon has been documented from numerous perspectives in dozens of novels, films, and so forth. However, an adequate elucidation of the brain mechanisms responsible for this dramatic and rapid behavioral change of millions has not yet been unearthed. It is worth considering that the manipulability of human behavior is obviously independent from the general cultural level of a society. For example, Germany produced numerous contributions to science and art and the average education level was well established already by World War I. Only one recruit out of 1,000 was illiterate in Germany, versus 330 in Italy, 220 in the Austrian-Hungarian Monarchy and 68 in France.

As a survivor of Auschwitz and the Dachau death train, I directly experienced a few typical representatives of manipulated fanatics (Dunn 1998). I have had ample time and primary experience to reflect upon the essential changes in the physiological manipulability of the human brain. *My life work was not a mere chance.*

When I started my behavioral studies in the early 1950s, I was planning to investigate how rats acquire an unnatural drive. I succeeded in developing a rat model to exactly study the nature of the brain changes in the course of the acquisition of an acquired drive from the start of training until its firm manifestation. *I soon realized that this cortical mechanism explains the manipulability of more sophisticated mammalian organisms.* Two enthusiastic students, Károly Kelemen and Berta Knoll, now distinguished scientists, joined me in this work. Since I began my own laboratory many decades ago, I never left it for longer than a month and worked with my best co-workers for decades.

In 1945, I matriculated at the Pázmány Péter University, Faculty of Medicine, Budapest (from 1951 University of Medicine, Budapest; from 1969 Semmelweis University of Medicine; and from 1999 Semmelweis University). In 1949 I was invited by Professor Bela Issekutz (1886-1979), Head of the Department of Pharmacology, to join his research staff. I began working there as a student in February 1949. After Professor Bela Issekutz retired, I was the Head of the Department from 1962 until 1992. I began working in 1949 in the original building of the Department of Pharmacology and we moved into a new building in 1978, where I am still working, now as Emeritus Professor of Pharmacology. The Department changed its name to the Department of Pharmacology and Pharmacotherapy in 1999.

Until the end of the 1960s, the role of acquired drives in the mammalian brain was in the center of my interest. I summarized the results after a 16-year research period in my first monograph (Knoll 1969).

In the 1970s, a new period of my research began with the discovery of the enhancer-sensitive regulations in the mammalian brain. I selected the catecholaminergic and serotonergic neurons as enhancer-sensitive models for detailed analysis. Two synthetic enhancers, specified to the selected models: (R)-N-methyl-N-(1-phenylpropan-2-yl)prop-2-yn-1-amine (Selegiline/(-)-deprenyl) (DEP), the β-phenylethylamine (PEA)-derived synthetic enhancer substance and (2R)-1-(1-benzofuran-2-yl)-N-propylpentane-2-amine (BPAP), the tryptamine-derived much more potent synthetic enhancer substance than DEP, were developed. We can define the enhancer-sensitive brain regulations as: the existence of neurons capable of changing their excitability in milliseconds and working on a higher activity level, due to natural or synthetic enhancer substances. After 36 years of research, I summarized the results of this new line of research in a second monograph (Knoll 2005).

We seek now to ascertain the full complexity of the enhancer-sensitive brain regulations, identify hitherto unknown regulations and further analyze how the synthetic enhancers counteract brain aging with the aid of synthetic enhancers. We demonstrated that during the developmental/young phase of life, from weaning until sexual maturity, the enhancer-sensitive neurons work on a significantly higher activity level and sexual hormones terminate the developmental phase and restore the enhancer regulations to the low, pre-weaning activity level. This is the beginning of the post-developmental/aging phase characterized by a slow, irresistible decline of the enhancer-sensitive brain regulations which continues until death. It is extremely important that the regressive effects of brain aging in mammals are primarily due to the age-related decline of the natural enhancer regulations.

The unique pharmacological spectrum of DEP allowed me to discover in the mid-1990s the enhancer regulation in the mammalian brain. The realization of the catecholaminergic and serotonergic neurons' enhancer-sensitivity and their selection as models to study the characteristics of the enhancer-sensitive brain regulations, brought to light unknown mechanisms of brain-aging in mammals during the post-developmental phase of their life.

Finally, the development of synthetic enhancers rendered the countering of brain-aging possible, which has always been the main practical aim of my research.

To demonstrate our recent success, I refer to our first experimental confirmation in a longevity study that life-long maintenance of rats from sexual maturity on a low dose of BPAP

(0.0001 mg/kg), *the selective, highly potent synthetic enhancer fully prevented aging of the dopaminergic neurons*. The study proved that the slowly progressing, aging-related loss of the natural enhancers was responsible for the regressive effects of brain-aging. In dopaminergic neurons, the aging-related loss of PEA, the natural enhancer of the catecholaminergic neurons, leads to the aging-related loss of dopamine. We know, for example, that in the healthy human population, the calculated loss of striatal dopamine (DA) is about 40% at the age of 75. Essentially, the same percentages apply to rats (Knoll 2005).

However, the enhancer-sensitive neurons do not age. This means that to prevent the aging of the dopaminergic neurons via substituting the lost natural enhancer with a synthetic enhancer substance after sexual maturity until death is a feasible possibility. We demonstrated this possibility in a longevity study with our new approach to fight brain aging.

As published in 2016, we treated a group of rats from sexual maturity until death, three times a week, subcutaneously, with 0.0001 mg/kg BPAP, the peak dose which exerts the specific enhancer effect in rats. After an 18-month treatment, we measured the learning ability of the rats in the shuttle box. In perfect agreement with our preliminary findings, the longevity study presented evidence that BPAP-treatment completely prevented aging of the dopaminergic neurons. The learning ability of the aged rats was equal with the performance of the young, 3-month old saline treated rats, which are peaking in their learning ability. Since due to aging, the catecholaminergic neurons lost already a lot of their natural enhancers, aged, 18-month-old saline-treated rats retain only 30% of their learning ability compared to their 3-month-old peers (Knoll and Miklya 2016).

This book is primarily devoted to analyzing the pharmacological profile of the two presently available, safe synthetic enhancers: DEP and BPAP. Prior to the discovery of the enhancer-sensitive brain regulations and the development of the synthetic enhancer substances, it was entirely unimaginable to safely counteract the regressive effects of brain aging. As it will be shown in detail in this monograph, the enhancer-sensitive catecholaminergic and serotonergic neurons work in human and animal models similarly, and regarding the mechanism of the enhancer effect, we could not detect any qualitative difference between DEP and BPAP.

The primary aim of my book is to motivate clinicians to test whether synthetic enhancers can counteract brain aging in healthy humans similarly as shown in our recent paper in aged rats (Knoll and Miklya 2016).

Abbreviations

AD	Alzheimer's disease
AM	amphetamine
BPAP	(2R)-1-(1-benzofuran-2-yl)-N-propylpentane-2-amine
CAE	catecholaminergic activity enhancer
CAR	conditioned avoidance response
CNS	central nervous system
CR	conditioned reflex
CS	conditioned stimulus
DA	dopamine
DATATOP	Deprenyl And Tocopherol Antioxidant Therapy of Parkinsonism
DEP	(R)-N-methyl-N-(1-phenylpropan-2-yl)prop-2-yn-1-amine, selegiline/(-)-deprenyl (Eldepryl, Jumex, Emsam, Zelepar)
DMI	desmethylimipramine
ECR	extinguishable conditioned reflex
EF	escape failure
HP	highest performing
ICR	inextinguishable conditioned reflex
IPAP	(-)-1-(indol-3-yl)-2-propylamino-pentane
IR	inter-trial response
LP	lowest performing
MAM	methamphetamine
MAO	monoamine oxidase
MAO-A	A-type monoamine oxidase
MAO-B	B-type monoamine oxidase
MDD	major depressive disorder
NE	norepinephrine
PEA	β-phenylethylamine
PD	Parkinson's disease
PPAP	(-)-1-phenyl-2-propylaminopentane
PSG	Parkinson Study Group
SAR	structure-activity-relationship
SE	serotonin
SOD	superoxide-dismutase
TA	trace amine
TBZ	tetrabenazine
TLS	technical lifespan
TLS_h	human technical lifespan
TMS	tumor-manifestation-suppressing
TRY	tryptamine
US	unconditioned stimulus
VMAT2	vesicular monoamine transporter 2

Chapter 1
Acquired drives - the mechanism of the manipulability of mammalian behavior

After preliminary experiments, I began research in the early 1950s with two students, Karoly Kelemen and Berta Knoll, which aimed to investigate the exact reason in cortical mechanisms explaining why rats (in striking contrast to mice! as I soon realized) are capable of fixing acquired drives.

We formulated a useful model, the "glass-cylinder-seeking" behavior, to study the development and the final, irreversible fixation of an acquired drive in rat brains and then tried, using the same device adjusted to mice, to measure exactly the basic difference in the behavior between these two closely related species.

Innate drives divide into two subgroups and are responsible for a limited number of indispensable (life important) goals: *drives that ensure the survival of the individual* - the urge to maintain internal stability (homeostasis); the urge to avoid anything that is endangering or unpleasing; and the urge to obtain water and food; and *drives that ensure the survival of the specie*s - the instinct to copulate and nurture offspring.

Acquired, unnatural drives are responsible for an unlimited number of dispensable goals. The capability to acquire an insuppressible urge for a goal, which is unnecessary for individual or species' survival, represents the most sophisticated function of the telencephalon. Humans are the only beings on earth whose life is predominantly based on acquired drives. To a certain extent, a minority of the mammalian species (monkey, dog, horse, dolphin, rat, etc.) possess this endowment which remains, under natural conditions, unexploited. Nevertheless, humans obviously discovered thousands of years ago, probably through a kind of serendipity, that the behavior of such animals can be modified by proper training, and thus began the progressive domestication of various species.

The development of an acquired drive always originates in some way in an innate drive. Though this relation becomes later unrecognizable, it is obvious that nothing exists in the human brain without a rational origin.

The course of evolution is progressing towards the perfectibility of various species. For example, rats are one of the most teachable mammals readily capable of acquiring an unnatural drive, but indocile mice are devoid of this ability. The successful demonstration in the significant differences in the EEG arousal reaction in rats with extinguishable (ECR) and inextinguishable (ICR) conditioned reflexes, and many more unexpected data (Knoll, Kelemen

and Knoll 1955a,1955b,1955c; Knoll 1956, 1957; Knoll, Kelemen and Knoll 1956; Kelemen, Longo, Knoll and Bovet 1961; Knoll 1961; Knoll 1968), inspired my realization that *the development of mammalian brains capable to fix unnatural drives created the manipulability in behavior which rendered community life possible*.

An acquired drive is fixable only on the basis of an innate drive, so our "glass-cylinder-seeking" drive was built on an unconditioned avoidance reflex (escape from a hot plate). The sound of a high-pitched bell served as a high-priority conditioned stimulus (CS). Rats were trained to vigorously search for a 30-cm-high glass cylinder and jump to the rim of it. The cylinder was open at bottom and top with diameters of 16 cm and 12 cm, respectively, and with a side opening through which a rat (up to 350-400 g body weight) could move inside the cylinder.

In the training procedure, rats (male or female) were pushed through the side opening of the glass cylinder which resulted in them standing on a metal plate heated to 60°C. The jumping reflex was then elicited for weeks, three times daily, for 10-50 repetitions at 10 second intervals with bell and heat stimulation until a chain of ICRs developed and the rat indefatigably displayed the jumping reflex without heat stimulation, even after 100 times in succession (Knoll, Kelemen and Knoll 1955a,1955b,1955c). This was a transient stage leading to the manifestation of the "glass-cylinder-seeking" drive (Knoll 1969).

The rats that performed best in this study acquired the "glass-cylinder-seeking" drive in a stable manner and, thereafter, maintained this unnatural urge for life. The rats showed the same high-grade adaptability and readiness in overcoming different obstacles during goal-attainment as those influenced by innate drives, such as hunger or sexual desire (Knoll 1956, 1957; Knoll, Kelemen and Knoll 1956). In the most efficiently trained, best performing rats, the acquired drive was so powerful that it prevailed over life important innate drives. When said rat had been deprived of food for 48 hours and then food was offered within the usual setup that contained the glass cylinder, the rat looked for the glass cylinder and left the food untouched. Similarly, when a receptive female was offered to a fully sexually active "glass-cylinder-seeking" rat in the usual setup, the male looked for the glass cylinder and neglected the receptive female.

The mouse, a rodent closely related to the rat, trained under the same experimental conditions as the rat, was unable to acquire the "glass-cylinder-seeking" drive, clearly demonstrating that the development of mammals with a brain capable to fix acquired drives possess a qualitatively higher developed brain than the mammals devoid of this ability (Kelemen, Londgo, Knoll an Bovet 1961; Knoll1961,1968).

Berta Knoll worked for seven years carefully analyzing the physiologically important differences in the unconditioned and conditioned stimuli-induced excitation processes between the mouse (males or females) and the rat. She used, with dimensions adjusted to mice, essentially the same device as applied to fix the "glass-cylinder-seeking" drive in rats. The unconditioned stimulus (US) was capable of inducing an intensive dominant state of excitation in mice and a jumping reflex could be elicited, within 1 second latency, with the CS (bell sound) alone. Thus, the mouse behaved as if it possessed a conditioned reflex (CR) highly resistant to inhibition, *but within a couple of hours, no sign of the behavioral change was detectable. A stable CR could not be established in mice, even when the number of associations was increased from 4 to 100 times daily and carried out over periods varying from one week to full lifetime.* In 1959, the highly interesting details regarding the nature of this phenomenon, termed by Berta Knoll a "pseudo-conditioned reflex," were published (Knoll 1959; Knoll 1969).

It is obvious that rats possess a qualitatively higher developed brain than mice; clearly demonstrating that the development of mammals capable to fix acquired drives, thus being in possession of behavioral manipulability, was the last qualitative change in development of the mammalian brain.

Vertebrates can be divided into three groups according to their brains' mode of operation: *(a) those that operate with only innate drives (the majority); (b) those with an ability to acquire drives (a minority); and (c) the "group of one" that operates almost exclusively with acquired drives (Homo sapiens sapiens)* (Knoll 2005).

With the evolution of brains capable of acquiring drives, species developed whose members could manipulate each other's behavior and act in concert. This was the condition *sine qua non* for the evolution of social living, a form of life that enabled species to qualitatively surpass the performance of any given individual. It goes without saying that training members in the skills needed to act in concert improved the quality of life. The learned behavior, for example, of five to six famished female lions act to collectively separate an animal chosen from its herd to consume, significantly increased the chances of capturing the prey. It was the evolution of the brain with the ability to acquire drives that made the appearance of life on earth so immensely variable.

With the development of the human brain, a functional network with more than 100 billion interrelated nerve cells and 10^{11}-bit capacity arose. With this system, whose operation is inseparably connected to conscious perception, life on earth reached its most sophisticated form. Furthermore, the human being, who is primarily a social creature, is a building block in the creation of a gigantic network: human society.

The function and capacity of society obviously exceeds the sum in activity of its members. With the practically inexhaustible capacity of the human brain to acquire drives, human society represents a qualitatively new, higher form of life. For example, a country, presently the most sophisticated form of a human community, consists of millions or even more than a billion humans and operates *de facto* as a huge living complex interacting with other similar entities, more than 200 at present.

The birth and development of human society, an insignificant moment and fleeting in the endless history of the universe, necessarily means everything to us. It can be taken for granted that during the birth of human society, probably somewhere in South-Africa, very small groups formed a micro-community, working together. Due to learning, practice and experience, their community life became more and more efficient, and the accumulation of basic knowledge opened the way for a more rapid development, truly reflected in population growth.

In the last phase of the Stone Age, about 8,000-9,000 years before our age, marked by the domestication of animals, development of agriculture, and the manufacture of pottery and textile, the human population on earth approached the *one-million* level. Thereafter, however, the population increased exponentially. By the beginning of the Common Era, it had reached *300 million*, grew to *1.6 billion* by 1900 and is today around *7.5 billion*. The world population is still growing, but this tendency is already in decline. For example, the forecast for the yearly increase in 2020 is 1.09 % and in 2050 only 0.57 %, which is promising for the future.

After numerous experiments with rats and mice, I concluded that *in the mammalian brain capable to acquire drives, untrained cortical neurons (Group 1) possess the potentiality to change their functional state in response to practice, training, or experience in either of three consecutive stages: (i) in an extinguishable conditioned reflex (ECR) (Group 2); or (ii) in an inextinguishable conditioned reflex (ICR) (Group 3); or (iii) in an acquired drive (Group 4).*

It was crucial to find an exact measurable test to demonstrate the functional difference between the ECR and ICR. An EEG study, performed in Daniel Bovet's department in Rome by Károly Kelemen in Vincenzo Longo's laboratory, presented the expected experimental evidence to better understand the unique changes in the rat's brain when it's behavior is fully under the control of the "glass-cylinder-seeking" acquired drive (Kelemen, Longo, Knoll and Bovet 1961; Knoll 1969).

The EEG experiments were performed by implanting two pairs of screw-electrodes into the rat's scull in locations corresponding to the sensorimotor and association surfaces of the cortex. The electrodes were fixed to the bone with dental cement. During the EEG experiments

the rats were kept in sound-proof chambers in shielded boxes measuring 60x80x100 cm that differed from the environment used for CR trials and rendered the performance of the learned reflex activity impossible. The bell was fixed above the box inside the chamber with the switch and the EEG apparatus outside. For EEG recording, the electrodes were connected with the device by flexible long-cables which did not interfere with the animal's movements. After 1-3-hour adaptation, during which the rats grew accustomed to the box, a control record was made and compared with the activities recorded during continuous or intermittent bell ringing. Under these conditions, freely moving rats habituated for 1-3 hours displayed predominantly synchronized activity on the EEG record. Prior to the application of a specific stimulus, there was no difference between the EEG records of controls and rats with ECRs or ICRs. Under the influence of various indifferent stimuli, slow potentials with high amplitudes were suddenly replaced by 20-25 c/s and low-voltage waves predominating sometimes for seconds after the cessation of the stimulus. Simultaneously, the animals displayed a typical orientatory-searching behavior. After applying the specific (conditioned) stimulus, however, the individual experimental groups displayed differences in behavior.

The effect of 20 min continuous bell ringing on the EEG arousal reaction was examined under the conditions described above. The controls responded to the bell sound in the usual way. When the sound began – a new stimulus! – desynchronization, i.e. excitation of the non-specific activation systems, began. This state lasted for a short period; after habituation to the stimulus, synchronized cortical activity was restored (Kelemen, Longo, Knoll and Bovet 1961; Knoll 1969).

The effect of continuous bell stimulus on EEG arousal in traditionally conditioned animals (ECR) hardly differed from the controls' effects. Although in this case the bell-stimulus was not indifferent, yet its desynchronizing effect on the activation system was only transitory. Habituation after EEG arousal set in at practically the same rate as in the controls. Thus, the conditioned reflex's typical functional property, the appearance of extinction, paralleled with the extinction in EEG arousal, as expected on the basis of literary data (Kelemen, Longo, Knoll and Bovet 1961; Knoll 1969).

In addition, an entirely different behavior was observed in rats whose ICR had been established during the procedure when the "glass-cylinder-seeking" acquired drive was fixed. Throughout the 20-min bell ringing, they displayed desynchronization, i.e., cortical activation. Thus, there was a parallelism between the absence of reflex extinction and the absence of habituation in the EEG arousal. Accordingly, the bell stimulus had a lasting capacity to cause

excitation in the non-specific activation system or, in other words, to influence the centrencephalic system (Knoll 1969).

We also soon realized and described unknown basic differences between the ECRs and ICRs in their sensitivity toward psychopharmacological agents. While the ECRs were readily inhibited by sedatives/hypnotics and major tranquilizers, the ICRs were conspicuously resistant to sedatives/hypnotics, displaying only selective sensitivity to major tranquillizers (Knoll and Knoll 1961,1964; Knoll, Nador, Knoll et al. 1961).

It is reasonable to conclude that the ability to acquire an irrepressible urge for a goal that is unnecessary for survival was the last step in the development of the mammalian brain. This is the most sophisticated function of the telencephalon.

By the end of 1953, experiments with the "glass-cylinder-seeking" rats led me to shape the working hypothesis that the appearance of the mammalian brain with the ability to acquire drives produced a species fit for domestic life, i.e., *to live in intimate association with and to the advantage of humans*. This ability of the mammalian brain ensured the interaction of the individual and the group and finally led to the evolution of the most sophisticated form of organized life: human society.

To develop the full possibilities of this approach, I thereafter clarified during a 36-year research period those key important brain mechanisms, which determine the life of mammalian species whose members, being capable to fix acquired drives, possess a brain with a higher level of manipulability than mammalian species whose cortex is missing this potential. I finally summarized my theory, *"The Brain and Its Self. A Neurochemical Concept of the Innate and Acquired Drives,"* in a second monograph (Knoll 2005).

The physiology of acquired drives furnishes knowledge about the most important brain mechanism: the manipulability of mammalian behavior. As discussed, an acquired drive is always built on one of the innate drives that ensure the survival of the individual or the species. However, after the acquired drive has been ultimately fixed, its origin, the innate drive, cannot be recognized anymore either in humans or in domesticated animals. A "glass-cylinder-seeking" rat will never acquire this drive under natural conditions. The experimenter consciously manipulated the rat's brain, making use of the innate potential to change a group of cortical neurons via proper training in a way that the rat is *fixing an acquired drive*. Finally, the "glass-cylinder-seeking" rat behaves as one possessing a "fanatical" desire for the glass cylinder. Essentially the same mechanism works in all mammals capable to acquire drives, including humans, who *possess the most manipulable brain among all living creatures on earth.*

The brain of a suicide killer is furtively manipulated. The properly acquired drive develops as a result of long-lasting training. The subject always acts under coercion, under severe mental pressure. Nevertheless, it is the nature of acquired drives that if manipulation is fully successful, then the individual ultimately behaves as one possessing a fanatical desire to reach the acquired-drive-motivated goal. Thus, current expert opinion that the global war on terror is a 7th-century ideology clashing with 21th-century weapons is certainly correct.

To terminate the still existing myth-based era of human society and arrive at the rationally-directed phase of human history is obviously a most imperative necessity. To reach this era is not only a most desirable necessity; its establishment and stabilization, which fully depends on scientific development, is unstoppable.

The main message of my theory is that human society is still in the trial-and-error phase of its development. It already seeks to bring to an end the myths-directed era, the first part of its history, and reach its final goal: the rationally directed human society in which behavioral modification induced by the family/school/society triad will be based, from birth until death, on the total knowledge of how the human brain works. In this way, communities will understand how the human cortex created with its chaos function the myths of supernatural forces. This was the ideology which enabled the creation of human society prior to the *sine qua non* knowledge of the creative and controlling forces in the universe needed to establish and maintain a rationally directed homogenous human society.

It is evident that the power of thinking in orderly rational ways, i.e., the capacity to understand the natural world (science), is that physiological reality which determines the conscious fight of the individual to find and fix acquired drives that optimally fit their natural endowments. *Homo sapiens sapiens* appeared around 150,000 years ago; reached full behavioral modernity around 50,000 years ago; and slowly accumulated proper knowledge regarding natural forces. Science's development finally brought to fruition the era of enlightenment, the spiritual revolution which dramatically changed the continued fate of the myths-directed human society. Separation of Church, by its nature the main creator and guardian force of the myths-directed era of human society, and State, interested by its nature in increasing support over time to rationally directed human activities, was more than 200 years ago the decisive step which enhanced with a previously unimaginable rapidity, the development of science and technology. Let me demonstrate the enlightenment-induced acceleration in science and technology with a convincing example described in one of my former monographs (Knoll 2016).

George Stephenson created the first steam engine in **1814**. (His passenger train completed in 1825 its first 39 kilometers at 24 kilometer/hour.) *Only 62 years later,* in **1876** Nikolaus Otto discovered the four-stroke combustion engine. Benz and Daimler created the automobile and Benz designed in **1885** the first usable automobile. *Only 18 years later*, in **1903,** the Wright brothers developed the first airplane: 274 kg in weight, flying at 48 kilometer/hour. *The rocket technique soon developed with dramatic speed.* The era of space research started. Gagarin, the first astronaut, left Earth on April 2, **1961,** *only 58 years after the first efficient airplane was created.*

Mankind used the horse for thousands of years and after the spiritual revolution of enlightenment, science and technology accelerated within 147 years (!) from the first steam engine to the era of space research. Science, the continuously developing human brain product, would benefit even more with the termination of the regrettable but inescapable myths-directed phase of human society's history. However, the dense ignorance of the overwhelming majority of the world population explains that despite the breath-taking acceleration of science and technology, more time, perhaps a few hundred years, is still necessary to firmly establish and stabilize the ratio-directed human society.

We all seek a brain, which acquires drives with an utmost ease through a permanently active state. The acquisition of proper acquired drives in the most sensitive developmental period of life was always and remains forever in any individual's life determinant. However, since we still live in the myths-directed era of human history, only a small segment of the world's population, primarily the most creative artists and scientists, are lucky enough to possess the drive to work day and night enthusiastically and indefatigably in full harmony with their natural endowments. The majority, as a matter of fact, assume, most often, coerced professional drives that will ensure their place in society. Conformity of innate faculties and acquired professional drives are keenly important for lifelong equilibrium.

However, not only is the permanently active brain an ambition, but also there are times when a drive might need to be altered or replaced. While even the most satisfying professional drive becomes redundant after its permanent, continuous use, there is yet the undoubted desire to maintain the brain in a satisfyingly active state. Inexhaustible forms of supplementary activities serve this aim. *Absolute dominance of a fully satisfying professional drive and the acquisition of beneficial-supplementary drives is the formula for a harmonious, well-balanced life.* Lack of full satisfaction in the acquired professional and supplementary drives generates an urge to flee from frustration and seek salvation in "Ersatz" activities: smoking, alcohol, drugs, etc.

Metaphorically, every human being is born tabula rasa with a telencephalon that resembles a book with more than 100 billion empty pages (untrained, naive cortical neurons, Group 1), and with the capacity to inscribe as much as possible in this book throughout life. In reality, cortical enhancer regulation – the modification of the presently still unknown chemistry of the cortical neurons through learning, aiming to establish cooperation between cortical neurons previously unacquainted with one another – is the essence of human life.

Whenever a drive is acquired, chains of ICRs are fixed in the brain and neurons responsible for emotions are coupled to the integral whole. Thus, cognitive/volitional consciousness is necessarily inseparable from an affective state of consciousness. The mechanism that binds emotions, appurtenances to any chain of ICRs, is crucially important to interpersonal communication. Cortical neurons belonging to Group 3 or 4 continuously synthesize their specific enhancer substance within their capacity. This means that even in the vigilant resting state (leisure), in the absence of a dominant drive, as well as in the non-vigilant resting state (sleeping), the cortical neurons representing the totality of the already fixed chains of ICRs and acquired drives are permanently under the influence of their specific enhancer substance.

Although the level of this *permanent, undulating activation* remains low, it is unpredictable as to when any group of cortical neurons will be influenced by enhancer substances on the level already inseparable from conscious perception. Thus, it is always unforeseeable what will suddenly come to our mind. As the totality of the cortical neurons belonging to Group 3 or 4 works continuously on an unconscious level, there is a steadily operating, chaotic background noise in the human telencephalon which can never cease to exist, but it never endangers the function of the dominant innate or acquired drives. Because of this phenomenon, *rational brain activity is always amalgamated with irrational brain activity and we are in every moment of our life in readiness for experiencing the coexistence of order and chaos in our brain.*

Thus, (a) whenever a chain of ICRs is fixed in the human brain, the proper cortical neurons remain, on an unconscious level, constantly active for life, and (b) if the proper method is used, even a chain of ICRs that had never been recalled after fixation can be activated to the level needed for conscious perception at any later date. The recalling of any chain of ICRs is necessarily inseparable from an affective state of consciousness, due to the emotions coupled as appurtenances to the cortical neurons when they synchronize with each other.

In summary,

(i) past experiences are irreversibly fixed in neurons belonging to Groups 3 and 4 that learned to cooperate with each other and constitute an integral whole;

(ii) proper stimulation of the cooperating neurons as an integral whole allows the fixed information to be later recalled. This is inseparable from conscious perception and thus, past experiences are vividly re-lived in a cognitive and affective manner; and

(iii) during the operation of a dominant drive the activity of the individual is primarily focused on reaching the goal represented by this drive (rational activity), but the ability to simultaneously, consciously revive past experiences that are outside the limits of the operating dominant drive (irrational activity) is a natural endowment of our brain.

Because of the theoretically immense variability of cortical enhancer regulation, any trial to develop a compound that will reasonably stimulate learning seems to be, from a physiological point of view, a hopeless undertaking. The natural method of behavioral modification – by means of experience, training, or practice – will likely remain not only the most effective, but also the only viable way to change the performance of the cortical neurons in species capable of acquiring drives, presumably forever. *Therefore, everything depends and will in all probability always depend on teaching, learning and education.*

As the background noise in the brain is never interrupted and can even become more accentuated during sleep than in the vigilant resting state, the dream world, the classic example of a human-created universe, has always given inspiration to art. Its ultimate explanation awaits the resolution of scientific problems: the chemistry of cortical enhancer regulation and the natural law that determines the operation of "The Brain and Its Self" and is responsible for the immense variability of human activities.

Human society – the sustainability of which has always required the proper manipulation of the brain of its members – still finds itself in a state of development. It seeks its final equilibrium: namely, that state in which behavioral modification induced by the family/school/society triad will be based, from birth until death, on the exact knowledge of the neurobiological mechanisms that determine human behavior.

In this way, members of the community will understand that the simultaneity of order and chaos in their brain is the physiological reality that determines human activity and they will consciously seek to find the acquired drives that optimally fit their natural endowments.

For the time being, those lucky enough to acquire the best fitting drives in due time, in the early uphill period of life, have had fair chances for success and happiness. In contrast,

those who for any reason have missed this opportunity will remain frustrated and look for "Ersatz." It seems reasonable to conclude that order and chaos are of equal importance in our brain.

Without the ability to adapt ourselves to the concrete (science), we would not be able to survive; without the ability which allows detachment from the concrete and explorations in the infinite (art), life would not be worth living.

The existence of a variety of animal species with extremely restricted abilities to fix ICRs and acquire drives marks Nature's long road of experimentation with the brain. The end result of this process, the human brain, has been the most perfect variety.

The limitless capacity of the human cortex to fix ICRs and acquire drives allowed, in conjunction with the development of language, an unmatched interpersonal communication. This unique facility made the cognitive/volitional and affective states of consciousness of the human brain, and as a consequence of it, human social life, unparalleled. Because animals lack similar developments, there is no way to obtain direct evidence regarding the nature of their psychic experiences. Nevertheless, the observation of the goal-seeking behavior of trained monkeys, dogs, horses, dolphins and so forth furnishes convincing indirect evidence that the operation of ICRs and acquired drives is inseparable, even in animals, from an archetype of consciousness.

The historic billions who remained untouched by wartime mass killings of their innocent peers and were ready to die in the name of "God," "Fatherland," "King" and so on, illustrates the consequences of the practically unlimited capacity of the human brain to fix acquired drives. Even in the dark history of humanity, the Holocaust – the extermination of millions within a few years with unprecedented success, due to a systematically planned and executed evil mass manipulation of a whole nation (in the 20th century!) – was a unique event.

It is worth considering that after the extinction of Pithecanthropus erectus, Sinanthropus pekinensis, Roanthropus dawsoni, Homo heidelbergiensis, Homo sapiens fossilis, etc., Homo sapiens is the unique, surviving member of the human family. *Humanity is born with a brain without any knowledge as to how the real world, the human body and the mind function. In contrast, we all are born with a brain capable of creating a "non-existing world." Thus, Homo sapiens created the still operating myths-directed society. However, in order to survive, it was compelled to discover how the real world functions. By the end of the 18th century, general knowledge progressed to a critical level, and since the age of enlightenment, science and technology developed from strength to strength. Due to the undreamed speed of sciences' development, humanity rapidly approaches the final state: the rationally organized human*

society, grounded fully on scientific knowledge, first of all in understanding how the human brain, the creator of science, works. There is little doubt that the agony of the myths-directed phase of human-society's history offers daily convincing proof that termination of this era, where one hand destroys what the other hand has created, is overdue.

S*ince "All the forces in the world are not so powerful as an idea whose time has come" (Victor Hugo: "Precious stones. Small anthology from Victor Hugo's thoughts"; Drágakövek. Kis antológia, Hugo Victor gondolataiból (in Hungarian), Gutenberg, Budapest. 1930), I firmly believe that the development of science will finally terminate the myths-directed, short, first phase of human society and stabilize the ratio-directed, hopefully longer, and happier phase of human history on earth.*

Chapter 2

Development of Selegiline/(-)-Deprenyl (DEP). The Discovery that the Catecholaminergic Brain Machinery is an Enhancer-Sensitive Regulation, β-Phenylethylamine (PEA) is a Natural Enhancer and DEP is a PEA-Derived Synthetic Enhancer

Brief History of DEP

Analyzing acquired drives in the 1950s, we regularly used amphetamine (AM) or methamphetamine (MAM) known at the time to be the best cortex activators. However, amphetamine-induced incalculable aimless hypermotility, due to catecholamine-release, disturbed our measured sensitive purposeful behavior and compelled me to start in the early 1960s a structure-activity-relationship (SAR) study to get rid of this side effect of the amphetamines.

In the early 1960s, monoamine oxidase (MAO) inhibitors represented a new type of central stimulation, so it seemed to me reasonable to begin the SAR study with MAM containing a propargyl-group attached to the nitrogen. This group was known to form a covalent binding with the flavin in MAO and irreversibly block the enzyme. Out of a series of newly synthesized patentable, racemic MAM derivatives, I selected (R)-N-methyl-N-(1-phenylpropan-2-yl)prop-2-yn-1-amine (code name: E-250) as the most promising to remove the catecholamine-releasing property, responsible for hypermotility. The first paper describing

the beneficial pharmacological profile of racemic E-250 was published in 1964 in Hungarian (Knoll, Ecsery, Kelemen et al. 1964) and in 1965 in English (Knoll, Ecsery, Kelemen et al. 1965).

The (-) isomer, Selegiline/(-)-deprenyl (DEP), marketed as Eldepryl, Jumex, Zelepar, Emsam, Anipryl, and today about 100 other trade names, was the ultimately developed drug. DEP is used to treat Parkinson's disease (PD), Alzheimer's disease (AD), and major depressive disorder (MDD). Since the maintenance on a low, 1 mg/day dose of DEP slows aging related decay of the catecholaminergic brain engine, DEP is successfully prescribed to the healthy, aging population to improve the quality of life in their middle or late years (Knoll 2012; Knoll 1983; Miklya 2011, 2016.)

After more than 50 years in research and therapy, it is timely to assess the still improper evaluation of the pharmacological spectrum of DEP and the possible controversies.

The Gradual Recognition of the Complicated Pharmacological Spectrum of DEP

The first phase: Recognition that DEP is the unique MAO inhibitor free of the "cheese effect"

In 1963, a calamitous number of clinical reports (Womack, Foster, Maan, Davies) appeared in *The Lancet* concerning patients treated with MAO inhibitors (tranylcypromine, nialamide, pargyline) who developed temporary clinical symptoms (hypertension, palpitation, neck stiffness, headache, nausea, vomiting), similar to a paroxysm produced by pheochromocytoma.

Blackwell realized that these hypertensive crises are associated with the ingestion of high amounts of tyramine in cheese and MAO inhibitors impede metabolism (a.k.a. the "cheese effect") (Blackwell 1963). For me, this was a highly important, thought-provoking perception, because when Blackwell's paper was published, I was working on the manuscript of the first DEP papers (at that time we still used the original code name: E-250) and the detailed pharmacological analysis clearly indicated, already in 1965, that *DEP is the first MAO inhibitor free of the "cheese effect"*; the prima-facie experimental evidence was published three years later (Knoll, Vizi and Somogyi 1968).

As a matter of fact, I selected E-250 for further development because I discovered the unique, surprising property of the new compound in the hope of which I started the SAR study. We already confirmed on isolated organs that E-250 is free of the "cheese effect," but since I

was waiting for a preliminary confirmation of our finding in humans, we published our pharmacological study with a rather long delay (Knoll, Vizi and Somogyi 1968). We noted in our first two DEP papers (Knoll, Ecsery, Kelemen et al. 1964, 1965) that Varga described in his first short publication DEP's effectiveness in depression and also published that in agreement with our experimental results, no symptoms indicating a tyramine potentiating effect was found in his first clinical trials with E-250 (Varga 1965). We also mentioned in the discussion of our paper that clinical investigators Kardos and Füredi and also Juhász, independently confirmed Varga's finding that DEP did not provoke the "cheese effect" in healthy volunteers (Knoll, Ecsery, Kelemen et al. 1965).

Unfortunately, 1960s Hungary was isolated from the Western world's mainstream science. Our results remained largely unnoticed. I asked Ervin Varga in 1964, who worked as a psychiatrist in our University Clinic, to test the antidepressant effect of racemic E-250 and to pay special attention also to the convincing lack of the "cheese effect."

Varga published a preliminary note (in German) on the promising results of the running clinical trial with racemic E-250 on depressed patients (Varga 1965). He also wrote with his coworker papers in English, describing racemic E-250 as an efficient, prompt-acting antidepressant (Varga and Tringer 1967; Tringer, Haits and Varga 1971).

In retrospect, it is surprising that although our first papers on racemic E-250, which proposed to use the new compound as an antidepressant, appeared in 1964/1965; the first clinical studies which supported the proposal were published by Varga in Hungary between 1965-1971; the first clinical trial abroad confirmed the antidepressant effect of DEP was published in the USA (Mann and Gershon 1980) and a couple of studies corroborated the finding thereafter (Knoll 2012). Nevertheless, selegiline (DEP) with the indication to treat major depressive disorder was *only first registered in 2006* in the United States. DEP, based on a transdermal selegiline study in outpatients (Bodkin and Amsterdam 2002), was marketed as the first transdermal antidepressant: Emsam.

Ervin Varga found that in harmony with our rat experiments, DEP is also free of the "cheese effect" in humans. As cited as a personal communication in the discussion of our 1968 paper, he stated: "Even provocative cheese consumption failed to produce headache or hypertensive crisis" (Knoll, Vizi and Somogyi 1968). Varga moved to the USA in 1968 and he discontinued his clinical studies with DEP. His convincing preliminary study, which confirmed that DEP is devoid of the "cheese effect," was never completed and remains unpublished. It marks the era in Hungary in the 1960s that the discussion of the Knoll, Vizi and Somogyi (1968) paper referred to other unpublished Hungarian studies (Kardos and Füredi; Juhász)

which confirmed that DEP was devoid of the "cheese effect." None of them were completed, but later studies with DEP corroborated these observations (Knoll 2016).

Finally, Merton Sandler (1978) acceptably confirmed that DEP is a MAO inhibitor free of the "cheese effect." Sandler and his co-workers in London demonstrated that parkinsonian volunteers pretreated with DEP, who had received levodopa or levodopa+carbidopa, suffered no adverse pressor reaction after challenged with oral tyramine in considerably greater amounts than the dose likely to be encountered in a normal diet (Elsworth, Glover, Reynolds et al. 1978; Sandler, Glover, Ashford and Stern. 1978). This aligned with our findings in animal experiments and preliminary studies by Hungarian clinicians.

Because of the serious side effects of levodopa in PD, Birkmayer and Hornykiewicz attempted to achieve a levodopa-sparing effect with the coadministration of levodopa with a MAO inhibitor. As such combinations frequently elicited hypertensive attacks, they soon terminated this line of clinical research (Birkmayer and Hornykiewicz 1962). Considering the peculiar pharmacological profile of DEP, Birkmayer in Vienna was the first clinician who dared to combine DEP with levodopa in PD. The trial, the first clinical study with DEP in the West, was successful. The levodopa-sparing effect was achieved in patients without signs of significant hypertensive reactions (Birkmayer, Riederer, Ambrozi and Youdim 1977). This study initiated, and a subsequent Lancet Editorial (1982) enhanced, the world-wide use of DEP in PD.

In the early 1970s, DEP achieved its place in research and therapy as the first selective inhibitor of MAO-B (Knoll and Magyar 1972).

The second phase: Recognition that DEP is the first selective inhibitor of MAO-B

In the same year that we published the unique behavior of DEP (Knoll, Vizi and Somogyi 1968), Johnston described clorgyline, which came into world-wide use as an experimental tool in MAO research (Johnston 1968). He realized that clorgyline preferentially inhibits the deamination of serotonin (SE) and this important finding was soon confirmed by Hall, Logan and Parsons (1969). Johnston proposed the existence of two forms of MAO, "type A" and "type B," the former being selectively inhibited by clorgyline and the latter relatively insensitive to it. Johnston's nomenclature has become widely accepted and is still in use. Clorgyline remained the classic experimental tool to analyze A-type monoamine oxidase (MAO-A).

That DEP is the only MAO-inhibitor free of the "cheese effect" was exactly demonstrated by us in a study published in 1988 in which we compared all known MAO inhibitors' response to tyramine on rabbit arterial strips (Abdorubo and Knoll 1988). Not only the best known and regularly used MAO inhibitors, but also all the newly published, at that time less known, compounds were studied: *Phenylethylamine derivatives*: tranylcypromine (Maass and Nimmo 1959); pargyline (Taylor, Wykes, Gladish and Martin 1960); clorgyline (Johnson 1968); DEP (Knoll, Ecsery, Kelemen et al. 1965); TZ-650 (Knoll, Ecsery, Magyar and Satory 1978); MLD-72145 (Bey, Fozard, McDonald et al. 1984); *indane derivatives*: J-508 (Knoll, Ecsery, Magyar and Satory 1978); AGN-1135 (Finberg, Tenne and Youdim 1981); *miscellaneous compounds*: U-1424 (Knoll, Ecsery, Magyar and Satory 1978); RO-16-6491 (Kettler, Keller, Bonetti et al. 1985). *The study revealed that several specific MAO-B inhibitors (TZ-650, J-508, AGN-1135, U-1424, MDL-72165 and RO-16-6491) potentiated the responses in a dose dependent manner*, similarly to pargyline, the semi-selective B-type monoamine oxidase (MAO-B) inhibitor, or to tranylcypromine, the non-selective MAO inhibitor. ***Only DEP inhibited the response to tyramine.***

For further studies, a selective inhibitor of MAO-B was strongly needed. Fortunately, DEP proved to be the missing, selective inhibitor of MAO-B. I presented the finding in my lecture at the First International MAO Meeting in Cagliari (Sardinia) in 1971. DEP is still the classic experimental tool to analyze MAO-B. The first paper which described this novel property of DEP (Knoll and Magyar 1972) had become a Citation Classic 10 years later.

For several years, the selective MAO-B inhibitory effect of DEP was at the center of my team's interest, yet it delayed the discovery of the drug's enhancer effect. Prior to the discovery of the catecholaminergic activity enhancer (CAE) effect of DEP (Knoll 1998), *it was my firm belief that the selective inhibition of MAO-B is responsible for the drug's beneficial therapeutic effects.* In my lecture at the "Strategy of Drug Research" IUPAC/IUPHAR Symposium in Noordwijkerhout, The Netherlands, in 1982, I presented experimental evidence that preventive daily administration of DEP during the post-developmental phase of life has an unexpected possibility to improve the quality and prolong the duration of mammalian life (Knoll 1982).

Since this was the first formulation of my concept, it is quite interesting to read the summary of the lecture and consider the new experimental findings backing the concept as it occurred:

> "In the aging brain, there is a loss of neurons, compensated for by a proliferation of glial cell. We might thus predict that dopaminergic and

> ‘trace-aminergic’ modulation in the brain declines in senescence because of neuronal loss and because of the increased MAO-B activity present in the glia. The hypothesis was forwarded that the significant increase of depression in the elderly, the age-dependent decline in male sexual vigor and the frequent appearance of parkinsonian symptoms in the latter decades of life might be attributed to a decrease of dopamine (DA) and trace-amines (TAs) in the brain. The possibility to counteract these biochemical lesions of aging by chronic administration of DEP, a selective inhibitor of MAO-B, which facilitates dopaminergic and trace-aminergic activity in the brain and is a safe drug in humans, was analyzed in detail. The restitution and long-term maintenance of full-scale sexual activity in aged males continuously treated with DEP was demonstrated as an experimental model in support of the view that the long-term administration of small doses of DEP may improve the quality of life” (Knoll 1982).

This concept was based on rat studies performed with 0.25 mg/kg DEP which selectively blocked MAO-B activity in the brain. We precisely measured the aging-related decrease of sexual vigor in male CFY rats. We measured the copulatory patterns (mounting, intromission and ejaculation) of 381 3-6-month-old and 137 12-18-month-old males coupled with receptive females (scored in the light phase between 11:00 a.m. and 2:30 p.m.). We found that among the (young) 3-6-month-old rats, 5.70% were sexually inactive; 5.24% showed mountings only; 36.75% displayed mountings and intromission; 20.47% displayed ejaculation in one test only; and 31.80 % displayed full-scale sexual activity. In contrast, among the (aged) 12-18-month-old rats, 19.71% were sexually inactive; 19.71% showed mountings only; 55.47% displayed mountings and intromission; 3.65% displayed ejaculation in one test only; and 2.19% displayed full-scale sexual activity. Since aging-related decline in mating performance is due to the aging of the dopaminergic neurons, it was reasonable to measure the aphrodisiac effect of DEP treatment. I showed in this lecture for the first time the true aphrodisiac effect of the repeated administration of 0.25 mg/kg DEP.

Male CFY rats weighing 650-750 g, which showed at least one intromission without any ejaculatory patterns out of four mating untreated tests, were chosen as sexually sluggish ones for the experiment. Thereafter, we treated the rats three times a week for 10 weeks either with saline (control) or with 0.25 mg/kg DEP. Copulatory tests were performed once a week on Tuesdays. For each test a female in oestrus, showing high receptivity, brought into heat by a subcutaneous injection of 30 µg estradiol monoproprionate, followed 48 hours after by 0.5

mg progesterone, was used 4-7 hours after the progesterone injection. DEP treatment exerted a highly significant, true aphrodisiac effect (Knoll 1982).

I ended the lecture showing an experiment with U-1424, at that time a newly developed indane-derived potent selective inhibitor of MAO-B (Knoll, Ecsery, Magyar and Satory 1978). We performed with U-1424 exactly the same experiment as with DEP on sluggish, aged rats and found that this compound was ineffective in this test (Knoll 1982). I concluded that DEP exerts its aphrodisiac effect by more than one mechanism. For me, this finding was a serious warning that we needed to clarify DEP's unknown mechanism, obviously unrelated to MAO-B inhibition.

We developed (-)-1-phenyl-2-propylaminopentane (PPAP), the DEP-analog containing instead of the propargyl-group, a propyl-group attached to the nitrogen. The propyl-group is unable to covalently bind with the flavin in MAO-B rather than the propargyl-group in DEP (Figure 2.1 and Table 2.1). Thus, PPAP leaves MAO-B activity unchanged, however, as a central stimulant PPAP proved to be as potent stimulant of the catecholaminergic neurons as DEP (Knoll, Knoll, Török et al. 1992).

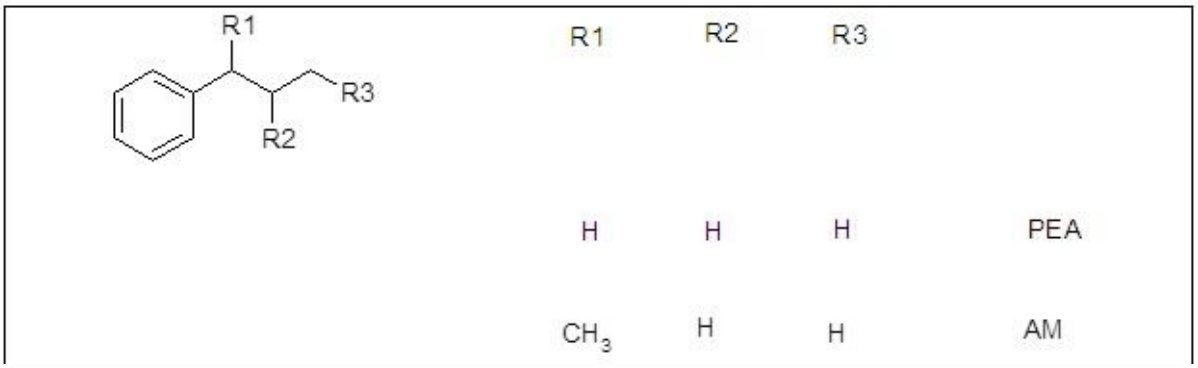


Table 2.1 Essential differences in the pharmacological spectrum of PEA and amphetamines versus DEP and PPAP

Name	Enhancer effect	Releasing effect	Relation to MAO
PEA	+	+	MAO-B substrate
AM	+	+	Week MAO inhibitor
MAM	+	+	Week MAO inhibitor
DEP	+	0	Selective MAO-B inhibitor
PPAP	+	0	0

This finding accelerated the discovery of the enhancer-regulation in the mammalian brain (Knoll 1994; Knoll and Miklya 1994) and the realization that: i) PEA is a natural CAE substance (Knoll, Miklya, Knoll et al. 1996a); ii) DEP is a PEA-derived synthetic CAE substance (Knoll, Miklya, Knoll et al. 1996b); and iii) tryptamine is a natural enhancer substance (Knoll 1994) which catalyzed the SAR study resulting in the development of (2R)-1-(1-benzofuran-2-yl)-N-propylpentane-2-amine (BPAP), the tryptamine-derived, presently known most potent synthetic enhancer substance (Knoll, Yoneda, Knoll et al. 1999).

Since DEP as a drug (selegiline) is classified in all textbooks only as the prototype of the selective inhibitor of MAO-B, it is still the universal belief among clinicians that selective inhibition of MAO-B in the brain is fully responsible for selegiline-treatment induced therapeutic benefits. This view is inconsistent with the already proven, primary important CAE effect of DEP and this is the controversy which still deserves careful consideration.

The third phase: Recognition that DEP is a PEA-derived synthetic enhancer substance

The high-pressure liquid chromatography (HPLC) method with electrochemical detection allows exact measurement of the continuously released catecholamines and SE from freshly excised brain tissue. This method ensured our obtaining experimental evidence regarding the operation of the enhancer regulation in the life-important catecholaminergic and serotonergic systems in the rat brain. We selected the catecholaminergic and serotonergic neurons as models to study the characteristics of the enhancer-sensitive brain regulations. In 1993, we began to use this technique to measure the amount of DA released from the striatum, substantia nigra and tuberculum olfactorium, as well as norepinephrine (NE) from the locus coeruleus and SE from the raphe. We had previously measured the nerve-stimulation induced release of [3H]-norepinephrine, [3H]-dopamine, and [3H]-serotonin, respectively, from the isolated brain stem of rats (Knoll, Miklya, Knoll et al. 1996a).

In 1994, we presented the results from the first series of experiments performed with the HPLC method which demonstrated that multiple, small dose (0.1 mg/kg) administration of DEP keeps the catecholaminergic, but not the serotonergic, neurons on a significantly higher activity level and demonstrated that DEP's peculiar enhancer effect is unrelated to MAO-B inhibition (Knoll and Miklya 1994).

To measure the biogenic amines released from freshly excised brain tissue by means of HPCL with electrochemical detection we treated rats of both sexes injecting them subcutaneously, daily for 21 days, either with saline or with a dose of one of enantiomers of the

selected compounds. We measured the effect of DEP and PPAP, the DEP-analog free of MAO-B inhibitory potency (Knoll, Knoll, Török et al. 1992). MAM is the parent compound of DEP and AM is the parent compound of PPAP.

The effect of DEP's two enantiomers were studied in five doses (0.01, 0.025, 0.05, 0.1 and 0.25 mg/kg), that of PPAP in two doses (0.05 and 0.1 mg/kg), others in 0.05 mg/kg. Treatment with DEP enhanced the release of DA from the striatum, substantia nigra and tuberculum olfactorium (significant in 0.05-0.25 mg/kg), while the release of SE from the raphe was diminished (significant in 0.05-0.25 mg/kg in males and 0.25 mg/kg in females). (+)-DEP was slightly less potent than (-)-DEP. PPAP acted like DEP; the (+)-enantiomer was more active than the (-)-enantiomer; (-)-MAM was as potent as (-)-DEP in enhancing catecholaminergic activity and more potent than (-)-DEP in diminishing serotonergic activity.

Since three of the five doses (0.01, 0.025, 0.05 mg/kg) are below the MAO-B inhibitory dose, the clear conclusion of this study was that DEP is enhancing with a hitherto unknown mechanism, already in very low doses, the activity of catecholaminergic neurons in the brain and the enhancer effect is unrelated the MAO inhibition. The expected success of this study confirmed my theory that the exploration of the unknown world of the enhancer-sensitive brain regulations is greatly promising in counteracting the regressive effects of brain aging which has always been the main practical aim of my research.

Since we use the HPLC method to measure the amount of catecholamines and SE released within 20 minutes from the freshly isolated, discrete brain regions, we measure the surviving spontaneously active neurons. Treating the rats with a proper low dose of DEP, we exactly measure the synthetic enhancer-treatment induced increase in the number of spontaneously active neurons. For example, whereas from the isolated striatum of male rats treated subcutaneously with 0.3 ml saline/100g daily measured 24 hours after the last injection the amount of released DA was 2.72±0.10 nmoles/g tissue, from rats treated with 0.05 mg/kg DEP the amount of DA released from the striatum increased to 4.42±0.09 nmoles/g. Thus, we measured the essence of the enhancer effect, the DEP-treatment induced transformation of silent neurons into spontaneously working neurons (Knoll 2016).

We investigated MAM's enhancer effect because it is like DEP, a synthetic PEA-derived enhancer substance with the same pharmacological spectrum as its parent compound, and PEA, a natural enhancer of the catecholaminergic neurons and in higher concentrations a potent releaser of catecholamines.

We measured the effect of DEP, free of the catecholamine-releasing property, and found that in higher concentrations it was a potent selective inhibitor of MAO-B. We also measured

the enhancer effect of PPAP, which acts like DEP, but leaves MAO-B activity unchanged (Knoll, Knoll, Török et al. 1992).

Like PEA, the synthetic PEA-derivatives are in low concentration potent CAE substances. As measured 24 hours after the last injection, a three-week daily treatment of male and female rats with 0.01 mg/kg DEP, or 0.1 mg/kg PPAP, or 0.05 mg/kg methamphetamine, kept the catecholaminergic neurons working on a significantly higher activity level, but they did not enhance the activity of the serotonergic neurons.

PEA, as an endogenous trace-amine (TA) in the brain was discovered by Fischer et al. and soon corroborated (Fischer, Spatz, Heller and Reggiani 1972; Saavedra 1974; Wilner, LeFevre and Costa 1974). Fischer, Heller and Miró (1968) claimed that urinary excretion of free PEA is reduced in depressed patients and suggested the hypothesis that a PEA-deficit is one of the biochemical lesions of depression. Sabelli and his coworkers hypothesized that PEA might play a role in affective behavior (Sabelli and Giardina 1973; Sabelli and Mosnaim 1974). Since Borowsky and his team identified a family of mammalian G-protein-coupled receptors and found that these receptors are activated by PEA and tryptophan (Borowsky, Adham, Jones et al. 2001), thus already identified natural enhancers (Knoll 2005). Because of PEA's effects, it was and has remained common knowledge that the TA is an indirectly acting sympathomimetic agent which displaces catecholaminergic transmitters from their storage sites. The enhancer regulation's discovery revealed that PEA is a CAE substance in low concentrations and releases catecholamines from their storage sites in higher concentrations (Knoll, Miklya, Knoll et al. 1996b). The releasing effect of PEA is detectable on isolated organ preparations with catecholaminergic innervation. A capacitance vessel preparation, the helical strip of the pulmonary artery of the rabbit, is particularly well suited to measure the displacement of NE molecules from their storage site by PEA and the CAE effect is undetectable (Knoll 2016). On this preparation, the cumulative administration of 1.4 μg/ml PEA in three consecutive small doses (0.2, 0.4 and 0.8 μg/ml) neither increased stimulation induced contractions (lack of CAE effect), nor smooth muscle tone (lack of release of NE). Following an additional 6 μg/ml PEA, there was a dose-related increase in the smooth muscle tone in the resting state due to the stoichiometric displacement of NE by PEA, but there was no sign of a CAE effect. The electric stimulation induced contractions remarkably decreased when the highest concentration was given.

Desmethylimipramine (DMI) (5 μg/ml), a tricyclic antidepressant, completely prevents the NE displacing effect of PEA by inhibiting the neuronal transport of this amine. Also, PPAP, the PEA-derivative with a potent CAE activity that is devoid of the NE displacing property, is

an efficient inhibitor of PEA's effect (ID50 = 1.22 x 10-6 M). The antagonism is due to competition for the neuronal transport system (Knoll, Knoll, Török et al. 1992).

The rabbit's perfused ear artery, a resistance artery preparation, is an exceptional sample of a vascular smooth muscle suitable for investigating the dual effect of PEA on catecholaminergic transmission outside of the brain (Knoll, Miklya, Knoll et al. 1996a).

There are two mechanisms which lead to the emission of high amounts of NE from the end organs in the preparation: NE's release via the exocytosis of vesicles in response to electric stimulation and NE's continuous outflow via the transmitter's displacement by an indirectly acting sympathomimetic agent, like for example PEA, which has to be measured in the resting state when exocytosis of vesicles does not occur. This dual effect of PEA is measurable on the perfused central ear artery of a rabbit.

The dose-related CAE effect of PEA to electric stimulation, with unchanged smooth muscle tone, is detectable in the low concentration range (0.2-0.8 μg/ml). Much higher concentrations of PEA 4-6 μg/ml were needed for displacing NE. DMI (5 μg/ml) prevented PEA induced displacement of NE, whereas the CAE effect remained detectable in the presence of DMI (Knoll 2016). Since the CAE effect is exactly measurable in the brain, we use the isolated rat's brain stem to measure sympathomimetic amines' CAE effect. AM and MAM, the PEA-derivatives not metabolized by MAO are, like PEA, CAE substances and in substantially higher concentrations they release catecholamines from their storage sites. The releasing effect of PEA concealed their CAE effect which remained undetected. DEP's development, the first PEA-derivative devoid of the catecholamine releasing property made the detection of PEA and amphetamines' CAE effect possible (Knoll 2016).

The Main Effect of DEP is the Enhancer Effect

It is unquestionable that since the early 1960s DEP's story would be full of surprises. DEP research catalyzed us to the discovery of the enhancer regulation in the mammalian brain, to the realization that the catecholaminergic and serotonergic neurons are enhancer-sensitive units and to the development of BPAP.

The discovery of the enhancer regulation in the mammalian brain and the development of synthetic enhancer substances were recently summarized (Knoll 2016). A recent study presents evidence that the enhancer effect of DEP and BPAP are responsible for the prolongation of mammalian life (Knoll and Miklya 2016). Rats treated three times a week with 0.0001 mg/kg BPAP, which is the peak dose exerting its specific enhancer effect, significantly

prolonged the life of rats. Figure 1 in this study also shows that the 0.25 mg/kg dose of DEP, used from the beginning in the longevity studies (Knoll 1988) has two effects: it is the peak dose which completely blocks MAO-B in the brain and is also the peak dose which exerts the non-specific enhancer effect of DEP.

Since the presently used 10 mg daily dose of DEP in therapy was originally selected as the one equivalent with the dose used in animals, it remains for the future to clarify the role of the non-specific enhancer effect of DEP in the therapeutic benefits observed in the last decades.

It is undoubtable that the main effect of DEP is the enhancer effect. As already quoted, 0.25 mg/kg DEP is the peak concentration eliciting in rats both MAO-B inhibition and the non-specific enhancer effect (Knoll and Miklya 2016). Since we established at the very beginning of the planned clinical trials with DEP that the 0.25 mg/kg dose of DEP, which selectively and completely blocks MAO-B activity in the rat brain, is equivalent with 10 mg/day DEP in humans, this is still the regularly used daily therapeutic dose of DEP. Considering DEP's already exactly verified pharmacological profile it is obvious that DEP exerts the same two effects in humans as in rats.

As a matter of fact, it was the "Deprenyl And Tocopherol Antioxidant Therapy Of Parkinsonism" (DATATOP) study's results that DEP has a beneficial influence on the natural history of PD, which clearly proved, in light of the discovery of the CAE effect, that only the enhancer effect of DEP can be responsible for this unexpected, unknown and unique benefit. Tetrud and Langston published in Science in 1989 their finding that DEP-treatment delayed the need for levodopa therapy. They found that the average time until levodopa was needed was 312.1 days in the placebo group and 548.9 days for patients in the DEP group (Tetrud and Langston 1989). This finding was confirmed by the Parkinson Study Group (PSG) in 1989 in an important publication (PSG 1989).

The original title of the first Science paper, the finding that DEP treatment is changing the natural history of PD, was soon further confirmed by important multicenter studies, such as the French Selegiline Multicenter Trial (FSMT) (Allain, Gougnard and Naukirek 1991), the Finnish Study (Myttyla, Sotaniemi, Vourinen and Heinonen 1992), the Swedish PSG study (Pålhagen, Heinonen, Hägglund et al. 1998) and the Norwegian-Danish Study Group (Larsen, Boas and Erdal 1999).

When the DATATOP study was planned, DEP's enhancer effect was unknown, so the organizers' hypothesis was that the activity of MAO and the formation of free radicals predispose patients to nigral degeneration and contribute to the emergence and progression of PD. In accordance with their working hypothesis, they expected that DEP, the MAO inhibitor,

α-tocopherol, the antioxidant, and the combination of the two compounds would slow the disease's clinical progression.

They selected patients with early, untreated PD and measured the delay in the onset of disability necessitating levodopa therapy. In the first phase of the trial, 401 subjects were assigned to α-tocopherol or placebo and 399 subjects were assigned to DEP, alone or with α-tocopherol. Only 97 subjects who received DEP reached the "end" of the trial (i.e., the onset of disability necessitating levodopa therapy) during an average 12 months of follow-up compared with 176 subjects who did not receive DEP. The risk of reaching the end of the trial was reduced by 57% for patients who received DEP and these patients also had a significant reduction in their risk of having to give up full-time employment (PSG 1989). Following the course of changes, the authors concluded in their next paper that DEP, but not α-tocopherol, delayed the onset of disability associated with early, otherwise untreated PD (PSG 1993). But over time, the DATATOP study also revealed that DEP did not reduce the occurrence of subsequent levodopa-associated adverse effects in patients. This fact needs serious consideration (PSG 1996).

Idiosyncratic prescribing of DEP in combination with levodopa already led to a false conclusion (Knoll 2012). Due to the inhibition of MAO-B, DEP-treatment allows for a 20-50% decrease in levodopa dose needed in PD. In patients who need levodopa, however, there is always a risk that the administration of DEP will enhance the side effects of levodopa which can only be avoided by properly decreasing the levodopa dose according to the individual sensitivity of the patient.

An example of a multicenter clinical trial with improper combination of levodopa which led to confusion and misinterpretation, was the one performed by the PD Research Group in the United Kingdom (PDRG-UK) (Lees 1995). Quite unexpectedly, this group published an alarming paper claiming that parkinsonian patients treated with levodopa combined with DEP show an increased mortality in comparison with the patients treated with levodopa alone. This finding was in striking contradiction to all other studies published in a variety of countries. Comments uniformly pointed to substantial overdosing of levodopa (Dobbs, Dobbs and Charlett 1996; Knoll 1996; Olanow, Godbold and Koller 1996).

The outcome of the DATATOP study, the finding that DEP delayed the need for levodopa therapy, but α-tocopherol fell short of expectation, clearly proved that DEP exerts an unknown pharmacological effect of basic importance and α-tocopherol is devoid of this effect. Now we know that DEP as a CAE substance is an enhancer of the impulse propagation mediated

release of catecholamines. A comparative pharmacological analysis of DEP and α-tocopherol proved that α-tocopherol is devoid of the enhancer effect (Miklya, Knoll and Knoll 2003). Since 0.25 mg/kg DEP selectively blocks MAO in the brain and also exerts in the same dose the non-specific enhancer effect (Knoll and Miklya 2016), it is obvious that DEP's CAE effect was responsible for the delayed levodopa need (Knoll 2102).

This conclusion was also supported by the clinical trial with rasagiline, performed by the PSG. The trial revealed that unlike the early selegiline trials, rasagiline failed to demonstrate a decreased need for levodopa (PSG 2002). Even the results of additional studies (Olanow and Rascol 2010; Ahlskog and Uitti 2010) led to the conclusion that "based on current evidence, rasagiline cannot be said to definitely have a disease-modifying effect" (Robottom 2011). Similar to α-tocopherol, neither lazabemide nor rasagiline, the two selective MAO-B inhibitors used in PD, are also devoid of the CAE effect of DEP (Miklya 2014).

Since the mid-1980s, further analysis of the characteristic enhancement of the catecholaminergic brain machinery in DEP-treated rats rendered probable that this effect is unrelated to the selective inhibition of MAO-B. The development of PPAP, the DEP-analog devoid of a MAO inhibitory property, and an equally active stimulant of the catecholaminergic neurons as DEP, verified this suggestion (Knoll 1992). The first study which demonstrated that multiple, low dose administration of DEP enhances catecholaminergic activity in the brain and this effect is unrelated to MAO-B inhibition allowed for the discovery of the enhancer sensitive brain regulations (Knoll and Miklya 1994). PEA and its best-known synthetic derivatives (AM and MAM) are strong releasers of catecholamines from their plasmatic pools. Since the catecholamine releasing effect conceals the detectability of the enhancer-sensitive nature of the catecholaminergic neurons (Knoll 2016), DEP's primary physiological function as a natural enhancer substance, as well as the fact that AM and MAM are, like DEP, PEA-derived synthetic enhancer substances, remains unknown.

The later realization that tryptamine is like PEA a natural enhancer (Knoll 1994), signaled the elaboration of BPAP as the most selective and potent synthetic enhancer substance currently known (Knoll, Yoneda, Knoll et al. 1999).

Chapter 3

(2*R*)-(-)-1-(1-Benzofurane -2-yl)-*N*-Propylamino – pentane - 2 – amine (BPAP), the tryptamine derived synthetic enhancer substance

Development of a tryptamine derived synthetic enhancer substance

β-Phenylethylamine (PEA), the short acting trace-amine (TA) in the mammalian brain, as well as amphetamines, PEA's long-acting synthetic analogues and releasers of catecholamines from the plasmatic pools, are classified as indirectly acting sympathomimetics. Due to its unique pharmacological spectrum, selegiline/(-)-deprenyl (DEP) rendered it possible to discover that:

> *the catecholaminergic neurons belong to the enhancer-sensitive brain regulations;*
>
> *PEA is a natural enhancer of catecholaminergic neurons;*
>
> *amphetamines are PEA-derived synthetic enhancers and, like PEA, their parent compound, primarily catecholaminergic activity enhancer (CAE) substances;*
>
> *and they release catecholamines from the plasmatic pools only in high concentration.*

Since the release of catecholamines from the plasmatic pools concealed PEA's CAE effect, the enhancer-sensitivity of the catecholaminergic neurons remained undetected until the development of DEP, still the only PEA-derivative free of the catecholamine-releasing property (Knoll 2016). Thus, because of DEP, it was an unexpected possibility to discover the first enhancer-sensitive brain-regulation in the mammalian brain.

The discovery in 1994 that tryptamine (TRY) is like PEA, a natural enhancer of the impulse propagation mediated release of catecholamines and serotonin the brain, initiated the structure-activity-relationship (SAR) study aiming to develop a new family of synthetic enhancer compounds, unrelated to PEA and amphetamines (Knoll 1994). We synthesized 66 TRY-derived patentable new compounds, tested their ability to enhance the nerve stimulation-induced release of norepinephrine (NE), dopamine (DA) and serotonin (SE), respectively, and BPAP was selected after DEP as the second synthetic enhancer substance for the analysis of the enhancer regulation in the mammalian brain (Knoll, Yoneda, Knoll et al. 1999).

Figure 3.1 shows the chemical structure and pharmacological spectrum of TRY, (-)-1-(indol-3-yl)-2-propylamino-pentane (IPAP), the simplest TRY-derived, synthetic enhancer substance; and BPAP, the eventually selected compound for development.

There is a remarkable quantitative difference between PEA and TRY in their effectiveness on serotonergic neurons. A lower concentration of TRY (1.3 µmol/l) proved much more potent in enhancing the stimulation-evoked release of serotonin than a much higher concentration of PEA (16 µmol/l). This indicates that, on a molecular level, the enhancer regulation in the catecholaminergic and serotonergic neurons are not identical (Knoll 2005).

Figure 3.1

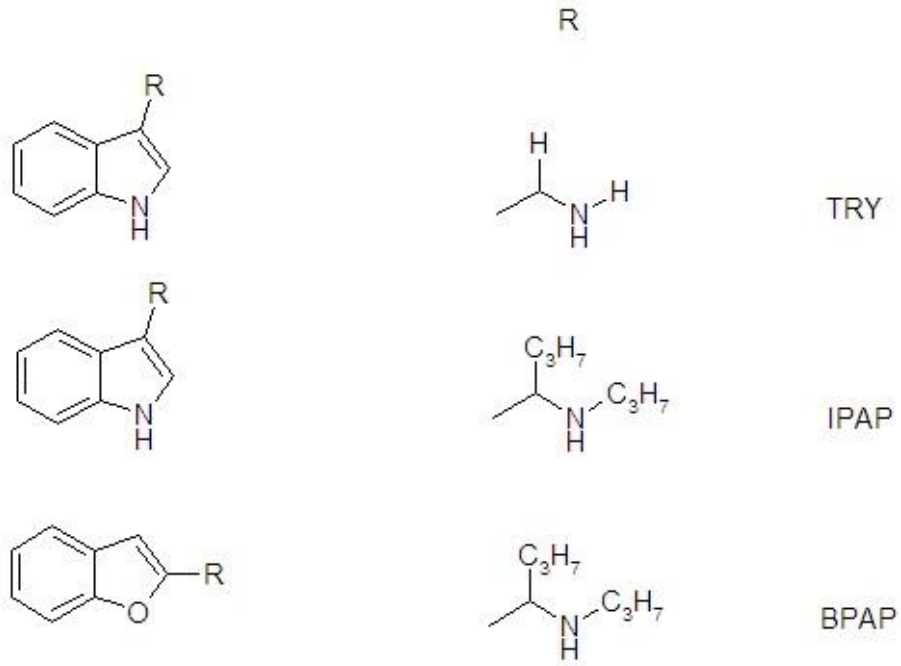


Figure 3.1 Schematic chemical structure of tryptamine (TRY), the natural enhancer substance of the serotonergic neurons; IPAP, the simplest TRY-derived synthetic enhancer substance; and BPAP, most potent TRY-derived synthetic enhancer substance (Knoll et. al. 1999)

Table 3.1 Pharmacological spectrum of TRY, IPAP and BPAP

Name	Enhancer effect	Releasing effect	Relation to MAO
TRY	+	0	MAO-A substrate
IPAP	+	0	Week MAO-A inhibitor
BPAP	+	0	Week MAO-A inhibitor

The bell-shaped concentration effect curve highly characteristic of the enhancer effect in both DEP and BPAP

Figure 3.2

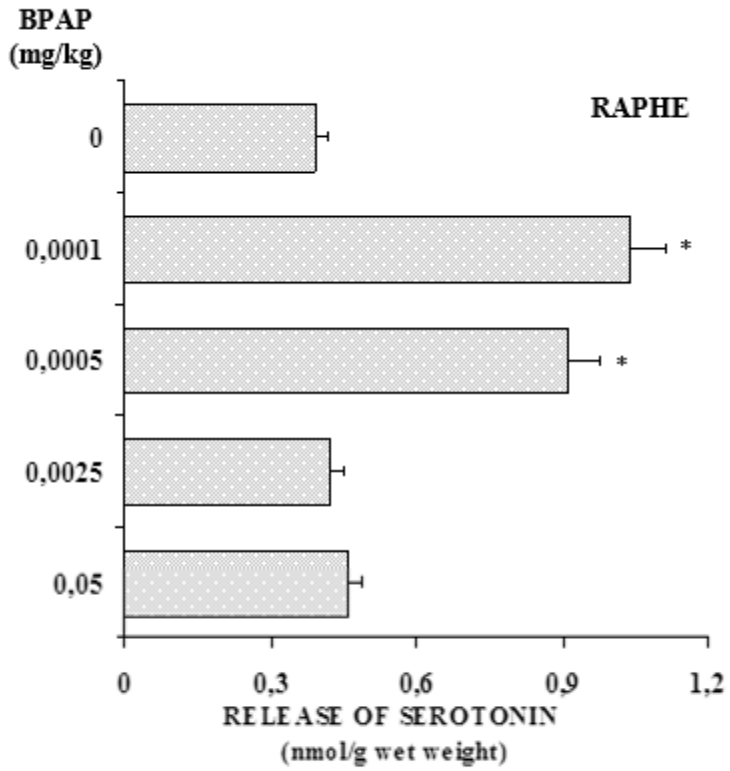


Figure 3.2 Illustration of the significant enhancement of the outflow of serotonin (SE) from the raphe of rats isolated 30 min after the subcutaneous administration of a single dose of BPAP. The amount of SE released from the tissue within 20 min following the administration of different doses of BPAP was measured according to Knoll and Miklya (1995). Paired Student's t-test. *P<0.01.

Figure 3.2 exemplifies that BPAP enhances the outflow of SE from the raphe of rats in a subcutaneous dose of 0.0001 mg/kg. Thus, BPAP is an extraordinarily potent enhancer of the serotonergic neurons, in contrast to DEP, which is practically ineffective on the serotonergic neurons. BPAP has a preference toward the serotonergic neurons and is a less potent enhancer of the catecholaminergic neurons, but is even (as a CAE substance) much more potent than DEP.

Note that Figure 3.2 shows the peculiar dose-dependency characteristic of the specific enhancer effect of BPAP: 0.0001 mg/kg is the peak dose; 0.0025 mg/kg is ineffective. This special behavior of the enhancer substances is so unique that it cannot be overstated. It is quite helpful in the identification of an unknown enhancer-sensitive brain regulation.

As an early example to illustrate the peculiarities of the enhancer effect of BPAP, I recall that BPAP exerted on cultured rat hippocampal neurons its specific enhancer effect with a peak at 10^{-14} M and its non-specific enhancer effect with a peak at 10^{-5} M (Knoll, Yoneda, Knoll et

al. 1999). BPAP exerted on the isolated locus coeruleus its specific enhancer effect with a peak at 10^{-13} M concentration and its non-specific enhancer effect with a peak at 10^{-6} M concentration (Knoll, Miklya and Knoll 2002). On learning performance, measured in the shuttle box on rats, 0.0001 mg/kg BPAP was the peak dose with the specific enhancer effect and 0.05 mg/kg BPAP was the peak dose with the non-specific enhancer effect (Knoll and Miklya 2016).

To date, BPAP is the most selective and potent experimental tool to investigate the enhancer regulation in the catecholaminergic and serotonergic neurons of the brain stem. The enhancer effect of BPAP can be uniformly detected following the subcutaneous administration of a single dose or a three-week treatment (Table 3.2), as well as following the addition of BPAP into the organ bath of freshly isolated discrete brain areas (Table 3.3). The data in Table 3.3 confirm that the peak concentration of BPAP's specific enhancer effect is at 10^{-13} M.

Table 3.2 Release of catecholamines and SE from selected discrete brain regions of male rats treated with BPAP

Amount of biogenic amine (nmol/g wet weight) released from the tissue within 20 min						
Series of experiments	**Dose mg/kg**	**Striatum**	**Substantia nigra**	**Tuberculum olfactorium**	**Locus coeruleus**	**Raphe**
		dopamine			***norepinephrine***	***serotonin***
Single dose treatment. Measurement 30 min after the sc. injection of the compound						
Saline	-	4.5±0.15	6.8±0.18	4.9±0.15	4.7±0.10	0.391±0.02
BPAP	0.0001	4.7±0.14	14.8±0.36****	7.2±0.23****	6.6±0.10***	1.040±0.03***
	0.0005	4.8±0.16	13.8±0.23****	6.7±0.08****	15.4±0.55****	0.914±0.03***
	0.0025	5.7±0.19***	13.1±0.21****	6.9±0.31****	3.9±0.05**	0.421±0.03
	0.0500	6.5±0.09****	10.9±0.11****	7.7±0.19****	4.3±0.25	0.457±0.01
Treatment for three weeks, once daily. Measurement 24 hours after the last sc. injection						
Saline	-	3.8±0.18	5.8±0.18	4.4±0.24	3.9±0.10	0.403±0.01
BPAP	0.0001	4.4±0.12*	8.8±0.28****	4.6±0.17	7.4±0.15***	0.870±0.02***
	0.0005	3.9±0.18	8.3±0.23	4.2±0.23	4.1±0.05	1.907±0.04****
	0.0025	5.5±0.05****	8.7±0.34****	6.0±0.30***	3.7±0.1	0.212±0.03***
	0.0500	4.8±0.12***	9.4±0.13****	6.3±0.09****	4.1±0.40	0.136±0.01***

*P<0.05 **P<0.02 ***P<0.01 ****P<0.001

Table 3.3 *In vitro* effect of BPAP on the release of catecholamines and SE from selected discrete brain regions of male rats						
Amount of biogenic amine (nmol/g wet weight) released from the tissue within 20 min						
Series of experiments	**Concentration (M)**	**Striatum**	**Substantia nigra**	**Tuberculum olfactorium**	**Locus coeruleus**	**Raphe**
		dopamine			***norepinephrine***	***serotonin***
Saline	-	3.0±0.06	6.1±0.32	3.2±0.07	4.0±0.15	0.364±0.01
BPAP	10^{-4}	3.4±0.19*	7.6±0.29**	4.1±0.26**	4.5±0.50	0.473±0.02
	10^{-8}	3.9±0.25**	9.9±0.35****	4.6±0.11****	5.4±0.05**	0.547±0.03***
	10^{-12}	4.3±0.19***	9.7±0.21****	4.4±0.10****	5.5±0.05**	1.716±0.02****
Saline	-	3.4±0.08	5.5±0.05	3.6±0.11	4.2±0.15	0.359±0.01
BPAP	10^{-5}	3.8±0.17	9.0±0.19****	4.6±0.20***	5.0±0.35	0.501±0.01**
	10^{-9}	4.9±0.13***	8.2±0.19****	5.1±0.13****	4.7±0.05	0.878±0.05***
	10^{-14}	4.4±0.23**	9.5±0.30****	5.0±0.27***	7.4±0.25***	0.454±0.05
Saline	-	3.0±0.22	6.1±0.29	3.3±0.22	4.4±0.15	0.351±0.03
BPAP	10^{-6}	3.7±0.19	9.5±0.17****	3.8±0.11	9.3±0.20***	0.910±0.03***
	10^{-10}	5.0±0.13***	9.4±0.25****	5.0±0.22***	4.6±0.25	1.462±0.07***
	10^{-13}	5.9±0.28***	15.1±0.38****	6.1±0.22****	8.2±0.35***	0.913±0.02***

*P<0.05 **P<0.02 *P<0.01 ****P<0.001

Considering the peculiar history of DEP's development, first as the unique MAO-inhibitor free of the cheese-effect, then as the first selective inhibitor of MAO-B, it is not random that the bi-modal, bell-shaped concentration effect curve, characteristic to DEP's enhancer effect has special practical consequences and forces clinicians to re-evaluate DEP's 50-year clinical history. As shown in our recent paper, 0.25 mg/kg is the peak dose of DEP with the non-specific enhancer effect and this is also the peak dose which selectively blocks MAO-B activity in the brain (Knoll and Miklya 2016). This means that the therapeutically used 10 mg/day DEP dose has two effects: it blocks MAO-B activity in the brain and exerts its non-specific enhancer effect, the scope of which still needs identification.

As was shown in Chapter 2, the unexpected outcome of the "Deprenyl And Tocopherol Antioxidant Therapy Of Parkinsonism" (DATATOP) study – the finding that DEP delayed the need for levodopa therapy, but α-tocopherol fell short of expectation – clearly proved that DEP exerts an unknown pharmacological effect of basic importance and α-tocopherol is devoid of this effect. Now we know that DEP, as a CAE substance, is an enhancer of the impulse propagation mediated release of catecholamines. A comparative pharmacological analysis of DEP and α-tocopherol proved that α-tocopherol is devoid of the enhancer effect (Miklya, Knoll and Knoll 2003). Since 0.25 mg/kg DEP selectively blocks MAO in the brain and exerts in the same dose also the non-specific enhancer effect, it is obvious that DEP's CAE effect was responsible for the delayed levodopa need (Knoll and Miklya 2016, Figure1). This conclusion was also supported by the clinical trial with rasagiline, performed by the PSG (2002). The trial revealed that unlike the early selegiline trials, rasagiline failed to demonstrate a decreased need for levodopa. It was shown that rasagiline, like α-tocopherol, is also devoid of the CAE effect of DEP (Miklya 2014).

Since the mid-1980s, further analysis of the characteristic enhancement of the catecholaminergic brain machinery in DEP-treated rats rendered probable that this effect is unrelated to MAO-B's selective inhibition. The development of (-)-1-phenyl-2-propylaminopentane (PPAP), the DEP-analog devoid of a MAO inhibitory property, and an equally active stimulant of the catecholaminergic neurons as DEP, verified this claim (Knoll, Knoll, Török et al. 1992).

The first study which demonstrated that multiple, low dose administration of DEP enhances catecholaminergic activity in the brain and this effect is unrelated to MAO-B inhibition called for the discovery of the enhancer sensitive brain regulations (Knoll and Miklya 1994). PEA and its best-known synthetic derivatives amphetamine (AM) and methamphetamine (MAM) are strong releasers of catecholamines from their plasmatic pools. Since the catecholamine releasing effect conceals the detectability of the enhancer-sensitive nature of the catecholaminergic neurons (Knoll 2016), PEA's primary physiological function as a natural enhancer substance, as well as the fact that AM and MAM are, like DEP, PEA-derived synthetic enhancer substances, remained unknown for some time.

The later realization that TRY is like PEA a natural enhancer (Knoll 1994), signaled the elaboration of BPAP. DEP is primarily a CAE substance and is a weak enhancer of serotonergic neurons. BPAP, as a TRY-derivative, is a highly potent enhancer of serotonergic neurons, but even as a CAE substance, it is significantly more potent than DEP. The catecholaminergic and

serotonergic neurons were studied as the first models of the enhancer-sensitive brain regulations (Knoll 2001, 2003, 2005). The fact that a bi-modal, bell-shaped concentration effect curve is characteristic of the enhancer substances was noted during our first experiments when we realized DEP's CAE effect. The exact analysis of the enhancer effect of BPAP, the selective and presently most potent enhancer substance, brought the distinction of the *"specific"* and *"non-specific"* enhancer effect to perfection. A comparison, for example, of the concentration effect curves of DEP (Knoll and Miklya 2016) and BPAP (Knoll and Miklya 2016) in our recent longevity study shows that the enhancer effect is responsible for the extension of lifespan and it clearly demonstrates that DEP is a less potent enhancer than BPAP. The peak dose of DEP exerting the specific enhancer effect was lower and the characteristic bell-shaped manner of the dose-effect curve was less pronounced as for BPAP.

A careful comparative analysis of the in vivo effectiveness of DEP's and BPAP's enhancer effect on the catecholaminergic and serotonergic neurons proved that DEP was in all tests a substantially less potent synthetic enhancer than BPAP (Knoll 2005).

The bi-polar, bell-shaped nature of the enhancer effect was also confirmed on cultured rat hippocampal neurons (Knoll, Yoneda, Knoll et al. 1999) and precisely analyzed on isolated locus coeruleus of rats (Knoll, Miklya and Knoll 2002). In these tests, BPAP enhanced the activity of the noradrenergic neurons in the femto/picomolar concentration range with a peak at 10^{-13} M ("specific" enhancer effect) and also in a 10 million times higher concentration range with a peak at 10^{-6} M ("non-specific" enhancer effect).

BPAP reverses tetrabenazine (TBZ) induced decrease of [^{3}H]-dopamine release from rat's striatum. A study of the molecular mechanism of the enhancer effect of BPAP

The dopaminergic (DA) machinery is the most rapidly aging neuronal system in our brain. The DA content of the human caudate nucleus decreases steeply, at a rate of 13% per decade over age 45. We know that symptoms of Parkinson's disease (PD) appear if the DA content of the caudate drops below 30% of the normal level. Experimental and clinical experiences show that daily dosages of DEP keep the brain engine's activity on a higher activity level in humans. From sexual maturity, a low daily dose of DEP (1 mg) is sufficient to significantly slow the pace of the aging-related decay of DA neurons. Even if we assume only a small protective effect of DEP in healthy humans against the age-related decrease in striatal DA, for example from 13% per decade to 10% per decade, this translates to a minimum 15-

year extension in an average lifespan and a considerable increase in the human technical lifespan (TLS_h), which is now estimated to be around 115 years (Knoll 1992).

Regarding the molecular mechanism of synthetic enhancer substances it was our first crucially important observation that both DEP and BPAP, in the peak dose which exerts their specific enhancer effect (0.001 mg/kg DEP and 0.0001 mg/kg BPAP, respectively), fully restored the learning ability of rats in the shuttle box when treated with 1 mg/kg tetrabenazine (TBZ) (Knoll, Yoneda, Knoll et al. 1999; Knoll, Miklya and Knoll 2002). TBZ-treatment reversibly blocks the vesicular monoamine transporter 2 (VMAT2) and within one hour depletes at least 90% of NE and DA from their transmitter-stores in the nerve terminals of the catecholaminergic neurons in the brain stem (Scherman, Jaudon and Henry 1983).

For analysis we used [^{3}H]-dopamine (dihydroxyphenylethylamine-3,4[^{3}H], specific activity: 28.0 Ci/mmol) and TBZ. We worked with male Wistar rats on a 12-hour light/dark cycle (6.00 a.m. on, 6.00 p.m. off) with food and water available ad libitum, housed five to a cage, in a temperature- and humidity-controlled animal facility. Rats were treated with TBZ (1 mg/kg sc.), BPAP (0.0001 mg/kg sc.) or with their combination 60 minutes prior to decapitation and the release of [^{3}H]-dopamine from striatal slices was determined. Control rats were injected sc. with saline.

Rats were decapitated by guillotine; the brains were removed and the striatum was dissected and sliced according to the Glowinski and Iversen method (Glowinsky and Iversen 1966). Striatal slices were collected and immersed in oxygenated (O_2 95%, CO_2 5%) Krebs-bicarbonate buffer at room temperature.

Rat striatal slices were loaded with [^{3}H]-dopamine (10 µCi) for 30 minutes in 1.5 ml aerated (O_2 95%, CO_2 5%, pH 7.4) and preheated (37 °C) Krebs-bicarbonate buffer (Harsing, Sershen and Lajtha 1992). After loading the tissues with [^{3}H]-dopamine, striatal slices were transferred into low volume (0.3 ml) superfusion chambers and superfused with aerated and preheated Krebs-bicarbonate buffer. The flow rate was kept at 1 ml/min by a Gilson multichannel peristaltic pump. The superfusate was discarded for the first 60-minute period of the experiments, and then 15 3-minute fractions were collected by a Gilson multichannel fraction collector. To evoke stimulated [^{3}H]-dopamine efflux, biphasic electrical field stimuli (40 V voltage, 10 Hz frequency, 2-msec impulse duration for 3 min in fraction 4) were delivered by a Grass S88 Electrostimulator.

At the end of the superfusion, tissues were collected from the superfusion chambers, weighed and solubilized in 0.4 ml Soluene-350. An aliquot (50 µl) was mixed with 5 ml of liquid scintillation reagent and subjected to liquid scintillation spectrometry for determination of tissue content of radioactivity. The tissue content of [^{3}H]-dopamine was expressed as kBq/g tissue.

To determine the radioactivity released from striatal slices, a sample (1 ml) of the superfusate was mixed with 5 ml of liquid scintillation reagent and subjected to liquid scintillation spectrometry. The efflux of [^{3}H]-dopamine was expressed as kBq/g/3 min fraction. To estimate the electrically induced [^{3}H]-dopamine overflow, the mean of the basal outflow determined before and after stimulation was subtracted from each sample and summed. The Quattro Pro and the GraphPad Prism computer programs were used for data calculation.

The Student t-statistics for two-means and the one-way ANOVA followed by the Dunnett's test were used for statistical analysis of the data as appropriate. A level of probability (p) less than 5% was considered significant. The mean±S.E.M. was calculated and the number of independent determinations was indicated with n.

Figure 3.3

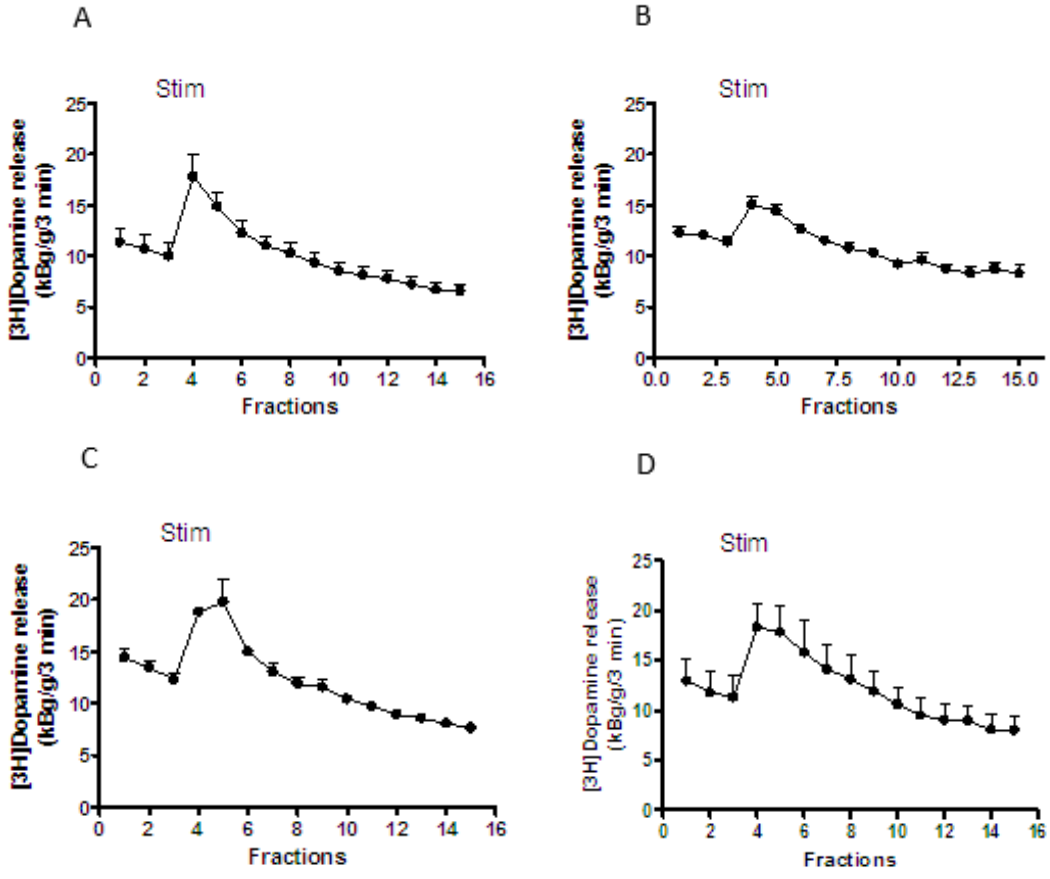


Figure 3.3 BPAP reverses TBZ-induced decrease of [^{3}H]-dopamine release in rat striatum.

A. Resting and electrical stimulation (40 V, 10 Hz, 2 msec for 3 min) induced [^{3}H]-dopamine release from rat striatal slices. Control experiments in which rats were treated with saline sc. 60 min prior to decapitation. Mean±S.E.M., n=8.

B. Effect of TBZ pretreatment (1 mg/kg sc. 60 min prior to decapitation) on resting and electrical stimulation-induced [^{3}H]-dopamine release from rat striatal slices. Note: TBZ pretreatment reduced electrical stimulation-evoked [^{3}H]-dopamine release. Mean±S.E.M., n=8.

C. Effect of BPAP pretreatment (0.0001 mg/kg sc. 60 min prior to decapitation) on resting and electrical stimulation-induced [^{3}H]-dopamine release from rat striatal slices. Mean±S.E.M., n=7.

D. Effect of combined administration of TBZ (1 mg/kg sc.) and BPAP (0.0001 mg/kg sc.) 60 min prior to decapitation on resting and electrical stimulation-induced [^{3}H]-dopamine release from rat striatal slices. Note: the electrical stimulation-evoked [^{3}H]-dopamine release was increased when TBZ and BPAP were administrated concomitantly compared to TBZ administration alone. Mean±S.E.M., n=7.

Figure 3.4

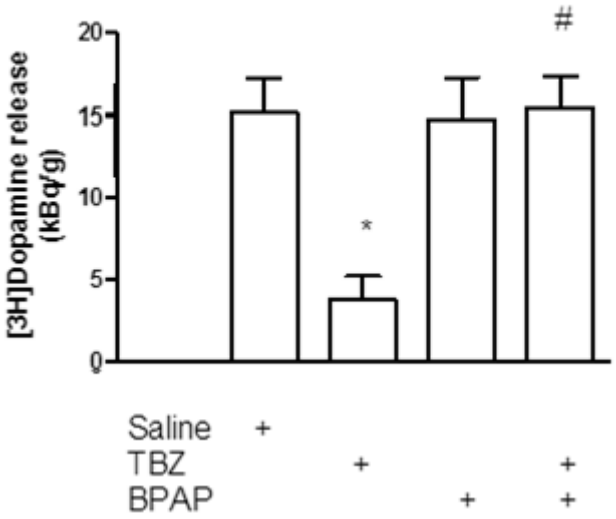


Figure 3.4 Electrical stimulation-induced [^{3}H]-dopamine release from rat striatal slices obtained from saline, TBZ (1 mg/kg sc.), BPAP (0.0001 mg/kg), and TBZ (1 mg/kg) + BPAP (0.0001 mg/kg) pretreated rats. Rats were injected with drugs sc. TBZ pretreatment reduced [^{3}H]-dopamine release, (one-way ANOVA followed by the Dunnett's test, $F(3,26)=8.617$, $p=0.0004$, saline vs. TBZ pretreated groups *$p<0.01$) and this decrease in the [^{3}H] dopamine release was reversed by BPAP administration (Student t-statistic for two means, $t=4.988$, $df=13$, #$p<0.0002$). Mean±S.E.M., n=7-8.

Figure 3.4 and Table 3.4 summarize the electrical stimulation-induced [^{3}H]-dopamine release from striatum obtained from saline, tetrabenazine, BPAP and TBZ plus BPAP-pretreated rats. As shown, the pretreatment of rats with TBZ reduced the [^{3}H]-dopamine release

evoked by electrical stimulation and this reduced release was reversed by concomitant injections of TBZ and BPAP.

Table 3.4 Electrical stimulation induced release of [^{3}H]-dopamine from striatal slices	
Treatment	**[^{3}H]-Dopamine release (kBq/g)**
1. Saline	15.19±2.05
2. TBZ	6.51±2.25*
3. BPAP	18.28±0.58
4. TBZ and BPAP	16.89±2.45#

Treatments: rats were injected with 1 mg/kg of TBZ or with 0.0001 mg/kg of BPAP sc. Drugs were injected 60 min before the experiment.
Rats were decapitated and striatal slices were prepared. The slices were loaded with [^{3}H]-dopamine and superfused. The resting and the electrical stimulation (40 V, 10 Hz, 2-msec for 3 min)-induced [^{3}H]-dopamine release was determined.
One-way ANOVA followed by the Dunnett's test, $F(3,26)=8.617$, $p=0.0004$, saline vs. TBZ pretreated groups *$p<0.0002$. Student t-statistic for two means, 2:4 #$p<0.05$. Mean±S.E.M., n=7-8.

Regarding the mechanism of action of the enhancer-substances (Figures 3.3 and 3.4 and Table 3.4), we have found that BPAP injected in a dose of 0.0001 mg/kg reversed the decrease in the electrical stimulation-induced [^{3}H]-dopamine release, evoked by 1 mg/kg of TBZ in superfused rat striatal slices. TBZ is a VMAT2 inhibitor proposed to interact with extravesicularly-located dihydro-TBZ binding site that is distinct from the DA uptake site on VMAT2. Moreover, TBZ also binds to intra-vesicular DA release sites of VMAT2, exhibiting high and low sensitivity in binding affinity. The peak dose of DEP exerting the specific enhancer effect was 0.001 mg/kg (Knoll and Miklya 2016). The characteristic bell-shaped manner of the dose-effect curve was less pronounced (Knoll and Miklya 2016), showing BPAP's effectiveness.

Both the extra- and intra-vesicular VMAT2 DA uptake and release sites may be involved in BPAP's effect. Furthermore, BPAP, acting as a substrate inhibitor of VMAT2, may compete with DA for uptake into the vesicle and may exhibit a low affinity binding to the DA uptake site on VMAT2, as suggested by its poor activity on resting [^{3}H]-dopamine release in superfused rat striatal slices (Horton, Nickell, Zheng et al. 2013).

The biphasic concentration-response curve for BPAP fits a two-site model of interaction and supports the interaction with two different intra-vesicular sites: a high affinity (picomolar) site and a low affinity (μmolar) DA release site.

A binding to the high affinity DA release site represents the specific catecholaminergic activity enhancer activity of BPAP (Knoll, Yoneda, Knoll et al. 1999), whereas the low affinity site is responsible for BPAP's non-specific enhancer effect on [^{3}H]-dopamine release. The two DA release sites may be linked to two pools of DA within the vesicles: a free pool and a pool associated with the ATP complex (Partilla, Dempsey, Nagpal et al. 2006).

Alternatively, these binding sites may regulate DA release from two distinct vesicular pools: the readily releasable DA stores and the long-term stores. It is also possible that the two binding sites evoke DA release with different mechanisms: the high affinity site mediates electrical stimulation induced release, whereas the low affinity site mediates the release of DA with reverse mode operation of VMAT2.

Taking into account the DA uptake and release sites on VMAT2, to which TBZ and BPAP bind, we concluded that the observed interaction of these two drugs in [^{3}H]-dopamine release may be related to a binding of BPAP to the high affinity intra-vesicular DA release site, which is also sensitive to TBZ, the vesicular inhibitor. All in all, currently verified details regarding the molecular mechanisms clarifying BPAP's highly characteristic bi-modal, bell-shaped concentration effect curves (Knoll, Miklya and Knoll 2002), testify that the discovery of the enhancer-sensitive brain regulations represents a promising new brain research domain.

The concept that the peculiar dose-dependency of natural enhancer substances might be responsible for the individual differences in behavioral performances

In our behavioral studies on rats we continuously observed the great individual variation in sexual activity and learning performance in any random population of mammals of the same strain. To illustrate the extreme individual differences in a random rat population I recall our second longevity study (Knoll, Yen and Miklya 1994).

We selected from a population of sexually inexperienced 1,600 Wistar-Logan male rats, the individuals born with the lowest and highest sexual potency. We found 94 males which did not display, in four consecutive weekly mating tests, any sign of sexual activity. This group of "non-copulators," classified as *low performing (LP) rats*, remained sexually inactive until they died. On the other hand, we found 99 *high performing (HP) rats*, which displayed at least one

ejaculation in each of the four tests.

The discovery of the bell-shaped concentration/effect curve of the enhancer substances, in pico/nano-molar concentration range, offers the first reasonable explanation for the great individual variation in behavioral performances. Since an *optimum* concentration of the enhancer substance was needed for the *optimum* performance, *I postulate that the substantial individual differences in behavioral performances are due to the peculiar dose-dependency of the endogenous enhancer substances.*

This approach offered us a new perspective on the results of our three longevity studies on rats: first, Knoll 1988 and Knoll, Dalló and Yen 1989; second, Knoll, Yen and Miklya 1994; and third, Knoll and Miklya 2016.

Considering the unique dose-related effect of an enhancer substance, we assume that out of the 1,600 rats, the *99 HP rats produced their endogenous enhancer substances responsible for sexual performance at the peak of the bell-shaped concentration/effect curve, while the 94 LP rats produced them at the least active part of the curve.* The overwhelming majority of the population (1,407 rats) fell in between these two extremes.

As outlined in Chapter 1, an analysis of the ability of rats to acquire the "glass-cylinder-seeking" drive is another example that convincingly illustrates the great individual differences in the behavioral performances of rats (Knoll 2005). Only two rats out of 100 maintained the acquired "glass-cylinder-seeking" drive with unchanged intensity throughout their life. The performance of these two rats was always fascinating, they worked indefatigably. Presumably the specific endogenous enhancer substances in the cortical neurons responsible for the operation of the "glass-cylinder-seeking" drive were mobilized in these two rats in the optimum concentration with never flagging zeal. Thus, we view these two rats as the most talented in the tested population regarding the measured function.

It is remarkable that the discovery of the enhancer-sensitive brain regulations and the development of the synthetic enhancers (DEP and BPAP) easily reframed our research. It is reasonable to remember that the development of brains capable to fix acquired drives was the last qualitative change in the development of the mammalian brain prior to the birth of Homo sapiens sapiens, the sole group that operates almost exclusively with acquired drives.

The discovery that PEA is a natural enhancer of the catecholaminergic and serotonergic neurons in the brain stem, and the fact that we successfully fabricated a much more potent and selective synthetic enhancer substance than DEP, is a strong argument for the thesis that

enhancer regulation operates in the catecholaminergic and serotonergic neurons in the brain and places at our disposal tools with which we can maintain the activity of enhancer-sensitive cells on higher activity level without changing their physiological milieu (Knoll 2012).

As it was shown earlier and will be discussed later in more detail, the enhanced activity of the catecholaminergic brain engine from weaning until full scale sexual maturity is primarily responsible for the most delightful phase of life, the glorious uphill journey (Knoll and Miklya 1995). Sexual hormones restore the enhancer regulation in the catecholaminergic and the serotonergic neurons in the brain to the pre-weaning level, thus terminating developmental longevity (Knoll, Miklya, Knoll and Dalló 2000). We need to begin counteracting brain aging as soon as sexual maturity is reached (Knoll 2012).

The dopaminergic machinery is the most rapidly aging neuronal system in our brain. We know that symptoms of PD appear if the DA content of the caudate drops below 30% of the normal level. Experimental and clinical experiences show that daily dosages of DEP keep the brain engine's activity on a higher activity level in humans. From sexual maturity, a low daily dose of DEP (1 mg) is sufficient to significantly slow the pace of the aging-related decay of the dopaminergic neurons. Even if we assume only a small protective effect of DEP in healthy humans against the age-related decrease in striatal DA, for example from 13% per decade to 10% per decade, this translates to a minimum 15-year extension in average lifespan and a considerable increase of TLS_h, which is now estimated to be around 115 years.

We demonstrated in earlier longevity studies that male rats injected with DEP preserved their learning ability longer, lost their ability to ejaculate later and lived longer than their placebo-treated peers. Assuming that the selective inhibition of MAO-B in the brain is responsible for these beneficial effects, we performed two longevity studies with 0.25 mg/kg DEP, the dose which completely blocks MAO-B activity in the brain (Knoll 1988; Knoll, Knoll, Dalló and Yen 1989; Knoll, Yen and Miklya 1994). The discovery that DEP is a CAE substance, and the development of BPAP, the more potent synthetic CAE substance than DEP, devoid of MAO-B inhibitory potency, directed our attention to this new subject (Knoll and Miklya 1994, 1995; Knoll 1998; Knoll, Yoneda, Knoll et al. 1999; Knoll 2001, 2003).

This new line of research confirmed that DEP, in contrast with my original opinion, is primarily a PEA-derived synthetic enhancer substance which exerts in rats in vivo its specific enhancer effect in the extremely low, 0.001 mg/kg dose, and blocks MAO-B activity in the brain in 0.25 mg/kg. *DEP also exerts in the same high, 0.25 mg/kg dose, its non-specific enhancer effect which means that it remains for the future to exactly identify the scope of DEP's*

non-specific enhancer effect in the therapeutic benefits displayed in patients treated with 1 mg DEP daily.

On the basis of natural enhancers (PEA and TRY, respectively), we developed DEP and BPAP, the first two synthetic enhancers with a previously unimagined pharmacological profile and we hope to extend with synthetic enhancers the short delightful period of life and counteract the regressive effects of the long, less desirable post-developmental phase of human life.

PPAP's enhancer effect is obviously responsible for the overwhelming majority of the beneficial therapeutic effects observed and described in the innumerable papers published on DEP's therapeutic effects (Knoll 2012). It is essential in this context that PPAP – devoid of the propargyl group, thus being free of MAO-B inhibitory potency – acts like DEP (Knoll, Knoll, Török et al. 1992), which in the 0.25 mg/kg dose has two effects: blocks MAO-B in the brain and exerts the non-specific enhancer effect (Knoll and Miklya 2016).

It is of great theoretical and practical importance that DEP and BPAP, as markers of unknown enhancer-sensitive brain regulations, detected the operation of an enhancer-sensitive tumor-manifestation-suppressing (TMS) brain-regulation with no prior literature. This discovery by itself is promising regarding the future of the enhancer-sensitive brain regulations. Presently, only the enhancer-sensitive catecholaminergic and serotonergic brain regulations were subject of detailed analysis and, since TMS-regulation shows similar sensitivity toward DEP and BPAP, it is easy interpret the findings to be presented in Chapter 9. Due to the specific pharmacological spectrum of DEP, the only synthetic PEA-derivative devoid of the catecholamine-releasing property which showed that the catecholaminergic neurons belong to the enhancer-sensitive brain regulations, guided the study of this hitherto unknown life important mechanism in the mammalian brain. As discussed, and briefly summarized in Chapter 2, we unveiled the pharmacological spectrum of DEP in three phases. We realized in the first phase that DEP is the unique MAO inhibitor free of the cheese effect and discovered in the second phase that DEP is the first selective inhibitor of MAO-B. Because of their immediately exploitable clinical significance, they received whole-hearted enthusiasm. However, the deeper we penetrated into the nature of the characteristic enhancement of the catecholaminergic brain machinery in DEP-treated rats, the stronger was the evidence that in striking contrast to my original view, the main therapeutic effect of DEP is unrelated to the selective inhibition of MAO-B. I realized that *DEP possesses a hitherto completely unknown pharmacological profile* signaling the existence of an unknown world in the mammalian brain: enhancer-sensitive brain regulations.

The development of PPAP, the DEP-analog devoid of MAO inhibitory property, being an equally active stimulant of the catecholaminergic neurons as DEP, was the first step forward supporting my suggestion (Knoll, Knoll, Török et al. 1992).

The second step was the foundation of the concept (Knoll 1994) which initiated the first study, also published in 1994, which demonstrated that multiple, low dose administrations of DEP enhances catecholaminergic activity in the brain and this effect is unrelated to MAO-B inhibition (Knoll and Miklya 1994).

PEA and its best-known synthetic derivatives (AM and MAM) are strong releasers of catecholamines from their plasmatic pools. Since the catecholamine-releasing effect conceals the detectability of the enhancer-sensitive nature of the catecholaminergic neurons (Knoll 2016), PEA's primary physiological function as a natural enhancer substance, as well as the fact that AM and MAM are, like DEP, PEA-derived synthetic enhancer substances, remained unknown.

A crucially important step forward was the realization that TRY is, like PEA, a natural enhancer of the serotonergic neurons (Knoll 1994). Thus, I found a reasonable experimental approach from a SAR study to create a second synthetic enhancer, hopefully acting on the serotonergic neurons, since we demonstrated that DEP was practically ineffective on the serotonergic neurons (Knoll 1994). It took me years until I found the proper TRY-derived synthetic enhancer and selected BPAP for further studies (Knoll, Yoneda, Knoll et al. 1999).

The discovery of the enhancer-sensitive TMS-regulation was our final convincing proof of the uniqueness of the enhancer-sensitive brain regulations. *Nevertheless, we still see only the beginning peak of this research.*

As recalled in Chapter 2, prior to the identification of the catecholaminergic activity enhancer effect of DEP and the discovery of the enhancer regulation in the mammalian brain, I proposed in 1981 that a progressively developing catecholaminergic and trace-aminergic deficiency is responsible for the biochemical lesion in the aging brain which leads to the age-related decline in sexual and learning performance and ultimately natural death (Knoll 1982). A further study proved that this effect of DEP is unrelated to the inhibition of MAO-B (Knoll and Miklya 1995).

Decades ago, I asked Walther Birkmayer, the only clinician who tested since the mid-1970s the effect of DEP on Parkinsonian patients, to make a retrospective analysis on the potential effect of DEP on longevity. Our analysis supported my concept. The long term (nine-

year) effect of treatment with Madopar alone (N=177) or in combination with Madopar+DEP (N= 564) revealed a significant increase in life expectancy in the Madopar+DEP group, regardless of the significant demographic differences between the two groups (Birkmayer, Knoll, Riederer et al. 1985).

Enhancer substances keep the catecholaminergic neurons on a higher activity level. For example: 6.8±0.18 nmol/g wet weight DA was released within 20 minutes from the substantia nigra isolated from saline treated rats and 14.8±0.36 nmol/g DA was released from rats treated with a single dose of 0.0001 mg/kg BPAP (Knoll 2016). *It is well established from studies with rodents and primates that dopaminergic neurons are silent or spontaneously active* (Marinelli, Rudick, Hu and White 2006). Treatment of rats with 0.0001 mg/kg BPAP transforms the silent catecholaminergic neurons into spontaneous firing entities, and thus, the discovery of the enhancer regulation explains the promptness of activation in assault/escape behavior (Knoll 2016). The therapeutic consequences that 0.0001 mg/kg BPAP is capable to dramatically transform dopaminergic neuron's operation cannot be overstated.

Chapter 4

The peculiar physiological role of the catecholaminergic neurons to keep the brain in a continuously active state

There are substantial reasons to assume that catecholaminergic neurons keep the higher brain centers continuously active and care for changes within broad limits, according to need. As signaled by the appearance of the EEG in an early phase of development, the catecholaminergic brain engine, responsible for the integrative work of the central nervous system (CNS), is ignited once spanning for a lifetime.

Due to aging, the maximum level of activation of the CNS, via the catecholaminergic system, decreases progressively over time. The blackout ("natural death") of the integrative work of the CNS, signaled by the disappearance of EEG, occurs when the catecholaminergic system's ability to activate the higher brain centers sinks below a critical threshold and an emergency incident transpires, when a high level of activation that is needed to survive and the CNS can no longer be properly activated. This explains why a common infection, a broken leg,

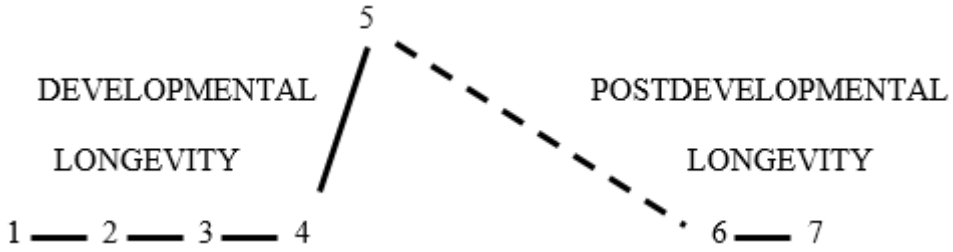
or any other normally minor challenge given fully active catecholaminergic machinery may cause death in old age.

The essence of this hypothesis is depicted in the Figure below. According to this schema the life of a mammalian organism can be divided, from a functional point of view, into six stages, each beginning with a qualitative change of crucial importance. The first stage starts with the fertilization of the ovum and lasts until the catecholaminergic system properly activates the higher levels of the brain, which then assume the lead and integrates the different parts of the organism into a highly sophisticated entity. The first stage of development of the mammalian organism is completed when the catecholaminergic engine of the brain is put into gear once and for all. This is the intrauterine birth of the unique individual. The appearance of EEG signals the transition from the first into the second stage of development.

Cells need oxygen, water, and food for life. These are first supplied, via the placenta, by the mother. The subsequent, highly complicated evolving process is devoted to ensuring independence from the mother.

The second stage of development ends with the passage of the fetus from the uterus to the outside world. From a functional point of view birth means the transition from fetal to postnatal circulation, with the newborn infant now supplying itself with oxygen.

The third stage lasts from birth until weaning and serves to develop the skills needed for the maintenance of integrity and for the infant to supply itself with water and food.



1) FUSION OF THE SPERMATOZOON WITH THE OVUM

2) THE INTEGRATIVE WORK OF THE CNS SETS IN;

APPEARENCE OF EEG

3) BIRTH OF THE FETUS

4) WEANING

5) SEXUAL MATURITY IS REACHED

6) THE INTEGRATIVE WORK OF THE CNS BLACKS OUT;

DISAPPEARANCE OF EEG; "NATURAL DEATH"

7) DEATH OF THE LAST CELL

Figure: Conception about essential changes during the lifetime of mammals (Knoll 1994).

The fourth stage lasts from weaning until the supreme goal in nature: full scale sexual maturity is reached. This is the most delightful phase of life, the glorious uphill journey. The individual progressively takes possession, on a mature level, of all abilities crucial for survival and maintenance of the species. It learns to avoid dangerous situations, masters the techniques for obtaining food and water, develops procreative powers for sexual reproduction and copulates. This is, at the same time, the climax of developmental longevity.

The discovery of the enhancer-sensitive brain regulations revealed that during *the interval from weaning (3rd week of life) until the end of the 2nd month of age, thus during the decisive period for development, enhancer-sensitive neurons work permanently on a significantly higher activity level then before weaning (in 2-week-old rats).* We measured the dopaminergic, noradrenergic and serotonergic activities in the brain during the crucial developmental phase, from weaning to sexual maturity (in 4- and 8-week-old rats) and in the early post-developmental phase of life (in 16- and 32-week-old rats). As an indicator of the basic activity of catecholaminergic and serotonergic neurons in the brain, we measured the release of dopamine (DA) from striatum, substantia nigra and tuberculum olfactorium; of norepinephrine (NE) from the locus coeruleus; and of serotonin (SE) from the raphe, in both male and female rats (Knoll and Miklya 1995).

The full, sexually mature individual fulfills its duty. Thus, to maintain the precisely balanced natural equilibrium among living organisms, the biologically "useless" individual has to be eliminated; according to the inborn program, the fifth, post-developmental (aging) stage of life begins. Since, from the discontinuation of breast feeding (end of the 3rd week of age) until the appearance of sexual hormones (end of the 2nd month of life) we measured in both male and female rats a significantly pronounced enhancer regulation in the dopaminergic, noradrenergic and serotonergic neurons, it was reasonable to deduce that sexual hormones play the key role in terminating the developmental phase of life (Knoll, Miklya, Knoll and Dalló 2000).

The essence of the fifth stage is the progressive decay of the catecholaminergic system's efficiency during the post-developmental lifespan, until at some point, in an emergency situation, the integration of the parts in a highly sophisticated entity can no longer be maintained and "natural death," signaled by the disappearance of EEG signal, sets in.

As parts of the organism remain alive, the sixth and last stage of life is the successive death of the different groups of cells, until the death of the last cell.

The hypothesis outlined suggests that quality and duration of life rests upon the inborn efficiency of the catecholaminergic brain machinery, i.e., a higher performing, longer-living individual has a more active, more slowly deteriorating catecholaminergic system than its low performing, shorter-living peer. To simplify this concept, we may say that a better brain engine allows for a better performance and a longer lifespan.

The concept clearly predicts that, as the activity of the catecholaminergic system can be improved at any time during life, it must essentially be feasible to develop a technique for transforming a lower-performing, shorter-living individual, to a better-performing, longer-living one. It therefore follows that a shift in the duration of life beyond the technical lifespan with a yet unpredictable limit must be possible in all mammals including the human species (Knoll 1994).

Because of the special role of the catecholaminergic neurons maintaining the brain permanently stimulated is the condition *sine qua non* for the indefatigability of the human-brain driven by an innate or an acquired drive. It is obvious that the ability to acquire an irrepressible urge for a goal not necessary for survival represents the most sophisticated function in the mammalian brain. Whenever a new drive is acquired, chains of inextinguishable conditioned reflexes are stabilized; neurons responsible for emotions are also coupled to the integral whole. Thus, cognitive/volitional consciousness is necessarily inseparable from an affective state of consciousness. The mechanism that binds emotions, appurtenances to any chain of inextinguishable conditioned reflexes is of crucial importance to interpersonal communication (Knoll 1969, 2005, 2010).

Since the era of enlightenment, the spiritual revolution which resulted in a previously unthinkable acceleration in the development of science and technology, it was foreseeable that the myths-directed first phase of human history is slowly arriving to its chaotic end phase. Dramatic changes since the beginning of the 20th century (the Great War, Second World War, Holocaust, as well as the Terror War in progress) are awful warnings that the myths-directed

first phase of human history is already unmaintainable and is fortunately slowly approaching its shameful end (Knoll 2001, 2003, 2005).

The ratio-directed future looks promising. Robots will work; science and art remain the main human activities. Thousands of anecdotes recall ingenious scientists/artists working day and night in perfect harmony and satisfaction toward their acquired drives. The higher the percentage of the population who find their best-suited acquired drives to their natural endowments, the better is the quality of life in a ratio-directed society.

The s*ame basic mechanism* manipulates the behavior of domesticated animals and humans. However, in striking contrast to the human brain with practically unlimited capacity to fix acquired drives, this ability in animals is strictly limited. In our animal model we manipulated the behavior of our rats to establish the "glass-cylinder-seeking drive." *Finally, the rat behaved as if possessing a fanatical desire to reach the unnatural goal (Chapter 1). In the properly manipulated rat the unnatural drive suppressed the life important natural drives.* The same applies to human beings. The brain of a suicide killer is furtively manipulated. The properly acquired unnatural drive develops as a result of long-lasting training. The subject always acts under coercion, under severe mental pressure. Nevertheless, it is the nature of acquired drives that if the manipulation was fully successful the individual ultimately behaves as one possessing a fanatical desire to reach the unnatural drive-motivated goal. Thus, the expert-opinion that the global war on terror is a 7th-century clash involving 21st-century weapons is correct.

Furthermore, since the development of science and technology continually accelerates, no matter how much time is still needed to arrive to the ratio-directed era of human history, *to achieve this final goal of development is a physiological necessity and cannot be stopped*.

Similar to humans, even animals capable of establishing acquired drives work with them passionately and untiringly. *In striking contrast, the innate drive works until the urge is satisfied.* The better we understand the enhancer-sensitive regulations in the human brain, get acquainted with the identity of their natural enhancers and develop proper synthetic enhancers, the better are our chances to counter the regressive effects of brain aging and improve the quality and duration of human life.

Unfortunately, for the time being, the ability of species to fix acquired drives as the mechanism of the manipulability of mammalian behavior is still not common knowledge. But all is not lost that is delayed. It is of crucial importance that with brains capable of acquiring

drives species appeared whose members could manipulate each other's behavior and act in concert. This was the condition *sine qua non* for the evolution of social living, a form of life that enabled species to surpass qualitatively the performance of any given individual.

Chapter 5

Longevity studies with DEP and BPAP

As already recalled, the selective B type monoamine oxidase (MAO-B) inhibitory effect of selegiline/(-)-deprenyl (DEP)was for several years at the center of our interest and delayed the discovery of the drug's enhancer effect. Getting acquainted with the peculiar pharmacological spectrum of DEP, it was reasonable to put forward the concept that the progressive decrease in brain catecholamines and the trace amines is an unavoidable biochemical lesion of aging, which can be counteracted by appropriate continuous medication (Knoll 1982).

The discovery of the bell-shaped concentration/effect curve of the enhancer substances, in the low pico/nano-molar concentration range, offers the first reasonable explanation for the great individual variation in behavioral performances. Since an optimum concentration of the enhancer substance was needed for the optimum performance, I concluded that the substantial individual differences in behavioral performances are due to the peculiar dose-dependency of the endogenous enhancer substances. This approach granted us a new perspective on the results of our three longitudinal studies performed on rats (Knoll 1988; Knoll and Miklya 2016; Knoll, Knoll, Dalló and Yen 1989, 1994).

In the years when we performed our first and second longevity studies and worked with the robust Wistar-Logan rats (first generation of Wistar males x Logan females), we observed that the males which completed their second year of life never did display in the weekly mating test a single ejaculation. We experienced later that the Sprague-Dawley CFY or Wistar (Charles-River) rats too lost this ability at this age. Our studies clarified that the aging-related irresistible decay of the dopaminergic brain machinery is responsible for this change. Saline-treated CFY male rats reached the stage of un-ability to ejaculate at an average of 112±9 weeks. Due to the anti-aging effect of DEP, rats injected subcutaneously,3-times a week with 0.25 mg/kg DEP, reached that stage at an average of 150±12 weeks (Knoll 1993).

The Longevity Studies Performed with the MAO-B Inhibitory Dose (0.25 mg/kg) of DEP

In our first longevity study we worked with 132 sexually inexperienced 2-year-old males. We tested their copulatory activity in four consecutive weekly mating tests during the 24th month of their life. According to their screening the rats were divided in three groups: 46 "non-copulators," 42 "mounting" rats and 44 "sluggish" rats (displaying mountings and intromissions). Thereafter we treated 66 rats with saline and 66 rats with 0.25 mg/kg DEP, three times a week, and observed their behavioral performances to the end of their life.

In the saline-treated group of the non-copulators died out first. The mounting rats lived longer. The longest living rats were in the sluggish group (Knoll 1988). Deprenyl treatment prolonged life in each group significantly. The 66 saline-treated rats lived in average 147.05± 0.56 weeks; the 66 DEP-treated rats lived in average 197.98± 2.31 weeks.

The fact that the saline-treated non-copulators died out first and the finding that DEP, the special stimulant of the catecholaminergic brain stem neurons, keeps the rats on a higher activity level and prolongs their life, suggested that the catecholaminergic engine of the brain, which is of crucial importance in activating the cortex (Chapter 4), is responsible for the lifespan-prolonging effect. Thus, the brain engine works in the 2-year-old non-copulators on a lower activity level than in the 2-year-old sluggish males.

Results of the first longevity study and the analysis of the peculiar physiological role of the catecholaminergic neurons to keep the brain in a continuously active state, as described in 1994 (Knoll 1994),determined the planning of the second longevity study.

As analyzed in detail in Chapter 4, the hypothesis out-lined in 1994 (Knoll 1994) suggested that quality and duration of life rests upon the inborn efficiency of the catecholaminergic brain machinery, i.e., a higher performing, longer-living individual has a more active, more slowly deteriorating catecholaminergic system than its low performing, shorter-living peer. To simplify this concept, we may say that a better brain engine allows for a better performance and a longer lifespan.

Thus, the concept clearly predicted that, as the activity of the catecholaminergic system can be improved at any time during life, it must essentially be feasible to develop a technique for transforming a lower-performing, shorter-living individual, to a better-performing, longer-living one. It therefore follows that a shift of the duration of life beyond the technical lifespan (TLS), with a yet unpredictable upper limit, must be possible in all mammals, including the human species.

To test this concept, we decided to perform our second longevity study on younger rats. We selected from a huge population of Wistar-Logan male rats the non-copulators and the sexually most active ones. We measured their sexual potency and learning ability until the end of their life and treated the rats with saline and DEP, respectively.

We started working with a random population of 28-week-old male rats and tested their sexual performance once a week. Rats that represented the two extremes in performance were selected for the study: the *ones that did not display a single intromission during the four consecutive weekly-mating tests used for selection and the ones which showed full scale sexual activity (mounting, intromission, ejaculation) in each of the four tests*.

Out of 1,600 sexually inexperienced 28-week-old Wistar-Logan male rats, that met a receptive female once a week during four consecutive weeks, 94 did not display a single intromission during the selection period and 99 displayed at least one ejaculation in each of the four tests. The former were taken for the sexually lowest performing (LP) and the latter for the highest performing (HP) rats. It is obvious that my main aim with the second longevity study was to test DEP-treatment's expected ability to transform an LP rat into a HP one.

After selection, we started to treat the 8-month-old rats subcutaneously with either 1 ml/kg 0.9% NaCl or with 0.25 mg/kg DEP, dissolved in 0.9% NaCl and given in the same volume, 3-times a week, until the end of their life.

Out of the 94 LP animals, 46 were saline-treated. Out of the 99 HP animals, 49 were saline-treated. The mating and learning performances of saline-treated LP and HP rats were tested during a period of 108 weeks. Copulatory activity was tested once a week.

The learning performance of the rats was tested in the shuttle box. The rats were trained for a five-day period once in three months with 20 trials a day. In this longevity study we trained our rats in the shuttle box, using instead of the optimal training conditions (100 trial) only 20 trials, to find more pronounced difference in the learning ability between HP and LP rats.

We found a highly significant difference in sexual and learning performances and in lifespan between LP and HP rats.

The saline-treated LP rats (n=44) never displayed ejaculation during their lifetime, they were extremely dull in the shuttle box and lived 134.58±2.29 weeks. The saline-treated HP rats (n=49) displayed 14.04±0.56 ejaculations during the first 36-week testing period and, due to

aging, they produced 2.47±0.23 ejaculations between the 73-108th week of testing. They lived 151.24±1.36 weeks, significantly (P<0.001) longer than their LP peers.

Maintenance on DEP-Enhanced Performance of Both LP and HP Rats Significantly Prolonged their Lifespan

The DEP-treated LP rats (n=48) became sexually active, their mating performance was substantially increased and they lived 152.54±1.36 weeks, significantly longer than their saline-treated peers and as long as the saline-treated HP rats. The DEP-treated HP rats (n=50) were sexually much more active than their saline-treated peers. They displayed 30.04±0.85 ejaculations during the first 36-week testing period and 7.40±0.32 ejaculations between the 73-108th week of testing. Also, their learning performance was substantially increased. They produced 113.98±3.23 conditioned avoidance responses (CARs) during the first 36-weektesting period and 81.68±2.14 CARs during the 73-108th week of testing. They lived 185.30±1.96 weeks, significantly more than their saline-treated peers and out of the 50 rats 17 lived longer than the estimated technical lifespan. Considering the unique dose-related effect of an enhancer substance, we assume that out of the 1,600 rats,*99 HP rats* produced their endogenous enhancer substances at the peak of the bell-shaped concentration/effect curve, while the*94 LP rats* produced them at the least active part of the curve. The overwhelming majority of the population (1,407 rats) fell between these two extremes.

The Second Longevity Study Verified that Life-long DEP Treatment Transforms a Lower-Performing, Shorter Living Rat, into a Better-Performing, Longer-Living One

An analysis of the ability of rats to acquire the glass-cylinder-seeking drive is another example that convincingly illustrates the great individual differences in the behavioral performances of the rat (Knoll 2005). We observed only in two rats out of 100 that the acquired glass-cylinder-seeking function operated lifelong with unchanged intensity. Presumably, the specific endogenous enhancer substances in the cortical neurons responsible for the operation of the glass-cylinder-seeking drive were mobilized in these two rats in the optimum concentration. Thus, regarding the measured function we may look upon these two rats as the most talented in the tested population.

There is a gleam of hope that better understanding of the enhancer regulation in the cortical neurons may finally allow definition on a molecular level of the physiological mechanism responsible of "man of talent/genius." As analyzed and discussed in detail in my monograph (Knoll 2005), since the natural endowments of the healthy human brain are identical, everybody is born with 100 billion neurons and 10^{11}-bit capacity, *everybody has necessarily brilliant abilities which remain unexplored, unutilized.*

As will be discussed in detail, the enhancer regulation of the catecholaminergic and the serotonergic neurons in the brain starts working on a significantly higher activity level after weaning and the intensified activity subsists until sexual maturity is reached; thereafter, activity returns to the pre-weaning level. Developmental longevity, the happy, uphill period of life, is a short phase between weaning and sexual maturity (Knoll and Miklya 1995).

As it will be shown in Chapter 7, sexual hormones dampen the intensified enhancer regulation in the catecholaminergic and serotonergic neurons and activity returns to the pre-weaning level. This is the transition from adolescence to adulthood. The post-developmental/downhill period of life begins with sexual maturity and lasts until natural death(Knoll, Miklya, Knoll and Dalló 2000).

During the post-developmental period, the enhancer regulation in the catecholaminergic brain machinery is on a slow continuous decline. The catecholaminergic neurons play a key role in the efficiency of learning performances, drive motivated behavior, etc. The continuous decline of their activity with the passing of time plays a crucial role in the behavioral consequences, the regressive effects of brain aging.

In this context, our early work with DEP, prior to the discovery of the enhancer effect, already called our attention to the peculiar nature of DEP-induced facilitation of striatal dopaminergic neurotransmission. We studied in detail the phenomenon that striata of rats treated with 0.25 mg/kg DEP daily for at least three weeks released significantly more DA in the resting state and in response to KCl stimulation than the striata of rats treated daily with 0.1 mg/ 100 g saline solution. The finding that DEP increases the firing rate of the nigrostriatal dopaminergic neurons was in complete agreement with previous data, proving the increased turnover rate of DA in the striatum of male rats treated for 2-4 weeks with a daily dose of 0.25 mg/kg DEP. *The facilitation of striatal dopaminergic neurotransmission by long term treatment was highly specific.* The turnover rate of NE was decreased and the level of this amine remained unchanged in the brain stem of male rats; and no change in the turnover rate of SE was detected (Knoll 1993).

S*ince, as it will be shown in Chapter 8,the aging-related continuous slow decay of the natural enhancers result in the regressive effects of brain-aging, but the enhancer-sensitive neurons do not age, and since we already developed proper synthetic enhancers, we are able to counteract brain aging.*

The daily preventive administration of a synthetic enhancer substance from sexual maturity until death can maintain the activity of the catecholaminergic and serotonergic neurons on a higher activity level and we can already slow aging-related decay of physical and mental welfare. *From a practical point of view, this is an essential recent message of our work*(Knoll and Miklya 2016).

The convincing experimental evidence that the 0.25 mg/kg dose of DEP, the peak dose which inhibits MAO-B in the brain, is also the peak dose with the non-specific enhancer effect and the increasing amount of data in support that the enhancer effect of DEP and (2*R*)-1-(1-benzofuran-2-yl)-*N*-propylpentane-2-amine (BPAP), is primarily responsible for the beneficial pharmacological effects of the synthetic enhancers initiated our recently published longevity study with low doses of DEP and BPAP (Knoll and Miklya 2016).

The discovery of the enhancer regulation in the mammalian brain and the study of the catecholaminergic and serotonergic neurons as enhancer-sensitive brain regulations (Knoll 1994, 2005, 2016);the identification of β-phenylethylamine (PEA) and tryptamine as natural enhancer substances (Knoll 1994, Knoll, Miklya, Knoll et al. 1996a; Knoll, Yoneda, Knoll et al. 1999); and the proof that DEP is the only PEA-derived synthetic enhancer substance devoid of the catecholamine releasing property (Abdorubo and Knoll 1988) revealed that PEA and amphetamines act as catecholaminergic activity enhancer (CAE) substances and release catecholamines from the plasmatic pools only in high concentrations (Knoll 1998). *Since the continued catecholamine-releasing effect of PEA and amphetamines concealed their CAE effect (Knoll 2016), PEA's natural enhancer function remained until the development of DEP undetected.* The development of BPAP, the tryptamine-derived *selective* synthetic enhancer substance (Knoll, Yoneda, Knoll et al. 1999) confirmed that the enhancer-sensitive brain regulations represent a promising new brain-research domain (Knoll 1992, 2012; Knoll, Miklya and Knoll 2002; Miklya 2016).

Taking all this into consideration, we decided to perform the third longevity study, with the aim to clarify the role of the enhancer effect in life extension (Knoll and Miklya 2016) *and to confirm that the enhancer sensitive neurons do not age.*

Proof that the Specific Enhancer Doses of DEP and BPAP are Responsible for Life Extension

We performed the third longevity study on male Wistar rats with selected peak doses of DEP and BPAP, in which the synthetic enhancers exerted their specific and non-specific enhancer effects and demonstrated that the enhancer effect of DEP and BPAP are responsible for life extension. We treated rats three times a week with 0.0001 mg/kg BPAP aiming to test their learning ability in the shuttle box as they completed their 18th month of age, in order to demonstrate that BPAP treatment prevents the enhancer-sensitive dopaminergic neurons from aging.

The longevity study demonstrated that the enhancer effect of DEP and BPAP are responsible for life extension(Knoll and Miklya 2016) *and presented also the first experimental evidence that life-long treatment of rats with0.0001 mg/kg BPAP counteracts brain aging*.

As shown before, DEP, *the unique PEA derivative free of the catecholamine releasing-property,* was the first experimental tool which enabled the revelation of the enhancer regulation in the mammalian brain. Devoid of MAO-B inhibitory effect, BPAP is, at present, the most selective and potent synthetic, tryptamine-derived enhancer substance available. We tested, during the last decades, the catecholaminergic and serotonergic neurons as potential enhancer sensitive ones. Deprenylis is a CAE substance, poorly acting on the serotonergic neurons. Much more potent than DEP, however, BPAP, even as a CAE substance, is an even more potent enhancer of the serotonergic neurons.

It is well known that DEP significantly prolongs the life of rats (Bickford, Adams, Boyson et al. 1997; Dalló and Koles 1996; Kitani, Kanai, Sato et al. 1993; Kitani, Minami, Isobe et al. 2002; Kitani, Kanai, Miyasaka et al. 2005; Knoll 1988; Knoll, Dalló and Yen 1989, 1994; Milgram, Racine, Nellis et al. 1990), mice (Freisleben, Lehr and Fuchs 1994; Archer and Harrison 1996), Syrian hamsters (Stoll, Hafner, Kranzlin and Muller 1997), beagle dogs (Ruehl, Bruyette, DePaoli et al. 1995)and acts even on Drosophila melanogaster (Jordens, Berry, Gillott and Boulton 1999). The first two longevity studies (Knoll 1988; Knoll, Dalló and Yen 1989, 1994) were performed with 0.25 mg/kg DEP, the MAO-B inhibitory peak dose of DEP. The Knoll and Miklya (2016) study was the first performed with low doses of DEP (0.001 and 0.1 mg/kg)and BPAP(0.0001 and 0.05 mg/kg)acting as peak doses eliciting the specific and non-specific enhancer effect, respectively.

The longevity study was performed with 200 male Wistar rats. Ten-week-old rats were randomly assigned into five groups and treated three times a week (Monday, Wednesday and Friday), subcutaneously, with saline, DEP and BPAP, respectively, until their natural death, as shown below:

Group	Treatment	Dose	N
1.	Saline	0.05 ml/100 g	40
2.	DEP	0.1 mg/kg	40
3.	DEP	0.001 mg/kg	40
4.	BPAP	0.05 mg/kg	40
5.	BPAP	0.0001 mg/kg	40

To select the optimal low doses of DEP and BPAP for the longevity study we used a modified version of the shuttle box, originally described in 1966 (Bovet, Bovet-Nitti and Oliverio 1966). The acquisition of a two-way conditioned avoidance reflex (CAR) was analyzed during five consecutive days. The rat was placed in a box divided inside into two parts by a barrier with a small gate in the middle and the animal was trained to cross the barrier under the influence of a conditioned stimulus (CS: light flash). If it failed to respond within 5s, it was trained with a foot-shock (1mA), the unconditioned stimulus (US). If the rat failed to respond within 5s to the US, it was classified as an escape failure (EF). One trial consisted of 10s inter-trial interval, followed by 20s CS. The last 5s of CS overlapped the 5s US. Rats received 100 avoidance trials/day. At each learning session, the number of CARs, EFs and inter-signal reactions (IRs) were automatically counted.

As was shown in Chapters 2 and 3, the bi-modal, bell-shaped concentration effect curve is characteristic to the CAE effect of the enhancer substances. We first took notice of this peculiar behavior in the course of our first experiments when we realized the CAE effect of DEP (Knoll 2005). Nevertheless, only the precise analysis of BPAP's enhancer effect, the selective and today's most potent enhancer substance, rendered the unquestionable distinction between the *specific* and *non-specific* enhancer effect possible. The bi-modal, bell-shaped nature of the enhancer effect was confirmed on cultured rat hippocampal neurons (Knoll, Yoneda, Knoll et al. 1999) and exactly analyzed on isolated locus coeruleus (Knoll, Miklya and Knoll 2002).

To select the optimal *specific* and *non-specific* enhancer doses of DEP and BPAP for our longevity study, we analyzed the acquisition of a two-way conditioned avoidance reflex (CAR) in a shuttle box using tetrabenazine (TBZ)-treatment. Tetrabenazine (1 mg/kg sc.) reversibly blocks the vesicular monoamine transporter 2 (VMAT2) and within one hour depletes at least 90% of NE and DA from their transmitter-stores in the nerve terminals of the catecholaminergic neurons in the brain stem (Scherman, Jaudon and Henry 1983). As quoted by Schreiber, Krieg and Eichhorn (1999), "noradrenergic neurotransmission – that is, neuronal noradrenaline depletion – can therefore be postulated to form one major origin of TBZ-induced depression. In line with this assumption, brain specific CAEs such as phenylethylamine have been shown to antagonize TBZ-induced depression-like behavior in rats" (Knoll, Miklya, Knoll et al. 1992). Due to the weak performance of the catecholaminergic brain engine, the activation of the cortical neurons remains below the required level for the acquisition of a CAR. According to our experience (we studied for years the drug families worth trying), TBZ-induced inhibition of learning performance can only be antagonized by administration of a synthetic CAE substance or by the complete inhibition of MAO-A, whereas selective inhibition of MAO-B or inhibition of the reuptake of catecholamines and/or SE is ineffective (Knoll, Miklya, Knoll et al. 1992).

Deprenyl, the first selective inhibitor of MAO-B, has been classified since the 1970s - in all papers and textbooks - as the reference compound to block this enzyme. Deprenyl's CAE effect, the significance of which was realized only in the late 1990s, is still waiting to be controlled and passed into common knowledge.

Figure 5.1 demonstrates that DEP exerts its enhancer effect in the bi-modal, bell-shaped manner characteristic to the CAE substances. It is of extreme importance that the dose of DEP which fully blocks MAO-B is also the optimum dose which elicits the non-specific CAE effect. Since rasagiline and lazabemide are devoid of the enhancer effect (Miklya 2011), it remains for the future to find out the role of the CAE effect in the therapeutic benefits observed for decades.

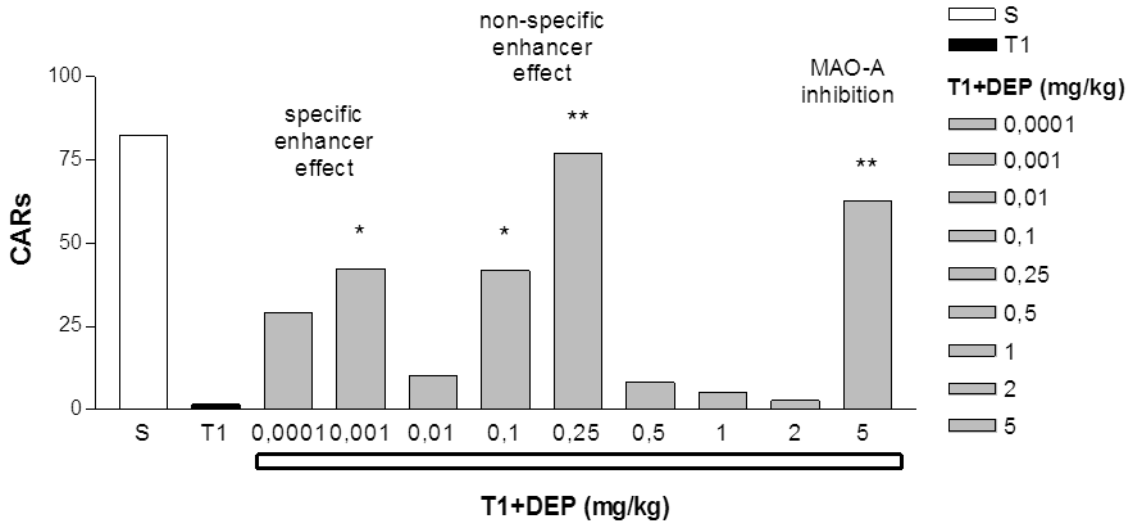


Figure 5.1. *Selection of optimal doses of DEP for the longevity study in the shuttle box.* Antagonism of TBZ-induced inhibition of learning performance in the shuttle box on the fifth day by DEP in the bi-modal, bell-shaped manner characteristic to the CAE substances. Measured: (S) the ability of saline-treated (control) rats to fix conditioned avoidance responses (CARs); (T1) the inhibition of the learning ability of rats treated subcutaneously with 1 mg/kg TBZ, one hour prior to training; (T1 + DEP) the ability of DEP to antagonize in a dose related manner the inhibitory effect of TBZ. Significance in the performance between the groups was evaluated by one-way ANOVA: followed by Newman-Keuls multiple comparison test. *$p<0.05$; **$p<0.01$.

A bi-modal, bell-shaped concentration effect curve is also characteristic to the enhancer effect of BPAP. Figure5.2 shows that BPAP enhanced the activity of the noradrenergic neurons in the femto/picomolar concentration range (*specific* enhancer effect) and also in a 10 million times higher concentration range (*non-specific* enhancer effect); BPAP acts only in a very high concentration on MAO-A (Figure 5.2) and is devoid of MAO-B inhibitory potency. Deprenyl, as shown in Figure 5.1, is a much less potent CAE substance than BPAP, but otherwise it exerts its *specific* and *non-specific* enhancer effect with the same characteristics as BPAP.

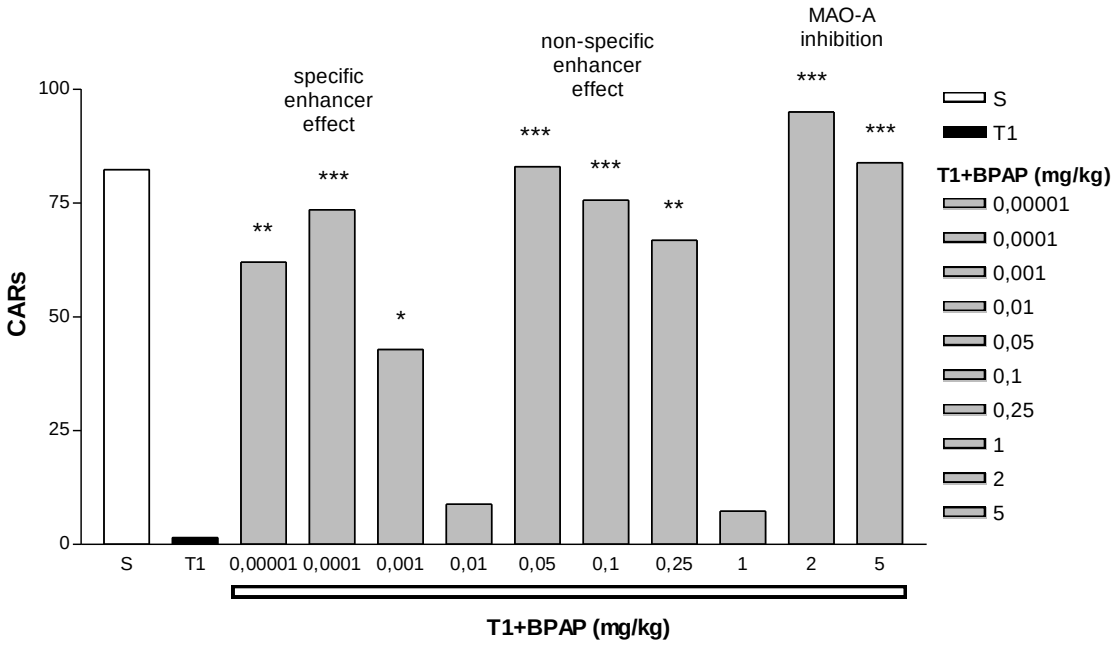


Figure 5.2. *Selection of optimal doses of BPAP for the longevity study in the shuttle box.* Antagonism of TBZ-induced inhibition of learning performance in the shuttle box on the fifth day by BPAP in the bi-modal, bell-shaped manner characteristic to the CAE substances. Measured: (S) the ability of saline-treated (control) rats to fix conditioned avoidance responses (CARs); (T1) the inhibition of the learning ability of rats treated subcutaneously with 1 mg/kg TBZ, one hour prior to training; (T1 + BPAP) the ability of BPAP to antagonize in a dose related manner the inhibitory effect of TBZ. Significance in the performance between the groups was evaluated by one-way ANOVA: followed by Newman-Keuls multiple comparison test. *p<0.01; **p<0.001, ***p<0.0001.

The longevity study with low doses of DEP and BPAP

Table 5.1. The first death (D)in the group treated with saline or an enhancer substance

Age of rats (months)	Saline 0.5 ml/kg	DEP 0.1 mg/kg	DEP 0.001mg/kg	BPAP 0.05 mg/kg	BPAP 0.0001 mg/kg
9	D				
11		D			
13			D		
14				D	
16					D

Table 5.1 shows the first rat death (D) in groups of rats treated with saline or with an enhancer substance, thus immediately suggesting the beneficial influence of BPAP on the lifespan of rats. The first rat died seven months later in the group of rats treated with 0.0001 mg/kg BPAP than the first rat in saline-treated group; BPAP is known to be a more potent enhancer substance than DEP (Knoll 2005) and acts in this test accordingly.

Table 5.2. The average lifespan of rats treated with saline, DEP and BPAP, respectively

TREATMENT	AVERAGE LIFESPAN IN WEEKS	
Saline	94.23 ± 3.48	[100%]
DEP 0.1 mg/kg	105.20 ± 3.07*	[112%]
DEP 0.001 mg/kg	101.60 ± 3.38	[108%]
BPAP 0.05 mg/kg	107.00 ± 3.45**	[114%]
BPAP 0.0001 mg/kg	107.00 ± 3.14***	[114%]

Student t-Statistic for two-means, *P<0.05; **P<0.02; ***P<0.01, mean±S.E.M., N=40. One-way ANOVA F(4/195)=2.632, P=0.0356 followed by Dunnet's multiple comparison test saline versus BPAP 0.05 and BPAP 0.0001 *p<0.05.

Table 5.2 shows average lifespan of rats treated with saline, DEP and BPAP, respectively. Due probably to the low dose treatment with DEP only three times a week, the specific dose of DEP (0.001 mg/kg) did not prolong lifespan significantly.

Table 5.3. Shortest and longest living rats treated with saline, DEP and BPAP, respectively.

TREATMENT	SHORTEST LIVING RAT (weeks)	LONGEST LIVING RAT (weeks)
Saline	36 [100%]	135 [100%]
DEP 0.1 mg/kg	46 [128%]	138 [102%]
DEP 0.001 mg/kg	61 [169%]	135 [100%]
BPAP 0.05 mg/kg	59 [164%]	153 [113%]
BPAP 0.0001 mg/kg	65 [180%]	145 [107%]

Table 5.3 compares the lifespan of the shortest and longest living rat in groups treated with saline, DEP and BPAP, respectively. The differences are remarkable. For example, in the group of rats treated with 0.05 mg/kg BPAP, the shortest living rat lived 59 weeks, 23 weeks (1.64 times) longer than its saline-treated peer (36 weeks). The longest living rat lived 153 weeks, 18 weeks longer than its saline-treated peer (135 weeks). In the group of rats treated with the extremely low dose of BPAP (0.0001 mg/kg), the shortest living rat lived 65 weeks, 1.8 times longer than its saline-treated peer (36 weeks) and the longest living rat in this group lived 145 weeks, 10 weeks longer than its saline-treated peer (135 weeks).

In the saline-treated group only 14 rats in the group treated with 0.1 mg/kg DEP 22 rats; in the group treated with 0.001 mg/kg DEP 19 rats; in the group treated with 0.05 mg/kg BPAP 22 rats; and in the group treated with 0.0001 mg/kg BPAP 23 rats lived longer than 2-years.

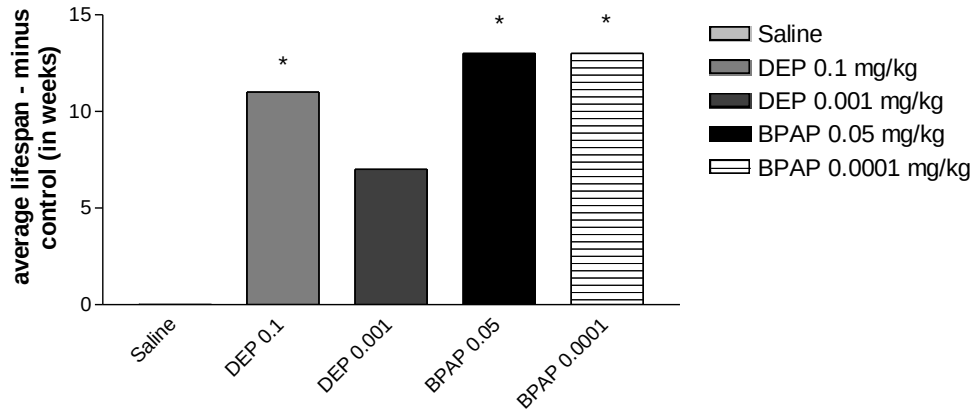


Figure 5.3. The average lifespan of rats minus control in weeks. One-way ANOVA: F(4/195)=2.0356 *p<0.05.

Figure 5.3 shows the average lifespan of rats minus control in weeks. The group of rats treated with 0.1 mg/kg DEP, 0.05 mg/kg and 0.0001 mg/kg BPAP lived significantly longer than their saline-treated peers. Figure 5.3 demonstrates that though the change was not statistically significant, even the rats treated with 0.001 mg/kg DEP lived longer than their saline-treated peers.

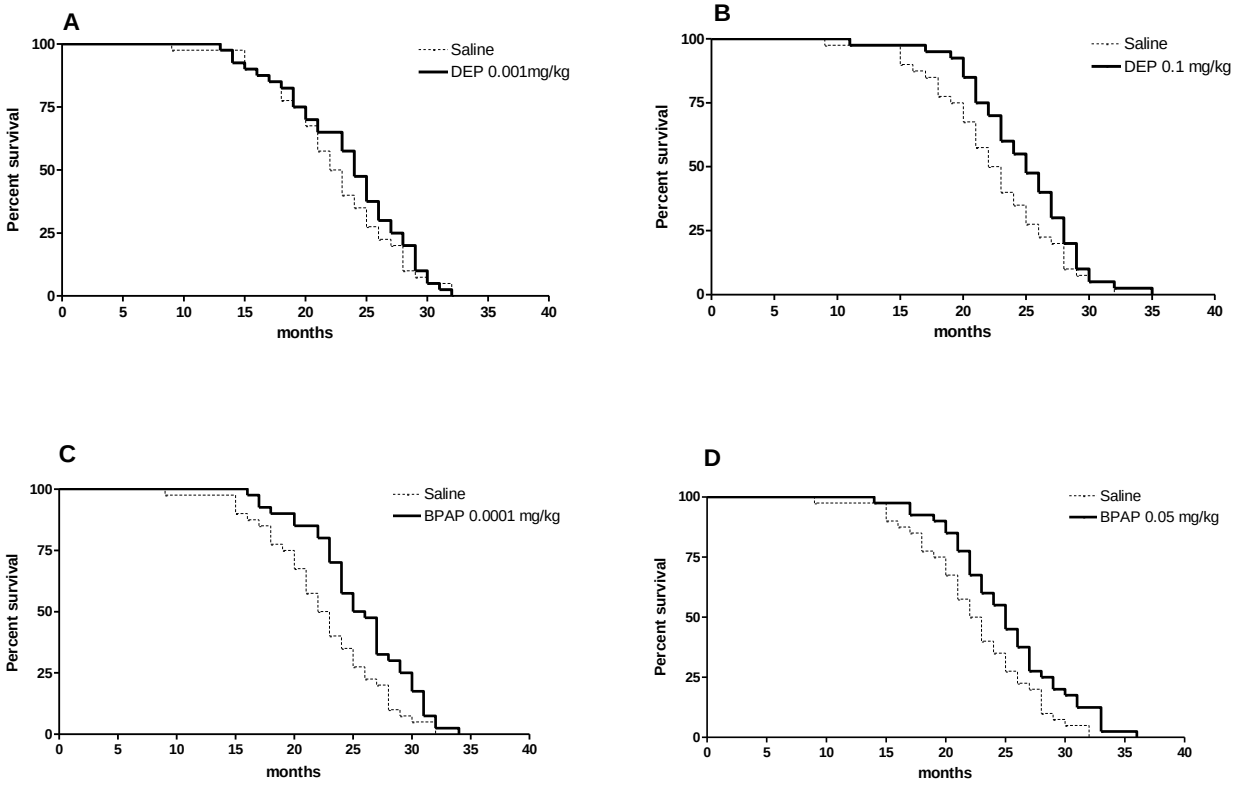


Figure 5.4. Life extension due to a low dose treatment with enhancer substances. Treatment with saline versus DEP (A, B) and BPAP (C, D) in doses selected in the shuttle box test for the longevity study. Kaplan-Meier test, A: DEP 0.001 mg/kg p=0.434 (ns), B: DEP 0.1 mg/kg p=0.0866 (ns); C: BPAP 0.0001 mg/kg *p<0.02 (P=0.011); D: BPAP 0.05 mg/kg *p<0.02 (P=0.011)

Further studies revealed that since aging of the natural enhancers is responsible for the regressive effects of brain aging, but the enhancer-sensitive neurons do not age, the life-long treatment of rats from sexual maturity with 0.0001 mg/kg BPAP prevents the aging-related decay of the dopaminergic neurons, which we used as the first model to present unequivocal experimental evidence that the enhancer-sensitive neurons remain sensitive toward BPAP, the selective and highly potent synthetic enhancer.

Chapter 6

The Physiological significance of the characteristic enhancer control during the developmental phase of mammalian life from weaning until sexual maturity

In the 1950s, we were measuring the hunger-induced orienting-searching reflex activity in rats when we noticed that rats in their late developmental phase of life (2 months of age) moved with unusually enhanced motility compared to their peers in their early post-developmental phase of life (4 months of age). This phenomenon pointed to the enhanced catecholaminergic activity during the developmental phase of life. After carefully reading all pertinent literature I was still unable to find any reasonable explanation regarding the mechanism of this unusual observation; it remained for some time inconceivable (Knoll 1957). The discovery of the enhancer regulation in the mammalian brain in the 1990s and our first paper demonstrating the peculiar enhancer control during the developmental phase of mammalian life finally clarified the responsible mechanism of this early finding (Knoll and Miklya 1995).

Figure 6.1 shows that if we measure the intensity of orienting-searching reflex activity of hungry rats in a new surrounding as a function of time elapsed from the last feeding, we observe the striking difference in activity between rats being in their uphill period of life

(2-month-old animals) and 4-month-old rats being already in their early post-developmental phase of life.

We also observed the awakening of sexual drive, maturation of spermatozoa and the development of the penis in male CFY rats. From the strain we used in this experiment, it was exceptional to find copulatory drive manifesting in males younger than six weeks. Although the appearance of copulatory patterns usually precedes maturation of spermatozoa and a fully developed penis, the overwhelming majority of the males reached full-scale sexual activity by the completion of their 2nd month of life.

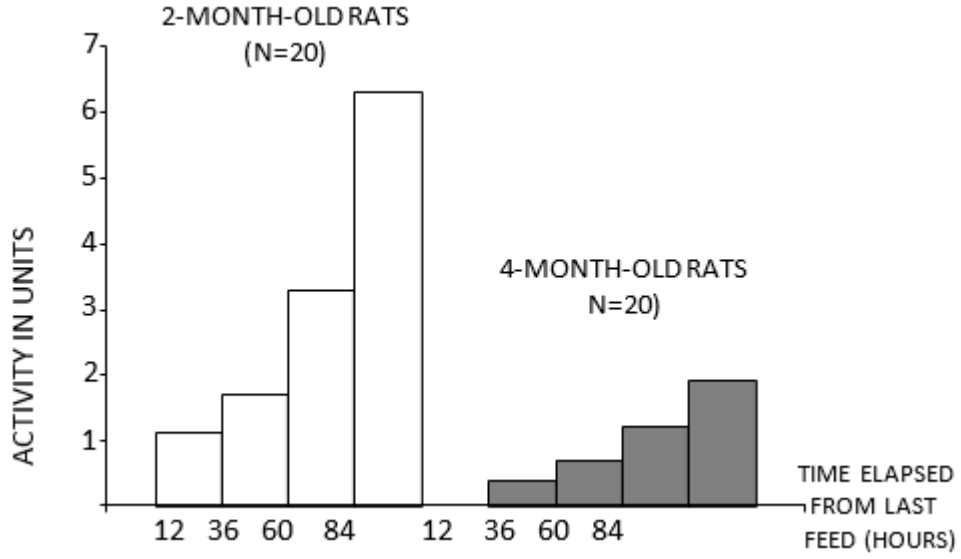


Figure 6.1. Intensity of orienting-searching reflex activity of hungry rats in new surroundings as a function of time elapsed from their last feeding. Activity was measured and expressed in units from 0 to 10 (Knoll and Miklya 1995).

The interval from weaning (3rd week of life) until the end of the 2nd month of age is the decisive period for the development of a rat. During this period the rat acquires abilities crucial for survival as an instinctive means to ensure the survival of the species. Based on the observation that 2-month-old famished rats are significantly more active than their 4-month-old peers, we measured their dopaminergic, noradrenergic and serotonergic activities in the brain before weaning (in 2-week-old rats), during the crucial developmental phase - from weaning to sexual maturity (in 4- and 8-week-old rats) - and in the early post-developmental phase of life (in 16- and 32-week-old rats). As an indicator of the basic activity of catecholaminergic and serotonergic neurons in the brain, we measured the release of dopamine (DA) from striatum, substantia nigra and tuberculum olfactorium; of norepinephrine (NE) from

the locus coeruleus; and of serotonin (SE) from the raphe, in both male and female rats (Knoll and Miklya 1995).

We found that from weaning until the second month of life, the rat's striatal dopaminergic system was significantly more active than either before or after that period.

Figure 6.2 demonstrates a dramatic increase in the release of DA from the striatum and tuberculum olfactorium after weaning (4th week) and the return of the release of DA to the pre-weaning level (2nd week) in sexually mature rats (32nd week).

This finding explains why, as demonstrated in Figure 6.1, food-deprived rats in their developmental phase of life were significantly more mobile in an open field than their peers already in their early post-developmental phase of life. Our finding regarding the age-related changes in the dopaminergic tone in the rat brain was confirmed on Long Evans Cinnamon rats (Samuele, Mangiagalli, Armentero et al. 2005).

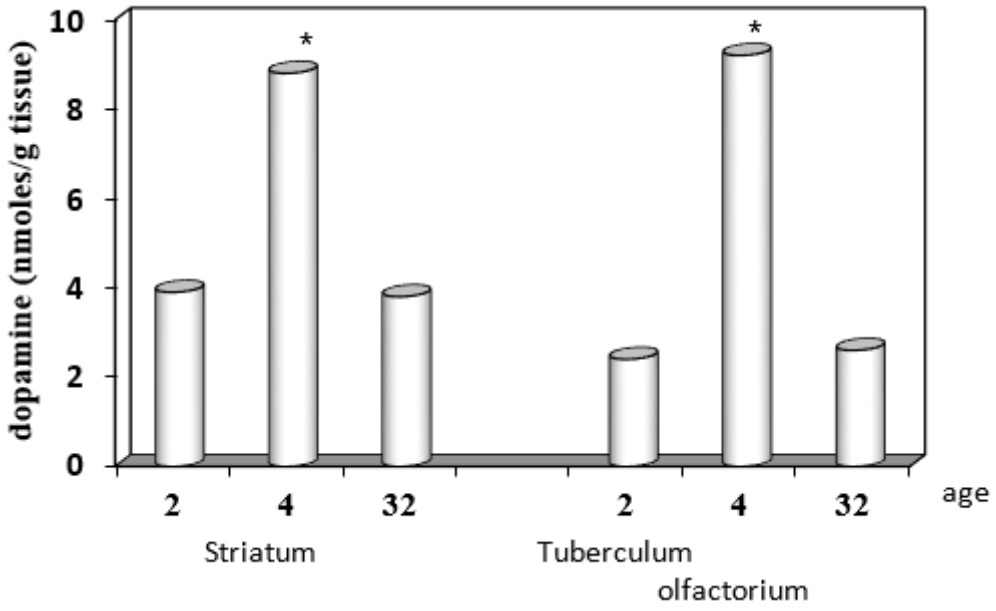


Figure 6.2. Release of DA from the striatum and tuberculum olfactorium, respectively, of male rats belonging to different age cohorts (age in weeks) N=12; *p<0.001 (Knoll and Miklya 1995).

The release of NE from the locus coeruleus (Figure 6.3) and the release of SE from the raphe (Figure 6.4) show the same dramatic increase after weaning and the return to the pre-weaning level in sexually mature rats as DA releases from the striatum and tuberculum olfactorium (Knoll and Miklya 1995).

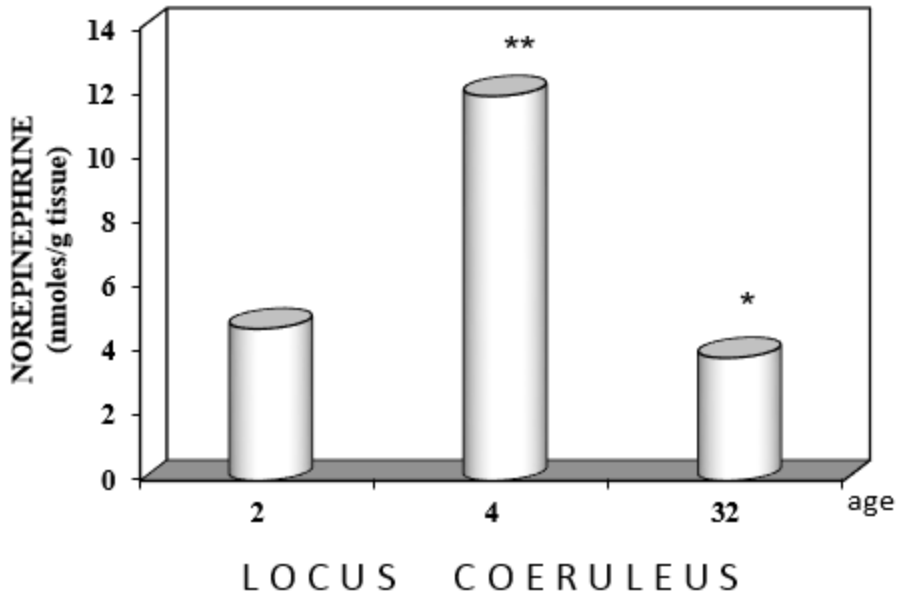


Figure 6.3. Release of NE from the locus coeruleus of male rats belonging to different age cohorts (age in weeks). N=12; *p<0.01, **p <0.001 (Knoll and Miklya 1995).

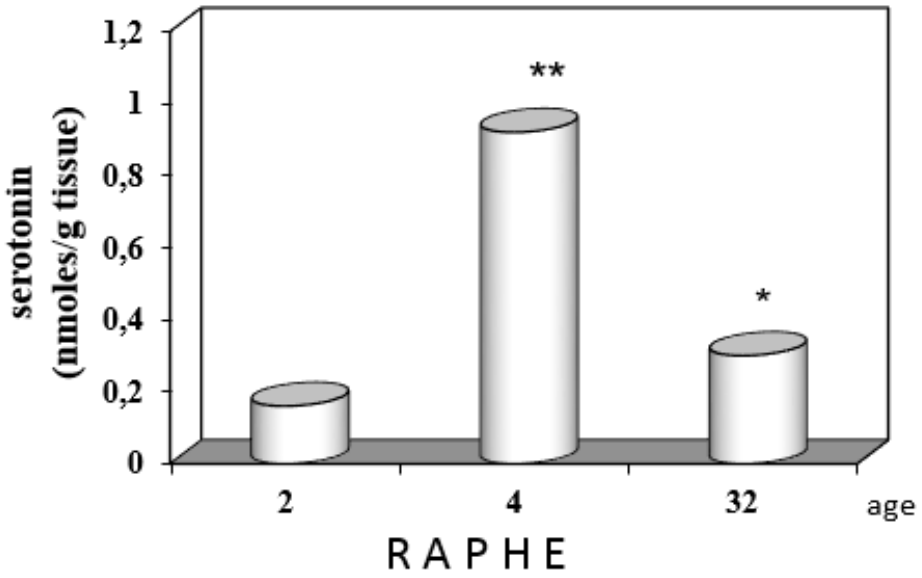


Figure 6.4. Release of SE from the raphe of male rats belonging to different age cohorts (age in weeks). N=12; *p<0.01, **p<0.001 (Knoll and Miklya 1995).

The discovery that the enhancer-regulation plays a key role in controlling the developmental period of mammalian life renders it possible to extend in the future the duration of the uphill period of life via the administration of a safe synthetic enhancer substance. This highly desirable aim is already safely realizable with a low dose DEP treatment.

From this study it was reasonable to deduce that sexual hormones play a key role in terminating the developmental phase of life.

Chapter 7

The Physiological Significance of the Characteristic Enhancer Control During the Post-Developmental Phase of Mammalian Life from Sexual Maturity to Death

Since, from the discontinuation of breast feeding (end of the third week of age) until the appearance of sexual hormones (end of the second month of life),we measured in both male and female rats a significantly pronounced enhancer regulation in the dopaminergic, noradrenergic and serotonergic neurons, it was reasonable to deduce that sexual hormones play the key role in terminating the developmental phase of life (Knoll, Miklya, Knoll and Dalló 2000).

The essence of the post-developmental phase of life is the slow, continuous decline of brain performances, due to the enhancer-sensitive brain regulations aging. The amount of natural enhancers decrease and the consequences are precisely measurable with the continuous, progressive decline of the appropriate brain functions.

The regulation of sexual hormones starts working in rats with full capacity at the end of the second month of age. This rapid decrease in norepinephrine (NE), dopamine (DA) and serotonin (SE) from selected discrete brain regions appeared synchronously with the completion of sexual maturity. Thus, it was reasonable to assume that sexual hormones dampen the enhancer regulation in the catecholaminergic and serotonergic brain stem neurons, and this is the mechanism which terminates developmental longevity as well.

In order to qualify these observations, we castrated three-week-old male and female rats and measured the release of DA, NE and SE from selected discrete brain regions at the end of the third month of their life. We found that in male rats the amount of DA, NE and SE released from the neurons was significantly higher in castrated than in untreated or sham operated rats, signaling that sex hormones inhibit enhancer regulation in the brain (Table 7.1).

To further analyze this effect of sex hormones, we treated male and female rats subcutaneously with oil (0.1 ml/rat), testosterone (0.1 mg/rat), estrone (0.01 mg/rat) and progesterone (0.5 mg/rat), respectively, and measured their effect on the enhancer regulation. Twenty-four hours after a single injection with the hormones, the release of NE, DA and SE was significantly inhibited in the testosterone- or estrone-treated rats (Table 7.2) but remained unchanged after progesterone treatment (Table 7.3).

In rats treated with a single hormone injection, testosterone in the male and estrone in the female was the significantly more effective inhibitor. Remarkably, the reverse order of potency was found in rats treated with daily hormone injections for 7 or 14 days (Table 7.4 and 7.5).After a two-week treatment with hormones, estrone was found in the male and testosterone in the female as the significantly more potent inhibitor of the enhancer regulation (Knoll, Miklya, Knoll and Dalló 2000).

The data prove that sex hormones terminate the hyperactive phase of life by dampening enhancer regulation in the catecholaminergic and serotonergic neurons. They initiate the transition from the developmental phase of life to post-developmental longevity, from adolescence to adulthood. This change is also simultaneously the beginning of the slow, continuous decay of the enhancer regulation in catecholaminergic and serotonergic neurons in the brain stem. As a consequence, the fixation of inextinguishable conditioned reflexes and the acquisition of drives are subject to an inevitable slowly progressing age-related decline until death.

Although the individual variation in decline of behavioral performances over time is substantial, the process developing in every brain and the decay in brain performances as well as the potential to manifest aging-related neurodegenerative diseases (Parkinson’s disease, Alzheimer’s disease) increases with the physiologically irrepressible aging of the brain. It is obvious that only the development of a safe and efficient preventive pharmacological intervention, starting immediately after the completion of sexual maturity, can significantly slow brain aging.

In our two longevity studies, performed with DEP on the robust Wistar-Logan rats, some lived beyond their estimated maximum age of death, showing promise to find in the future efficient means to prolong human life beyond the technical lifespan. This would be a groundbreaking example of man's endeavor to outwit Nature by understanding the laws of its operation.

Table 7.1. The release of catecholamines and SE from selected discrete brain regions isolated from the brain of 3-month-old male and female rats, untreated, sham operated or castrated at the age of 3-weeks.

	Amount of biogenic amine (nmoles/g tissue) released from the tissue within 20 min				
	Dopamine			Norepinephrine	Serotonin
MALES	Striatum	Substantia nigra	Tuberculum olfactorium	Locus coeruleus	Raphe
Untreated	3.4±0.008	4.8±0.17	3.5±0.15	3.9±0.12	0.334±0.01
Sham operated	3.3±0.11	5.2±0.34	3.5±0.16	3.9±0.09	0.329±0.02
Castrated	4.4±0.17**	7.4±0.21**	4.7±0.12**	5.5±0.22**	0.921±0.02**
FEMALES					
Untreated	3.0±0.14	4.5±0.14	2.9±0.05	3.1±0.07	0.337±0.01
Sham operated	2.9±0.13	4.3±0.17	2.8±0.18	3.0±0.05	0.339±0.01
Castrated	4.6±0.29**	8.3±0.18**	3.7±0.06**	4.40±0.05**	0.491±0.03*

Paired Student's t-test. N=16. *p<0.02; **p<0.001.

Table 7.2. The release of catecholamines and SE from selected discrete brain regions isolated from the brain of 4-week-old male and female rats 24 hours after a single subcutaneous injection with oil (0.1 ml/rat), testosterone propionate (0.1 mg/rat) and estrone (0.01 mg/rat), respectively.

	Amount of biogenic amine (nmoles/g tissue) released from the tissue within 20 min				
	Dopamine			Norepinephrine	Serotonin
MALES	Striatum	Substantia nigra	Tuberculum olfactorium	Locus coeruleus	Raphe
Vehicle (A)	6.6±0.23	11.8±0.23	6.8±0.21	9.6±0.19	1.178±0.14
Testosterone (B)	4.7±0.19	10.8±0.34	4.8±0.13	3.4±0.21	0.581±0.11
Estrone (C)	5.8±0.21	11.6±0.26	5.8±0.20	4.2±0.35	0.918±0.04
	A:B ****	A:B *	A:B ****	A:B ****	A:B **
	A:C *	A:C –	A:C ***	A:C ****	A:C –
	B:C **	B:C –	B:C ***	B:C –	B:C *
FEMALES					
Vehicle (A)	7.7±0.27	11.8±0.26	7.9±0.17	9.0±0.26	1.120±0.07
Testosterone (B)	6.8±0.45	11.4±0.21	7.1±0.35	4.7±0.37	0.815±0.09
Estrone (C)	5.5±0.16	11.2±0.39	6.3±0.39	3.7±0.32	0.377±0.11
	A:B –	A:B –	A:B –	A:B ****	A:B *
	A:C ****	A:C –	A:C ***	A:C ****	A:C ***
	B:C ***	B:C –	B:C –	B:C –	B:C *

Paired Student's t-test. N=16. p>0.05; *p<0.05; **p<0.02; ***p<0.01; ****p<0.001.

Table 7.3. The release of catecholamines and SE from selected discrete brain regions isolated from the brain of 4-week-old male and female rats, 24 hours after a single subcutaneous injection with oil (0.1 ml/rat) and progesterone (0.5 mg/rat), respectively.

	Amount of biogenic amine (nmoles/g tissue) released from the tissue within 20 min				
	Dopamine			Norepinephrine	Serotonin
MALES	Striatum	Substantia nigra	Tuberculum olfactorium	Locus coeruleus	Raphe
Vehicle	5.9±0.27	10.4±0.22	6.2±0.31	9.9±0.70	1.071±0.11
Progesterone	5.7±0.20	10.6±0.33	5.9±0.08	10.0±0.05	1.026±0.07
FEMALES					
Vehicle	5.8±0.13	10.5±0.29	6.4±0.21	10.8±0.10	1.080±0.02
Progesterone	5.8±0.15	10.1±0.30	6.2±0.22	10.4±0.80	1.470±0.03

Paired Student's t-test. N=16. p>0.05.

Table 7.4. The release of catecholamines and SE from selected discrete brain regions isolated from the brain of male and female rats injected once daily for 7 days subcutaneously with oil (0.1 ml/rat), testosterone propionate (0.1 mg/rat) and estrone (0.01 mg/rat), respectively.

	Amount of biogenic amine (nmoles/g tissue) released from the tissue within 20 min				
	Dopamine			Norepinephrine	Serotonin
MALES	Striatum	Substantia nigra	Tuberculum olfactorium	Locus coeruleus	Raphe
Vehicle	5.9±0.27	10.4±0.22	6.2±0.31	9.9±0.70	1.071±0.11
Progesterone	5.7±0.20	10.6±0.33	5.9±0.08	10.0±0.05	1.026±0.07
FEMALES					
Vehicle	5.8±0.13	10.5±0.29	6.4±0.21	10.8±0.10	1.080±0.02
Progesterone	5.8±0.15	10.1±0.30	6.2±0.22	10.4±0.80	1.470±0.03

Treatment started on 3-week-old rats. Brain samples were isolated 24 hours after the last injection. Paired Student's t-test. N=16. p>0.05; *p<0.05; **p<0.02; ***p<0.01; ****p<0.001.

Table 7.5. The release of catecholamines and SE from selected discrete brain regions isolated from the brain of male and female rats injected once daily for 14 days subcutaneously with oil (0.1 ml/rat), testosterone propionate (0.1 mg/rat) and estrone (0.01 mg/rat), respectively.

	Amount of biogenic amine (nmoles/g tissue) released from the tissue within 20 min				
	Dopamine			Norepinephrine	Serotonin
MALES	Striatum	Substantia nigra	Tuberculum olfactorium	Locus coeruleus	Raphe
Vehicle (A)	5.8±0.24	14.3±0.30	7.6±0.13	6.5±0.40	1.090±0.01
Testosterone (B)	6.4±0.28	13.0±0.19	5.8±0.24	5.6±0.10	0.415±0.01
Estrone (C)	4.6±0.21	9.8±0.27	5.6±0.21	2.0±0.10	0.213±0.02
	A:B ¯	A:B ***	A:B ****	A:B ¯	A:B ***
	A:C **	A:C ***	A:C ****	A:C ***	A:C ***
	B:C ***	B:C ****	B:C ¯	B:C ***	B:C **
FEMALES					
Vehicle (A)	5.1±0.06	11.7±0.13	6.2±0.15	6.7±0.25	1.007±0.01
Testosterone (B)	4.4±0.18	10.8±0.36	4.5±0.15	3.8±0.15	0.218±0.02
Estrone (C)	5.7±0.23	10.2±0.34	5.6±0.20	6.5±0.30	0.607±0.01
	A:B ***	A:B ¯	A:B ****	A:B ***	A:B ****
	A:C ¯	A:C ***	A:C *	A:C ¯	A:C ****
	B:C ***	B:C ¯	B:C ***	B:C **	B:C ***

Treatment started on 3-week-old rats. Brain samples were isolated 24 hours after the injection. Paired Student's t-test. N=16. ¯p>0.05; *p<0.05; **p<0.02; ***p<0.01; ****p<0.001.

Chapter 8

The anti-aging effect of DEP and BPAP

We can define enhancer-regulations as: the existence of enhancer-sensitive neurons capable of changing their excitability in milliseconds and working on a higher activity level, due to natural or synthetic enhancer substances (Knoll 2005, 2016).

Chapters 3 and 4 showed that the catecholaminergic and serotonergic neurons were identified and analyzed in detail as the first models of life important enhancer-sensitive brain regulations.

Analyzing the molecular mechanism of (2*R*)-1-(1-benzofuran-2-yl)-*N*-propylpentane-2-amine (BPAP)'s specific and non-specific enhancer effects we clarified that the interaction with

distinct sites on the vesicular monoamine-transporter-2 (VMAT-2) is the main mechanism of action of the enhancer substances. This finding elucidates BPAP's highly characteristic bi-modal, bell-shaped concentration-effect curves.

The discovery that natural enhancers maintain biological vigor during the developmental-phase of life, from weaning until sexual maturity, on a hyperactive level (Knoll and Miklya 1995); sexual hormones return the enhancer's activity back to its pre-weaning low level (Knoll, Miklya, Knoll and Dalló 2000); and due to post-developmental continuously progressing diminishment in the natural stimulation of the enhancer-sensitive brain regulations, mammals life is exactly limited to their technical life span (Knoll 2012).

As summarized in detail in Chapter 6, we presented unequivocal experimental evidence that as soon as sexual hormones terminate the developmental/uphill phase of life; the slow, continuous, progressively aging enhancer-sensitive brain-regulations begin and last until death. The amount of the natural enhancers shows, over time, a downward tendency and the consequences are exactly measurable with the continuous, progressive decline of the appropriate brain functions.

We finally published in 2016 the first longevity study demonstrating that the enhancer effect of the DEP and BPAP are responsible for life extension, and using the enhancer-sensitive dopaminergic neuron as experimental model, we presented the first experimental evidence in a longevity study that since enhancer-sensitive neurons do not age we can significantly extend the life expectancy of mammals by maintaining them during their post-developmental phase of life, on a low daily dose of a synthetic enhancer substance (Knoll and Miklya 2016).

DEP's pharmacological spectrum is unique. It is worthwhile to briefly recall that prior to the discovery of the enhancer-sensitive brain regulations the peculiar mode of DEP's effect on the nigrostriatal dopaminergic neurons appeared to represent a hitherto unknown brain regulation. This view was supported by a successful biochemical analysis.

We found in the early 1980s that the striata of rats treated with 0.25 mg/kg DEP daily for three weeks released five times more dopamine (DA) in the resting state and seven times more DA in response to KCl stimulation than the striata removed from rats treated daily with 0.1 ml/100 g saline. The striata were removed 24 hours after the last injection of saline or DEP, respectively. DEP increased the rate of utilization of DA in the striatum of DEP-treated rats. The increase in the turnover rate of DA in the striatum was due to the enhancement of the fractional rate constant of

DA efflux and the significant increase in the DA content. We soon realized that the facilitation of striatal dopaminergic neurotransmission by long term treatment is highly specific. With regard to noradrenaline a significant decrease in the turnover rate and unchanged level of this amine in the brain stem was found, and no change in the turnover rate of serotonin (SE)-treated daily with 0.25 mg/kg DEP for two weeks was detected (Zsilla and Knoll 1982; Zsilla, Szekely and Knoll 1986).

Now we know that DEP is a PEA-derived almost specific synthetic CAE substance, which being the only one free of the catecholamine-releasing property of PEA, its natural parent compound, made it possible to discover the operation of the enhancer-sensitive regulations in the mammalian brain (Knoll 1998).

The discovery of the enhancer-sensitive brain regulations and the development of DEP and BPAP, the synthetic enhancers, allowed a new approach to better understand the essence of brain aging and elaborate a previously unimaginable, simple and safe method to prevent the manifestation of the regressive effect of brain aging. To characterize the essence of brain aging we analyzed the aging-related decline of two dopaminergic functions: sexual activity and learning ability.

The Aging-related Decline of Sexual Activity

It is fascinating to compare the astonishing similarity in human and rat males in the aging-related decline of the mesencephalic dopaminergic system and realize the same functional consequences, the progressing weakening and final extinction of the ejaculatory activity during their postdevelopmental phase of life .

Sexual activity in the human male is known to be influenced by a number of factors, such as good health, stable marriage, satisfactory sexual partner(s) and adequate financial and social status. But even in the males who meet all the requirements for retention and maintenance of sexual functioning, there is an age-related decrease in sexual vigor.

In the Baltimore Longitudinal Study of Aging, coital activity was studied as function of age. They interviewed 628 members of the Washington-Baltimore area, varying from 20-95 years of age, white, married, urban residents in good health. According to this study the median coital

activity was highest, ***2.1*** events/week, between ages of 30-34, and decreased progressively with increasing age, sinking to ***0.2***/week in the age-group 65-69.

It is common knowledge that individual variation in sexual vigor is enormous. In this study the mean frequency of total sexual activity in 159 males was found to be 520 sexual events/5 years in the age-group 20-39, including young males performing below 100 sexual events/5 years and those with frequencies of total sexual activity over 1000 sexual events/5 years. In the age-group 65-79, the mean frequency of total sexual activity decreased to 75 sexual events/5 years, but even in this group subjects producing 400-700 sexual events/5 years were registered (Martin 1977).

In a number of longitudinal studies performed on male rats we observed that the age-related decline of coital activity in male rats and the striking individual differences in sexual performance in different age cohorts are essentially the same as in human males (Knoll 1988, 1989, 1990; Knoll, Dalló and Yen 1989; Martin 1977). Because of brain aging, even the most sexually high performing males may lose their potency to ejaculate if they live long enough. In our studies on male CFY rats, we followed the sexual performance of the animals once a week from sexual maturity until death. We measured three patterns: mounting, intromission and ejaculation. We found that in response to brain aging even the best performing individuals lost their potency to ejaculate no later than the completion of their second year of age (Knoll 1990). The results of our first longevity study clearly proved in retrospect that the age-related decline of the sexual performance of male rats signals the decay of the enhancer regulation in the dopaminergic neurons over time (Knoll 1988, 1989; Knoll, Yen and Miklya 1994).

In this series of experiments, we selected 132 aged, *2-year-old* male rats and measured in four consecutive weekly mating tests their sexual performance: mounting, intromission and ejaculation. Due to aging, the ability to ejaculate ceased in 2-year-old CFY rats. We classified the rats according to their sexual performance in the testing period as non-copulators (no sign of sexual activity), mounting rats (displayed mounting only) and sluggish rats (displayed mounting and intromission). Of the 132 rats 46 were found to be non-copulators (Group 1), 42 displayed mounting only (Group 2) and 44 rats proved to be sluggish (Group 3). After the selection period, we started to treat half of the rats with saline (1 ml/kg) and half with DEP (0.25 mg/kg) three times a week, until they died. We tested their sexual performance once a week. The dying out of the 66 saline-treated rats showed that lifespan was proportional to their sexual performance. As sexual

performance is directly proportional to the functional state of the enhancer regulation in the dopaminergic neurons, we assume that rats die when the age-related decline in mesencephalic enhancer regulation reached a critical threshold. With regard to sexual performance: Group 1 < Group 2 < Group 3, thus, rats belonging to Group 1 are the closest to exceeding the critical threshold resulting in natural death and die out first; rats in Group 2 live longer; and rats in Group 3 live the longest.

The age-related decline in mesencephalic enhancer regulation during the post-developmental phase of life in male rats can be further recognized by comparing the individual variation in sexual performance of 3-6-month-old male rats with the performance of their 2-year-old peers. Whereas 52.49% of 3-6-month-old male rats displayed ejaculations during the four consecutive mating tests, only 5.80% of 12-18-month-old males ejaculated and none of the 24-month-old males were endowed with this faculty any longer (Knoll 2005).

Moreover, the age-related change in the percentage of animals belonging to the "non-copulator" group clearly proved that enhancer regulation in the dopaminergic neurons is in continuous decline during the post-developmental phase of life. Only 5.51% of the 3-6-month-old males were sexually inactive, but 19.56% of the 12-18-month-old rats and 34.84% of the 24-month-old rats belonged to this group.

Due to the striking similarities between human and rat males in the age-related decline of their sexual activity it is hard to deny that the decay of the dopaminergic machinery over time plays the key role on the final loss of the ability to ejaculate, from which there is no escape. We demonstrated with a series of experiments that the treatment of male rats with DEP significantly enhanced their sexual activity and with the preventive administration of a small dose of DEP the loss of the ability to ejaculate was substantially shifted in time (Knoll 1988, 1989, 1990, 1993; Knoll, Dalló and Yen 1989; Knoll, Yen and Miklya 1994; Martin 1977).

In Table 8.1, a brief summary of the results of our first longevity study shows that the anti-aging effect of DEP was decisive even in a series of experiments performed on two-year-old rats which had already lost their ability to ejaculate.

Classification of the groups according to sexual performance before treatment	Number of animals	Total number of mountings (M), intromissions (I) and ejaculations (E) of the groups during treatment		
		M	I	E
		Saline-treated rats		
Non-copulators	23	37	0	0
Mounting rats	21	425	54	0
Sluggish rats	22	477	231	0
		DEP-treated rats		
Non-copulators	23	997	544	190
Mounting rats	21	1129	662	172
Sluggish rats	22	1696	1257	481

Table 8.1. Illustration of the antiaging effect of DEP-treatment on 2-year-old rats. Data taken from the first longevity study (Knoll, Yen and Miklya 1994).

A second example demonstrates on young male CFY rats the DA-dependency of sexual performance and the significant anti-aging effect of DEP-treatment. We selected 90 males possessing full-scale sexual activity. Half of the population was treated with saline (1 ml/kg), the other half with DEP (0.25 mg/kg), three times a week, from the 25th week of age. The rats' sexual performance was tested once a week. In this study, the loss in the ability to ejaculate was selected as the age-related end stage. Saline-treated rats reached this stage at an average of ***112±9*** weeks. In contrast, DEP-treated rats reached it at an average of ***150±12*** weeks (P<0.001) (Knoll 1993). As sexual performance is a dopaminergic function, it became obvious that the enhanced activity of the mesencephalic dopaminergic neurons was responsible for the significantly retarded loss of the ability to ejaculate in the DEP-treated group.

The Dopamine-dependent Age-related Decline of Learning Ability

In a modified version of the shuttle box, originally described by Bovet, Bovet-Nitti and Oliverio (1966), the acquisition of a two-way conditioned avoidance reflex (CAR) was analyzed

over five consecutive days. The rat was put in a box divided inside into two parts by a barrier with a small gate in the middle and the animal was trained to cross the barrier under the influence of a conditioned stimulus (CS, light flash). If it failed to respond within 5s, it was punished with a foot shock (1mA), the unconditioned stimulus (US) and it was classified as an escape failure (EF). One trial consisted of 10s inter-trial interval, followed by 20s CS; the last 5s of CS overlapped the 5s US. At each learning session, the number of CARs, EFs and inter-signal reactions (IRs) were automatically counted and evaluated by multi-way ANOVA.

The dopaminergic machinery is the most rapidly aging neuronal system in our brain. The dopamine content of the human caudate nucleus decreases steeply, at a rate of 13% per decade over age 45. We know that symptoms of Parkinson's disease (PD) appear if the DA content of the caudate sinks below 30% of the normal level. Experimental and clinical experiences show that daily DEP-dosages keep the brain engine on a higher activity level in humans. From sexual maturity, a low daily dose of DEP (1 mg) is sufficient to significantly slow the pace of the aging-related decay of the dopaminergic neurons. Even if we assume only a small protective effect in healthy humans against the age-related decrease in striatal DA, for example from 13% per decade to 10% per decade, this translates to a minimum 15-year extension in average lifespan and a considerable increase in lifespan, which is now estimated to be around 115 years (Knoll 1992).

Due to aging of the dopaminergic neurons, saline-treated 3-month-old rats are significantly better learners than their saline-treated 1-year-old peers (Figure 8.1). Since synthetic enhancers keep the dopaminergic neurons working on a higher activity level, rats treated with 0.1 mg/kg DEP (Figure 8.2), or 0.0001 mg/kg BPAP (Figure 8.3), showed no sign of aging-related decay in the learning ability

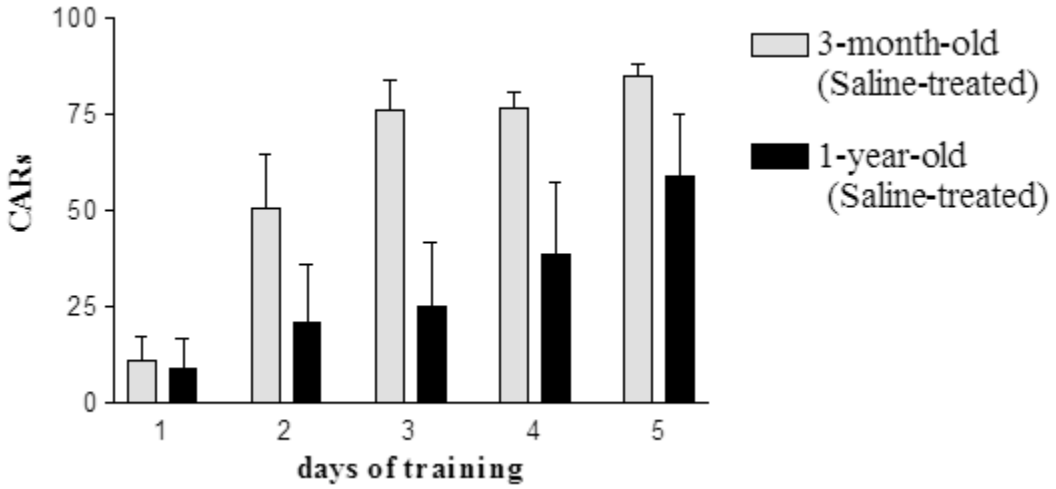


Figure 8.1. Experimental evidence that 3-month-old rats are significantly better learners than their 1-year-old peers ($P<0.001$). Significance in the performance between the groups was evaluated by multi-factor analysis of variance (ANOVA). Rats were trained in the shuttle box with 100 trials per day. Conditioned avoidance responses (CARs).

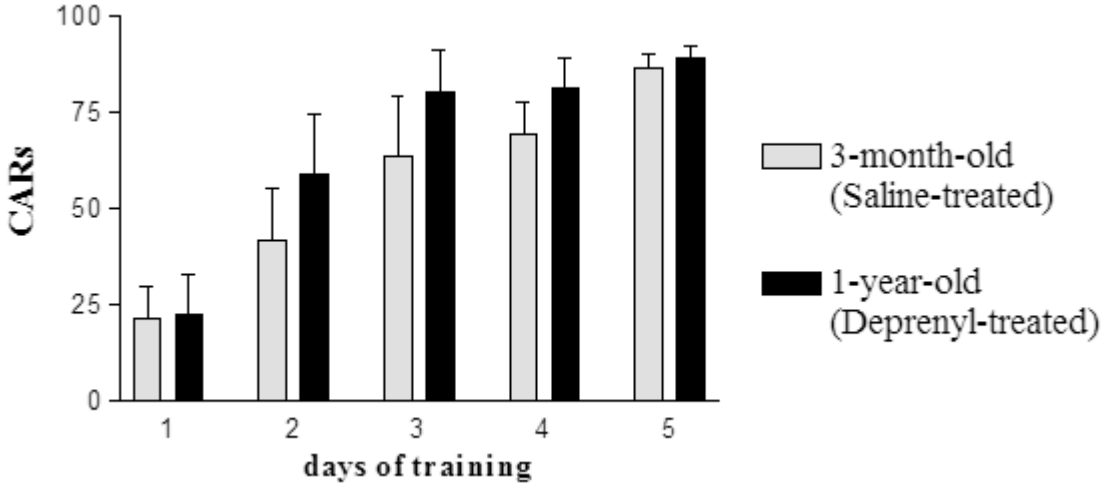


Figure 8.2. Experimental evidence shows that in rats treated with 0.1 mg/kg DEP there is no sign of aging-related decay in the learning ability. Rats were trained in the shuttle box with 100 trials per day. Significance in the performance between the groups was evaluated by multi-factor analysis of variance (ANOVA). There was no significant difference in the acquisition of conditioned avoidance responses (CARs) between the 3-month-old rats treated with saline and 1-year-old rats treated with DEP.

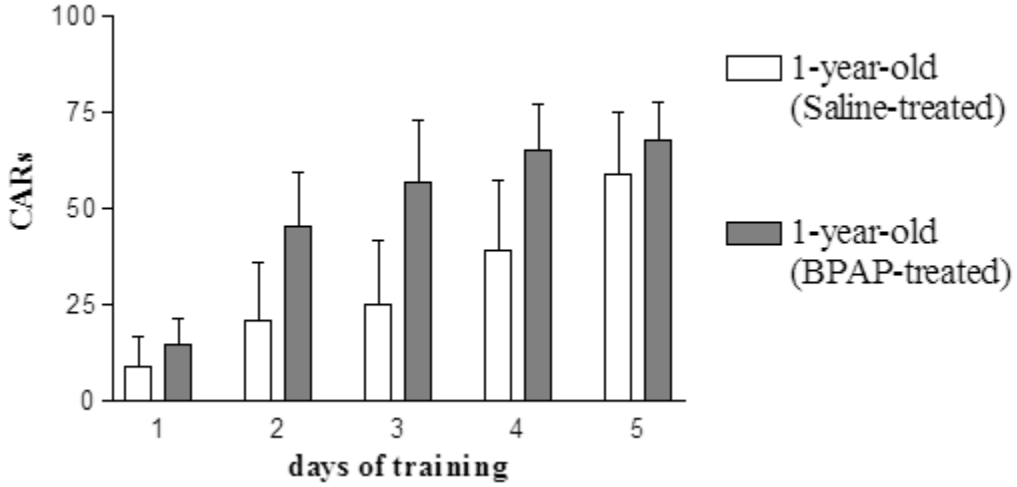


Figure 8.3. The anti-aging effect of 0.0001 mg/kg BPAP. 1-year-old rats were treated for 10 months subcutaneously, 3-times a week, with 0.0001 mg/kg BPAP. Their performance was compared to saline-treated rats. Rats were trained in the shuttle box with 100 trials per day. Significance in the performance between the groups was evaluated by multi-factor analysis of variance (ANOVA). BPAP treated rats performed significantly better than their saline-treated peers ($p<0.05$).

The Enhancer-sensitive Neurons Do Not Age

We realized in preliminary studies that in contrast to the rapidly aging natural enhancers, the enhancer-sensitive neurons do not age, thus the synthetic enhancers are capable to substitute the lost natural enhancers, thus to prevent the regressive effects of brain aging. To present unequivocal experimental evidence that enhance-sensitive neurons remain sensitive toward synthetic enhancers, we tested 3 monthly in selected naïve group of rats treated with saline, DEP and BPAP, respectively, the aging-related changes in learning ability as significant measure of the anti-aging effect of the enhancer substances (Knoll and Miklya 2016).

The shuttle box data were analyzed by two-way ANOVA followed by Bonferroni post-hoc test; and the fifth day results were calculated by one-way ANOVA followed by Newman-Keuls or Dunnet's multiple comparison test. Differences were considered significant at $p<0.05$.

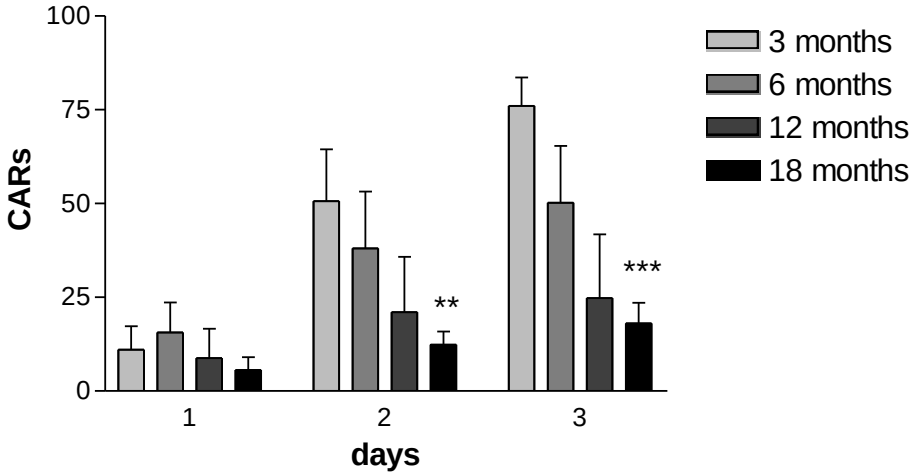


Figure 8.4. The age-related physiological decline in the learning ability of 3-, 6-, 12- and 18- month-old saline-treated rats. CARs-conditioned avoidance responses. Two-way ANOVA followed by Bonferroni post-hoc test: CAR age $F(3/48)=5.961$ $^{**}p<0.01$; days $F(2/48)=8.746$.

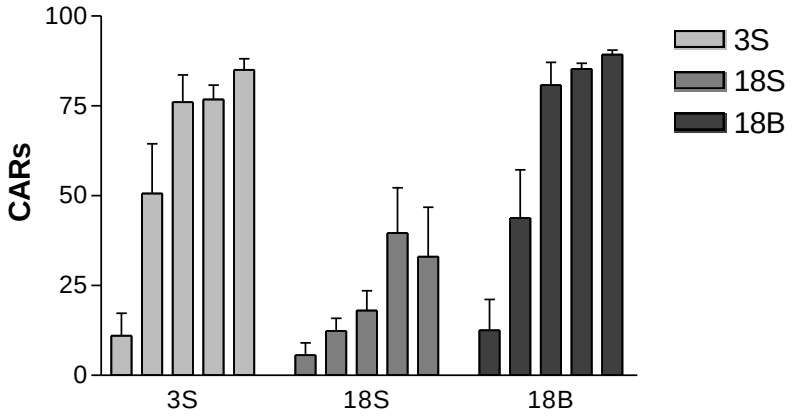


Figure 8.5.
3S: The full capacity of 3-month-old saline-treated rats in their ability to fix a condition avoidance response in the shuttle box during training for 5 consecutive days.
18S: The aging-related serious decline in the ability of saline-treated 18-month-old rats in building a condition avoidance response in the shuttle box during 5-day training.
18B: In contrast to saline-treated 18-month-old rats the longevity treatment with 0.0001mg/kg BPAP dramatically changed the ability of rats to fix in a 5-day consecutive training of a conditioned avoidance response. The performance of the18-month-old BPAP-treated rats was equivalent with the 3-month-old rat's performance. Two-way ANOVA; $F(8/55)=2.284$ $^{*}p<0.05$.

Figure 8.4 shows the conditioned avoidance responses in groups of 5 saline-treated rats selected from the longevity study. The young, 3-month-old rats showed their normal performance;

the 6-month-old group of rats already showed a considerable decline in their learning performance. The 12-month-old rats gave evidence of further loss in their ability to build CARs, and nevertheless, the difference according to the Bonferroni post-hoc test is still not significant, obviously due to the small number of the tested rats (N=5).

This longevity study was the first demonstration that lifelong treatment with 0.0001 mg/kg BPAP, the peak dose with the specific enhancer effect, completely prevented aging of learning ability. We used the learning test as a highly sensitive model to measure the aging-related decay of the dopaminergic neurons. The BPAP-treated 18-month-old rats performed in the shuttle box like the saline-treated 3-month-old rats. This finding is an unprecedented, convincing proof that the enhancer-sensitive dopaminergic neurons do not age. Thus, the development of the first synthetic enhancer substances guides the way to prevent the regressive effects of brain aging.

Chapter 9

Mechanisms of DEP's and BPAP's Anti-Aging Effect

Chapters 3 and 4 showed that the catecholaminergic and serotonergic neurons were identified and analyzed in detail as the first models of life-important enhancer-sensitive brain regulations.

Analyzing the molecular mechanism of BPAP's specific and non-specific enhancer effects, we clarified that the interaction with distinct sites on the VMAT2 is the main mechanism of action of the enhancer substances. This finding elucidates BPAP's highly characteristic bi-modal, bell-shaped concentration-effect curves (see Chapters 4 and 10).

Natural enhancers maintain biological vigor during the developmental-phase of life, from weaning until sexual maturity, on a hyperactive level (Knoll and Miklya 1995). Sexual hormones return the enhancer's activity back to its pre-weaning low level (Knoll, Miklya, Knoll and Dalló 2000) and due to post-developmental continuously progressing diminishment in the natural

stimulation of the enhancer-sensitive brain regulations (Knoll 2012). Mammals' life is exactly limited to their TLS.

As summarized in detail in Chapter 6, we presented experimental evidence that as soon as sexual hormones terminate the developmental/uphill phase of life, the slow, continuous, progressively aging enhancer-sensitive brain-regulations begin and last until death. The amount of the natural enhancers shows over time a downward tendency and the consequences are exactly measurable with the continuous, progressive decline of the appropriate brain functions.

We finally published in 2016 the first longevity study demonstrating that the enhancer effect of the DEP and BPAP are responsible for life extension. Using the enhancer-sensitive dopaminergic neuron as experimental model, we presented the first experimental evidence in a longevity study that since enhancer-sensitive neurons do not age (Knoll and Miklya 2016) *we can significantly extend the life expectancy of mammals by maintaining them during their post-developmental phase of life, on a low daily dose of a synthetic enhancer substance.*

DEP's pharmacological spectrum is unique (Chapter 3). It is worth to briefly recall that prior to the discovery of the enhancer-sensitive brain regulations the peculiar mode of DEP's effect on the nigrostriatal dopaminergic neurons appeared to represent a hitherto unknown brain regulation. A successful biochemical analysis supported this view.

We found in the early 1980s that the striata of rats treated with 0.25 mg/kg DEP daily for three weeks released five times more dopamine (DA) in the resting state and seven times more DA in response to KCl stimulation than the striata removed from rats treated daily with 0.1 ml/100 g saline. The striata were removed 24 hours after the last injection of saline or DEP, respectively. DEP increased the rate of utilization of DA in the striatum of DEP-treated rats. The increase in the turnover rate of DA in the striatum was due to the enhancement of the fractional rate constant of DA efflux and the significant increase in the DA content. We realized that the facilitation of striatal dopaminergic neurotransmission by long term treatment is highly specific. With regard to NE a significant decrease in the turnover rate and unchanged level of this amine in the brain stem was found (Zsilla and Knoll 1982) and no change in the turnover rate of serotonin (SE) treated daily with 0.25 mg/kg DEP for two weeks was detected (Zsilla, Szekely and Knoll 1986).

DEP, being the only PEA-derivative, free of the catecholaminergic property, made the discovery of the enhancer-sensitive regulation in the mammalian brain possible (Knoll 1998).

The discovery of the enhancer sensitive brain regulations and the development of DEP and BPAP, the synthetic enhancers, allowed a new approach to better understand the essence of brain aging and elaborate a previously unimaginable, simple and safe, method to prevent the manifestation of the regressive effect of brain aging. To characterize the essence of brain aging we analyzed the aging-related decline of two dopaminergic functions: sexual activity and learning ability.

The Age-related Decline of Sexual Activity

It is fascinating to compare the astonishing similarity in human and rat males in the age-related decline of the mesencephalic dopaminergic system and realize the same functional consequences, the progressing weakening and final extinction of the ejaculatory activity during their post-developmental phase of life.

Sexual activity in the human male is known to be influenced by a number of factors, such as good health, stable marriage, satisfactory sexual partner(s) and adequate financial and social status. But even in the males who meet all the requirements for retention and maintenance of sexual functioning, there is an age-related decrease in sexual vigor.

In the Baltimore Longitudinal Study of Aging, coital activity was studied as function of age. They interviewed 628 members of the Washington-Baltimore area, varying from 20-95 years of age, white, married, urban residents in good health. According to this study the median coital activity was highest, **2.1** events/week, between ages of 30-34, and decreased progressively with increasing age, sinking to **0.2**/week in the age-group 65-69.

It is common knowledge that individual variation in sexual vigor is enormous. In this study, the mean frequency of total sexual activity in 159 males was found to be 520 sexual events/5 years in the age-group 20-39, including young males performing below 100 sexual events/5 years and those with frequencies of total sexual activity over 1000 sexual events/5 years. In the age-group 65-79, the mean frequency of total sexual activity decreased to 75 sexual events/5 years, but even in this group subjects producing 400-700 sexual events/5 years were registered (Martin 1977).

In a number of longitudinal studies performed on male rats we observed that the age-related decline of coital activity in male rats and the striking individual differences in sexual performance in different age cohorts are essentially the same as in human males (Knoll, Dalló and Yen 1989; Knoll 1988, 1989, 1990, 1993). Because of brain aging, even the most sexually high performing males may lose their potency to ejaculate if they live long enough. In our studies on male CFY rats, we followed the sexual performance of the animals once a week from sexual maturity until death. We measured three patterns: mounting, intromission, and ejaculation. We found that in response to brain aging even the best performing individuals lost their potency to ejaculate no later than the completion of their second year of age (Knoll 1993). The results of our first longevity study (Knoll 1988; 1993) clearly proved in retrospect that the age-related decline of the sexual performance of male rats signals the decay of the enhancer regulation in the dopaminergic neurons over time.

As shown in Chapter 6, in this series of experiments, we selected 132 aged, *2-year-old* male rats and measured in four consecutive, weekly mating tests their sexual performance: mounting, intromission and ejaculation. Due to aging, the ability to ejaculate ceased in 2-year-old CFY rats. We classified the rats according to their sexual performance in the testing period as non-copulators (no sign of sexual activity), mounting rats (displayed mounting only), and sluggish rats (displayed mounting and intromission). Of the 132 rats 46 were found to be non-copulators (Group 1), 42 displayed mounting only (Group 2), and 44 rats proved to be sluggish (Group 3). After the selection period, we started to treat half of the rats with saline (1 ml/kg) and half with DEP (0.25 mg/kg) three times a week, until they died. We tested their sexual performance once a week. The dying out of the 66 saline-treated rats showed that lifespan was inversely proportional to their sexual performance. As sexual performance is directly proportional to the functional state of the enhancer regulation in the dopaminergic neurons, we assume that rats die when the age-related decline in mesencephalic enhancer regulation reached a critical threshold. With regard to sexual performance: Group 1 < Group 2 < Group 3, thus, rats belonging to Group 1 are the closest to exceeding the critical threshold resulting in natural death and die out first, rats in Group 2 live longer, and rats in Group 3 live the longest.

We compared during the post-developmental phase of life in male rats the individual variation in sexual performance of 3-6-month-old male rats with the performance of their 2-year-old peers. Whereas 52.49% of 3-6-month-old male rats displayed ejaculations during the four

consecutive mating tests, only 5.80% of 12-18-month-old males ejaculated, and none of the 24-month-old males were endowed with this faculty any longer.

Moreover, the age-related change in the percentage of animals belonging to the "non-copulator" group clearly proved that enhancer regulation in the dopaminergic neurons is in continuous decline during the post-developmental phase of life. Only 5.51% of the 3-6-month-old males were sexually inactive, but 19.56% of the 12-18-month-old rats and 34.84% of the 24-month-old rats belonged to this group.

Due to the striking similarities between human and rat males in the age-related decline of their sexual activity it is hard to deny that the decay of the dopaminergic machinery over time plays the key role on the final loss of the ability to ejaculate, from which there is no escape. We demonstrated with a series of experiments that the treatment of male rats with DEP significantly enhanced their sexual activity and with the preventive administration of a small dose of DEP the loss of the ability to ejaculate was substantially shifted in time (Knoll, Dalló and Yen 1989; Knoll 1988, 1989, 1990, 1993; Yen, Dalló and Knoll 1982).

In Table 9.1, a brief summary of the results of our first longevity study, shows that the anti-aging effect of DEP was decisive even in a series of experiments performed on two-year-old rats which had already lost their ability to ejaculate.

Table 9.1 Illustration of the antiaging effect of DEP treatment. Data taken from the first longevity study (Knoll, Yen and Miklya 1994)				
Classification of the groups according to sexual performance before treatment	**Number of animals**	**Total number of mountings (M), intromissions (I) and ejaculations (E) of the groups during treatment**		
		M	**I**	**E**
Saline-treated rats				
Non-copulators	23	37	0	0
Mounting rats	21	425	54	0
Sluggish rats	22	477	231	0
DEP-treated rats				
Non-copulators	23	997	544	190
Mounting rats	21	1129	662	172
Sluggish rats	22	1696	1257	481

A second example demonstrates on young male CFY rats the dopamine-dependency of sexual performance and the significant anti-aging effect of DEP-treatment. We selected 90 males possessing full-scale sexual activity. Half of the population was treated with saline (1 ml/kg), the other half with DEP (0.25 mg/kg), three times a week, from the 25th week of age. The rats' sexual performance was tested once a week. In this study, the loss in the ability to ejaculate was selected as the age-related end stage. Saline-treated rats reached this stage at an average of **112±9** weeks. In contrast, DEP-treated rats reached it at an average of **150±12** weeks (P<0.001) (Knoll 1989). As sexual performance is a dopaminergic function, it became obvious that the enhanced activity of the mesencephalic dopaminergic neurons was responsible for the significantly retarded loss of the ability to ejaculate in the DEP-treated group.

The Dopamine-dependent Age-related Decline of Learning Ability

In a modified version of the shuttle box we analyzed the acquisition of a CAR over 5 consecutive days. The technique, originally published by Bovet, Bovet-Nitti and Oliverio in 1966, was described in Chapter 6.

Due to aging of the dopaminergic neurons, saline-treated 3-month-old rats are significantly better learners than their saline-treated 1-year-old peers (Figure 9.1). Since synthetic enhancers keep the dopaminergic neurons working on a higher activity level, rats treated with 0.1 mg/kg DEP (Figure 9.2), or 0.0001 mg/kg BPAP (Figure 9.3) showed no sign of aging-related decay in the learning ability.

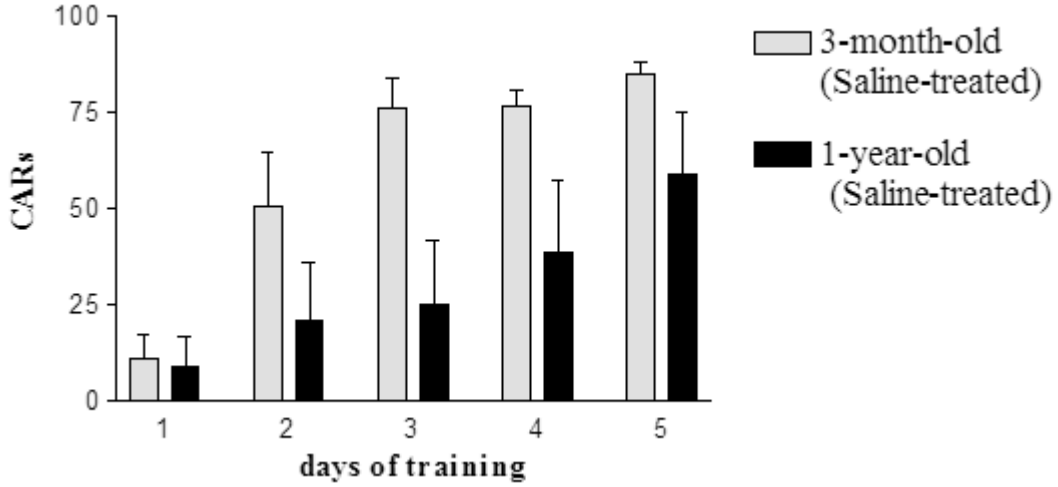


Figure 9.1. Experimental evidence that 3-month-old rats are significantly better learners than their 1-year-old peers ($P<0.001$). Significance in the performance between the groups was evaluated by multi-factor analysis of variance (ANOVA). Rats were trained in the shuttle box with 100 trials per day. Conditioned avoidance responses (CARs).

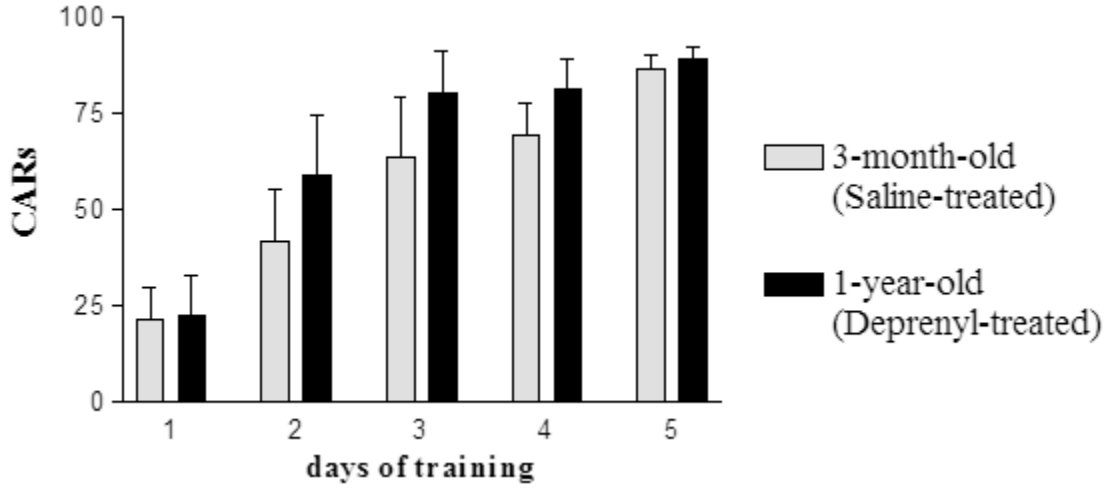


Figure 9.2. Experimental evidence shows that in rats treated with 0.1 mg/kg DEP there is no sign of aging-related decay in the learning ability. Rats were trained in the shuttle box with 100 trials per day. Significance in the performance between the groups was evaluated by multi-factor analysis of variance (ANOVA). There was no significant difference in the acquisition of conditioned avoidance responses (CARs) between the 3-month-old rats treated with saline and 1-year-old rats treated with DEP.

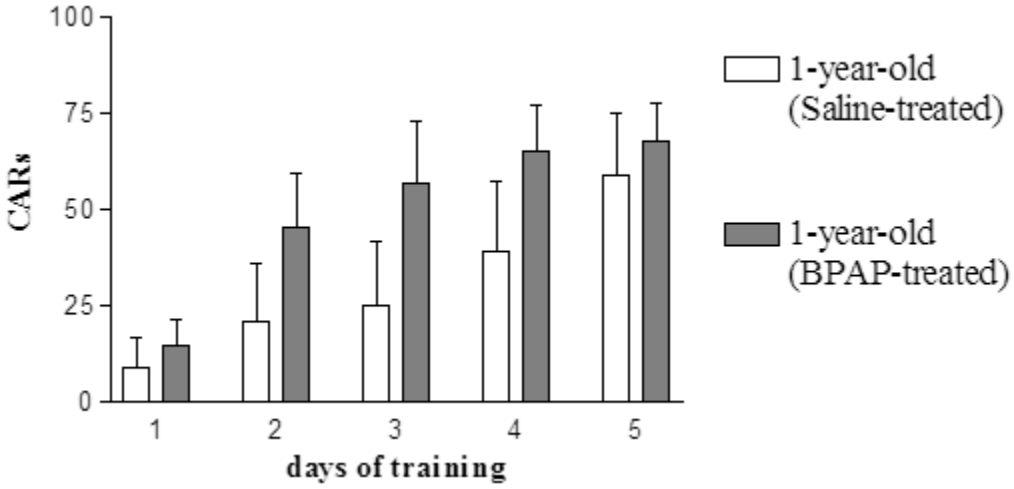


Figure 9.3. The anti-aging effect of 0.0001 mg/kg BPAP. 1-year-old rats were treated for 10 months subcutaneously, 3-times a week, with 0.0001 mg/kg BPAP. Their performance was compared to saline-treated rats. Rats were trained in the shuttle box with 100 trials per day. Significance in the performance between the groups was evaluated by multi-factor analysis of variance (ANOVA). BPAP treated rats performed significantly better than their saline-treated peers ($P<0.05$).

The first longevity study with the low enhancer doses of DEP and BPAP, shown in Chapter 6 (Figures 6.5, 6.6 and 6.7), presented experimental evidence that the synthetic enhancers' primary important therapeutic effect is their unique ability to counteract brain aging (Knoll and Miklya 2016).

This longevity study was the first demonstration that lifelong treatment with 0.0001 mg/kg BPAP, the peak dose with the specific enhancer effect, completely prevented aging of learning ability. We used the learning test as a highly sensitive model to measure the aging-related decay of the dopaminergic neurons. The BPAP-treated rats performed in the shuttle box like the saline-treated 3-month-old rats. This finding is an unprecedented, convincing proof that the enhancer-sensitive dopaminergic neurons do not age. Thus, the development of the first synthetic enhancer substances guides the way to prevent the regressive effects of brain aging.

Chapter 10

Discovery of an enhancer-sensitive, tumor-manifestation suppressing (TMS) regulation in rat brain

The first longevity study performed with the low, enhancer doses of DEP and BPAP gave us an astonishing, unforeseeable surprise: the discovery of the operation of a hitherto unknown enhancer-sensitive tumor-manifestation suppressing (TMS) regulation in the rat brain. The realization of the existence of a previously unknown enhancer-sensitive TMS regulation in a mammalian brain was a convincing unexpected confirmation that we penetrated into an unexplored world of mammalian brain (Knoll, Baghy, Eckhardt et al. 2017).

Around 50% of the Wistar rats used in our strain spontaneously manifest a rapidly growing subcutaneous *fibromyxosarcoma* during their lifetime. We usually detect the appearance of the first tumor during the last quarter of their first year and due to aging, more and more members of the group develop the tumor.

As shown in Chapter 6, we performed our first longevity study on male Wistar rats with low doses of DEP and BPAP, exerting their specific and non-specific enhancer effect, respectively (Knoll and Miklya 2016). In our longevity study with BPAP, we unexpectedly noticed the phenomenon that whereas in the saline-treated group the malignant tumors appeared as expected, in the group of rats treated three times a week with 0.0001 mg/kg BPAP, tumor-manifestation significantly decreased. Thus, BPAP unexpectedly uncovered the operation of an unknown malignant TMS regulation in the rat brain. This was the first proof that BPAP is an efficient specific marker of unknown enhancer-sensitive brain regulations.

We performed a strategic longevity study with low, enhancer doses of DEP and BPAP to measure exactly the effectiveness of the synthetic enhancers in suppressing the manifestation of the fibromyxosarcoma.

Selection of the optimal doses of DEP and BPAP for the longevity study using the shuttle box technique

The acquisition of a conditioned avoidance reflex (CAR) was analyzed during five consecutive days in the shuttle box. The optimal doses of DEP and BPAP for the longevity study were selected according to the method described in Chapter 6.

We performed the longevity study with 200 male Wistar rats, treating 10-week-old rats until death. Groups of 40 rats were injected subcutaneously, three-times a week (Monday, Wednesday and Friday) with saline, DEP and BPAP, respectively - Group 1: Saline (0.9% NaCl) 0.05 ml/100 g; Group 2: DEP 0.1 mg/kg; Group 3: DEP 0.001 mg/kg; Group 4: BPAP 0.05 mg/kg; Group 5: BPAP 0.0001 mg/kg.

A bi-modal, bell-shaped concentration effect curve is characteristic to the enhancer substances (Chapter 4). This peculiar behavior was noted in the course of our first experiments when we realized the CAE effect of DEP (Knoll 1994; Knoll and Miklya 1994). Nevertheless, only the exact analysis of the enhancer effect of BPAP, the selective and presently most potent enhancer substance, rendered the distinction of the *"specific"* and *"non-specific"* enhancer effect possible. The bi-polar, bell-shaped nature of the enhancer effect was confirmed on cultured rat hippocampal neurons (Knoll, Yoneda, Knoll et al. 1999); and exactly analyzed on isolated locus coeruleus of rats. In this test BPAP enhanced the activity of the noradrenergic neurons in the femto/picomolar concentration range with a peak at 10^{-13} ("specific" enhancer effect) and also in a 10 million times higher concentration range with a peak at 10^{-6} ("non-specific" enhancer effect) (Knoll, Miklya and Knoll 2002). The *in vivo* effectiveness of DEP and BPAP was exactly analyzed earlier with the shuttle box technique (Chapter 9). For the longevity study, DEP's and BPAP's optimum doses with the "specific" and "non-specific" enhancer effect, respectively, were selected from the dose-effect curves published in a recent longevity study (Knoll and Miklya 2016).

Immuno-histochemical identification of the fibromyxosarcoma in Wistar rats

To prove the tumors' origin, immune-histochemical reactions were carried out on formalin-fixed, paraffin embedded sections. Following deparaffinization and rehydration, the slides were incubated by the following primary antibodies against vimentin (Dako, Glostrup, Denmark, 1:1200 dilution), smooth muscle antibodies (SMA, Dako, 1:400 dilution), desmin (Dako, 1:300 dilution), Ki67 (Dako, 1:100 dilution). The reactions were carried out in a Ventana Benchmark XT automated immunohistochemical staining system (Ventana Medical System Inc., Tucson, AZ) with the HRP Multimer based, biotin-free detection method. Reagents and secondary antibodies were obtained from Ventana (iView DAB Detection Kit, Ventana).

On several rats selected from each group, histological analysis was performed post mortem. The subcutaneous tumors were measured by the two largest diameters. The tumors were removed

and fixed immediately after removal in 10% neutral formalin (in PBS, pH7.0) for 24 hours at room temperature, dehydrated and embedded in paraffin; 3-4 micrometer thick sections were cut and stained by hematoxylin and eosin (HE).

The tumors were greyish-white and of a soft consistency. Occasionally, hemorrhagic and necrotic areas of various degrees could be detected. Histologically, the tumor cells were round or elongated with round or oval nuclei and eosinophilic cytoplasm. Sometimes, mitotic figures were seen. The cells were embedded in a pale partly eosinophilic, partly basophilic loose matrix which contained areas of collagen fibers. The tumor infiltrated the subcutaneous tissues and the striated muscles.

Immuno-histochemistry proved the mesenchymal origin of the tumor cells, which were strongly stained with vimentin; however, reactions for SMA and desmin were negative. Ki67 was positive in up to 5% of the tumor cells, indicating the proliferation of tumor cells. The final histological diagnosis was *fibromyxosarcoma* in the subcutaneous tissue.

Perception of an enhancer-sensitive TMS-regulation in the brain of Wistar rats

From the beginning of the second year of treatment it seemed increasingly more likely that rats in the enhancer-treated groups would manifest the fibromyxosarcoma with a lower frequency as compared to the saline-treated group. The enhancer-treatment-induced highly significant suppression in the manifestation of the fibromyxosarcoma was first undeniable after 18-month treatment with 0.0001 mg/kg BPAP.

Table 10.1 shows the individual differences in the time span of tumor-manifestation in groups of 40 rats treated with saline or different doses of DEP and BPAP, respectively. In the saline-treated group of rats, the first tumor appeared in the 10^{th} month of treatment and by the end of the 18^{th} month of treatment 14 rats had already manifested the tumor. In striking contrast, none of rats treated with 0.0001 mg/kg BPAP manifested the tumor prior to the 19^{th} month of treatment. Thus, it was already clear in middle of the longevity study that an enhancer-sensitive TMS-regulation works in the rat brain.

Table 10.1 Individual differences in the time span of tumor-manifestation in groups of rats, treated with saline, or different doses of DEP and BPAP, respectively. N=40					
MANIFESTATION OF TUMORS IN THE GROUPS					
Age of rats (months)	**Saline** 0.5 ml/kg	**DEP** 0.1 mg/kg	**DEP** 0.001mg/kg	**BPAP** 0.05 mg/kg	**BPAP** 0.0001 mg/kg
10	1				
11	2		1		
12	2		1	1	
13	2		2		
14	1		2		
15		1			
16					
17	1				
18	3	1			
19	2	1	2		2
20	1	1	1		1
21	1	1	2	1	
23	1	3		1	
24	1	1			
25		1		2	1
26					
27	2		3	1	
28					1
29			1	1	2
30					
31					1
32		1			
Total number of tumors	**20**	**11**	**15**	**7**	**8**

Figure 10.1 (A-D) shows that low dose treatment with an enhancer substance suppresses tumor manifestation. The percentage of tumors manifested in rats treated with 0.1 mg/kg DEP was already significantly ($P<0.01$) lower than in saline-treated rats (Figure 10.1B). Both in the lower, 0.0001 mg/kg dose ("specific" enhancer effect) (Figure 10.1C) and in the higher, 0.05 mg/kg dose ("non-specific" enhancer effect) (Figure 10.1D), BPAP was more effective than DEP in suppressing the manifestation of the fibromyxosarcoma.

The data clearly demonstrates that the enhancer-sensitive TMS-regulation is decreasing with age in a similar way as the enhancer-sensitive catecholaminergic and serotonergic brain mechanisms, which work from weaning until sexual maturity, on a significantly higher activity level (Knoll and Miklya 1995). Sexual hormones (estrone, testosterone) then return the enhancer regulation to its pre-weaning level and the aging-related slow decay of the enhancer regulation continues until death (Knoll, Miklya, Knoll and Dalló 2000).

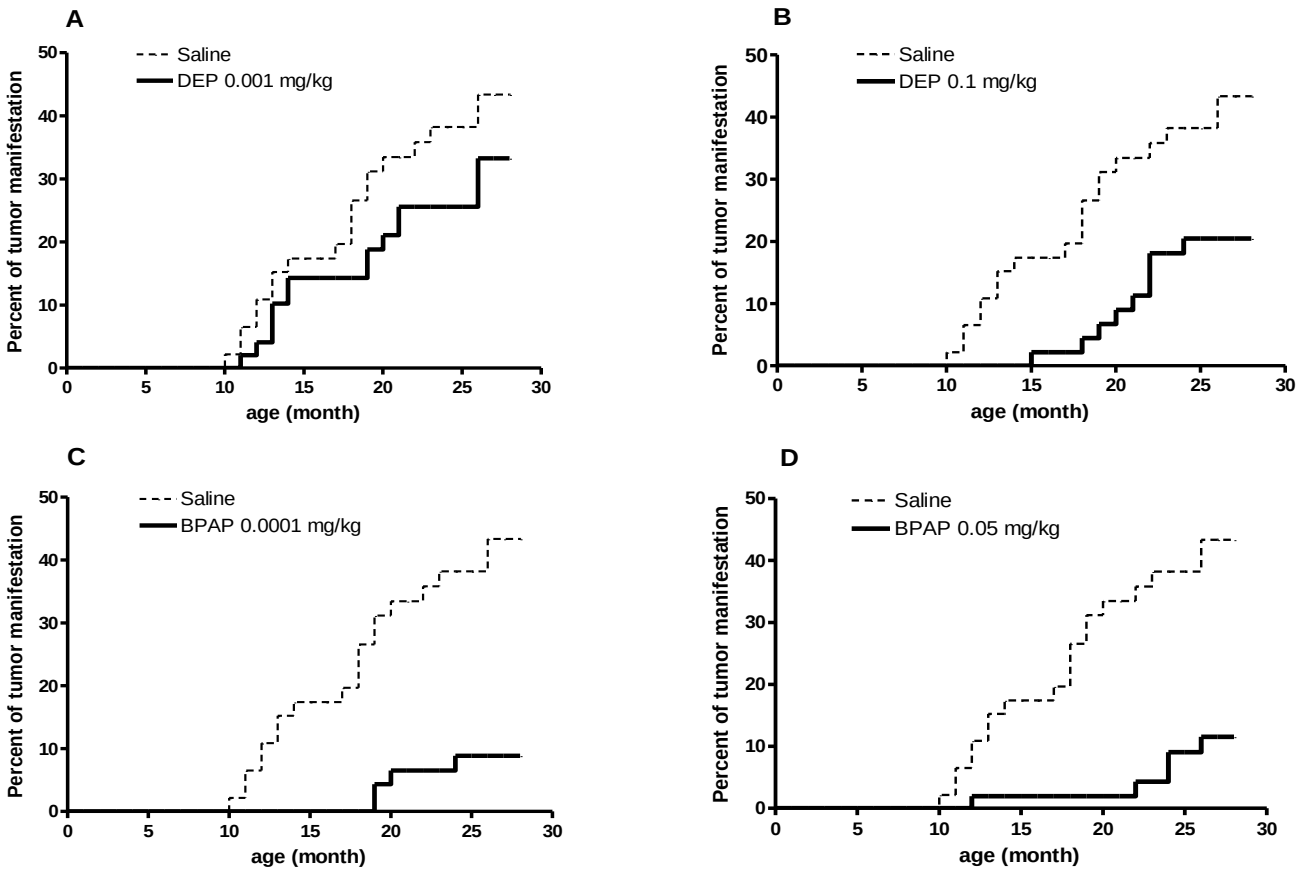


Figure 10.1 Suppression of tumor manifestation due to low dose treatment with enhancer substances. Treatment with saline versus DEP (A, B) and BPAP (C, D) in doses selected in the shuttle box test for the longevity study (see Methods). Statistics: Kaplan-Meier test, A: DEP 0.001 mg/kg P=0.1155 (ns), B: DEP 0.1 mg/kg *P<0.01 (P=0.0054); C: BPAP 0.0001 mg/kg **P<0.001 (P=0.0005), D: BPAP 0.05mg/kg **P<0.001 (P=0.0003).

Figure 10.2 shows that since the enhancer substances keep the TMS-regulation on a higher activity level, a lower number of rats manifested the tumor in their lifetime in the enhancer-treated groups and the fibromyxosarcoma appeared later than in the saline-treated group. In comparison to saline-treatment, enhancer-treatment with 0.0001 mg/kg BPAP, the peak dose with the "specific" enhancer effect, was the most efficient in this respect.

Developing tumors in surviving enhancer-treated rats is proof that, due to aging, the decline of TMS-regulation arrived at a critical level and ceased to operate. Accordingly, there is no difference in the microscopy and histology of the fibromyxosarcoma developed in saline-treated or enhancer-treated rats.

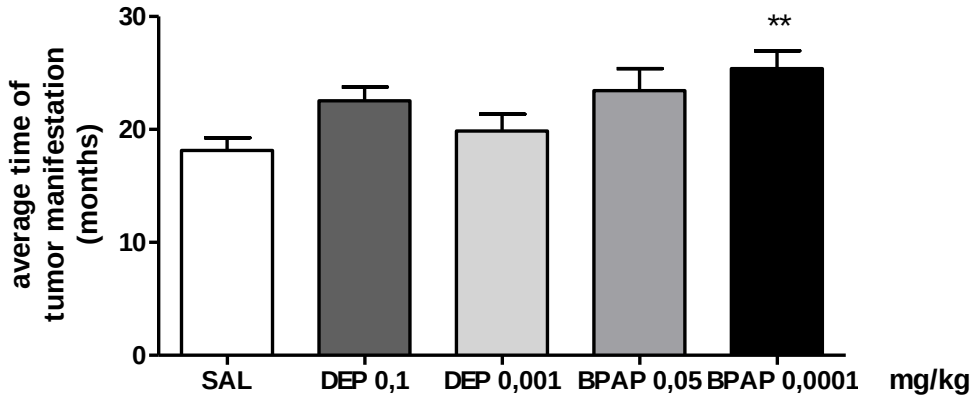


Figure 10.2 Enhancer-treatment-induced delayed tumor manifestation. One-way ANOVA followed by Neuman-Keuls post hoc test: Sal v. 0.0001 mg/kg BPAP **P<0.01.

Cell culture studies

Cell lines: Human medulloblastoma cell line, HTB-186 (Daoy) was purchased from ATCC and UW-228-2 was obtained via the courtesy of Professor Silber (University of Washington, Seattle).

Maintenance: HTB-186 (Daoy) and UW-228-2 cell lines were maintained in culture medium (each 500ml Minimum Essential Medium Eagle, Alpha Modification (M8042, Sigma, St. Louis) with 50 ml FCS (Gibco), 40 mg Gentamycin (Sandoz), 5 ml sodium-pyruvate (S8636, Sigma, St. Louis), 5 ml non-essential-amino acid solution (M7145, Sigma, St. Louis), 10 ml L-glutamine (Sigma, St. Louis) at 37°C in humified 5% CO_2.

Proliferation assays: In each well $3x10^3$ HTB-186 or UW-228-2 cells were seeded in 96-well plates (Sarstedt), solved in 100 µl of its own medium with 10% FCS. 24 hours after seeding, cells were treated for 72 hours by drugs solved in further 100 µl medium. First, both cell lines were treated by DEP and BPAP in monotherapy to determine its dose-effect curves in concentration of 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12}, 10^{-13} and 10^{-14} M. In combined treatment 10^{-3}, $3.3x10^{-4}$, $1.1x10^{-4}$ and $3.7x10^{-5}$ M of temozolomide (Schering Plough, NJ) or 0,04, 0.2, 1, 5 µM of cisplatin (Ebewe Pharma, Austria) or 0,04, 0.2, 1, 5 µM of etoposide (Ebewe Pharma, Austria) or 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} µM (UW228-2) or 0.001, 0.005, 0.025 and 0.125 µM (HTB-186) of vincristine (Richter Gedeon, Hungary) were applied in monotherapy or combined with 10^{-13} or 10^{-8} M of DEP and BPAP.

Cell proliferation was evaluated by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (M5655, Sigma) after a 72-hour treatment by the method described in the manufacturer's protocol.

We tested the effect of BPAP and DEP on the proliferation of tumor cells in two types of human cultured medulloblastoma cell lines: Daoy HTB-186 originating from desmoplastic cerebellar medulloblastoma (Jacobsen, Jenkyn and Papdimitriou 1985) and UW-228-2 originating from posterior fossa medulloblastoma with a diploid DNA content (Keles, Berger, Srinivasan et al. 1995). Considering the bi-modal, bell-shaped concentration effect curve characteristic to the enhancer effect of DEP and BPAP (Knoll 1994) we investigated the enhancers' effect within the 10^{-14} and 10^{-6} M range, in nine concentrations.

DEP and BPAP influenced *in vitro* the cultured tumor cells in a negligible degree and also in an opposite direction. The Daoy cell line was slightly inhibited by both DEP and BPAP; proliferation of UW-228-2 cells was slightly increased. *Thus, BPAP and DEP are devoid of any notable direct cytotoxic effect on tumor cells.*

Moreover, the enhancer substances did not change the direct cytotoxic effectiveness of temozolomide, cisplatin, etoposide, or vincristine on the two human-cultured medulloblastoma cell lines.

Colon carcinoma liver metastasis model

Colon 38 (C38) mouse colon carcinoma cell line maintained subcutaneously was utilized for the present study. C38 cells were isolated from subcutaneous tumor tissue by collagenase digestion (0.7 mg/ml) with subsequent filtration and washing steps. Afterwards, 30 000 tumor cells were inoculated into the spleen of each animal; liver metastases appeared after 23 days.

A total of 42 C57Bl/6 mice were used for the following experimental set-up. *Group 1*: Control; treatment: saline sc. from the following day after tumor cell inoculation (N=10). *Group 2*: BPAP; treatment: daily 0.0001 mg/kg sc., from the following day after tumor cell inoculation (N=8). Animals were terminated by cervical dislocation in ether anesthesia. Macroscopic metastases were counted, and liver samples were fixed in 10% formalin and embedded in paraffin for histological analysis.

Statistical analyses were made by Graphpad Prism 4.03 software. Significance of change between control and BPAP-treated groups were assessed by using Mann-Whitney U-test. Significance was declared at the standard $p<0.05$ level.

BPAP inhibits in vivo metastasis formation of colon carcinoma cells in mice. We inoculated 30,000 mice colon carcinoma cells (C38) into the spleen of C57BI/6 mice. In the saline-treated group, the average number of macroscopic liver metastasis on Day 23 after inoculation was **14** tumors/liver. In a group of mice pretreated daily, subcutaneously, for one week with 0.0001 mg/kg BPAP prior to the tumor cell inoculation, and further treated until the end of the experiment (23rd day), only **2** tumors/liver appeared ($p<0.05$). This finding clearly indicates that TMS regulation also works in the mouse-brain.

*

Our present study was the first example showing how DEP and BPAP as markers detected the operation of an unknown enhancer-sensitive TMS regulation in rodents' brain. The unknown regulation showed similar sensitivity toward DEP and BPAP as the catecholaminergic and serotonergic neurons.

The discovery an enhancer-sensitive TMS regulation in a mammalian brain is further convincing evidence that the discovery of the enhancer-sensitive brain regulations and the development of the first synthetic enhancers open a new highly promising area of brain research.

DEP and BPAP, specific markers of hitherto unknown enhancer sensitive brain regulations, detected the operation of an enhancer-sensitive TMS-regulation in the rat brain (Knoll, Baghy, Eckhardt et al. 2017). The existence of a physiological malignant tumor-manifestation-suppressing regulation in a mammalian brain *was never even considered*. This enhancer-sensitive brain regulation works presumably also in other mammalian brains, including the human brain.

Considering the safeness of the synthetic enhancer substances it seems reasonable to test, *in a well-designed multicenter clinical study*, the potential tumor-manifestation-suppressing effect of DEP and BPAP. I refer, as an example, to the DATATOP multicenter study in the USA. The Parkinson Study Group's finding in 1989 founded the indication for using DEP/selegiline in patients with early, untreated Parkinson's disease. Their discovery (Tetrud and Langston 1989; Parkinson Study Group 1989) was confirmed by further important multicenter studies (Allain, Gougnard and Naukirek 1991; Myttyla, Sotaniemi, Vourinen and Heinonen 1992; Pålhagen, Heinonen, Hagglund et al.1998; Larsen, Boas and Erdal 1999) and DEP is still generally used in *de novo* Parkinson's disease. We presented experimental evidence that the enhancer effect is responsible for this unique therapeutic benefit of DEP (Knoll and Miklya 2016; Knoll 2016).

A similar clinical trial could establish material proof that synthetic enhancers can counter via a previously unknown enhancer-sensitive TMS regulation the manifestation of a malignant tumor.

Chapter 11

Conclusion

Personal endeavors motivated me to plan rat and mouse experiments in order to investigate the mechanism responsible for the manipulability of mammalian behavior. The realization in the 1950s that *the inborn ability of rats' cortex to fix acquired drives is responsible for the manipulability of their behavior* was the first significant discovery in my research.

In contrast to *innate drives* with a limited number of indispensable (life important) goals, *acquired drives* are responsible for an unlimited number of dispensable goals. The capability to acquire an insuppressible urge for a goal which is not necessary for survival of the individual or species represents the most sophisticated function of the telencephalon.

Vertebrates can be divided into three groups according to their brains' mode of operation: *(a) those that operate with only innate drives (the majority); (b) those with an ability to acquire drives (a minority); and (c) the "group of one" that operates almost exclusively with acquired drives (Homo sapiens). The mouse, a rodent closely related to the rat, trained under the same experimental conditions as the rat, was unable to acquire the glass-cylinder-seeking drive, demonstrating that the development of mammals with a brain capable to fix acquired drives possess a qualitatively higher developed brain than the mammals devoid of this ability.*

The development of an acquired drive always originates in one way or another in an innate drive proving that nothing exists in the human brain without a rational origin

With the evolution of brains capable of acquiring drives, species appeared whose members could manipulate each other's behavior and act in concert. This was the condition *sine qua non* for the evolution of social living, a form of life that enabled species to surpass qualitatively the performance of any given individual. Unquestionably, members in the skills needed to act in concert with each other improved the quality of life. The evolution of mammalian brains with the ability to acquire drives made the appearance of life on earth so immensely variable (Knoll 1969).

The discovery of the enhancer-sensitive brain regulations; the selection of the catecholaminergic and serotonergic neurons to analyze their characteristics; the identification of β-phenylethylamine (PEA) for the catecholaminergic neurons and tryptamine for the serotonergic neurons as natural enhancers; and the development of selegiline/(-)-deprenyl (DEP), the PEA-derived synthetic catecholaminergic activity enhancer (CAE) substance and (2R)-1-(1-benzofuran-2-yl)-N-propylpentane-2-amine (BPAP) the tryptamine-derived synthetic enhancer substance, catalyzed the discovery of the unique ability of the synthetic enhancers to prevent brain-aging.

The average human lifetime is rapidly lengthening and is already around 90 years in developed countries. It is imperative to counteract the regressive effects of brain aging which embitter the life of the aged. To reach this aim, it was an important discovery to realize, on the one

hand, that the fading stimulation of the natural enhancers plays the key role in brain aging, and on the other hand, that the enhancer-sensitive neurons do not age. Due to permanently intensified enhancer-sensitive brain regulations, young mammals are vigorously mobile during the developmental period of life from weaning until sexual maturity. Sexual hormones immediately restore the pre-weaning low level of the enhancer-sensitive brain regulations and thus put the post-developmental (aging) phase in motion. Due to slow, unbroken loss of the natural enhancers over time, brain functions are subject to an aging-related continuous decline and the regressive effects of brain-aging grow until death. Since we clarified that the aging-related loss of natural enhancers are responsible for brain-aging, but the enhancer-sensitive neurons do not age, DEP and BPAP, the already developed synthetic enhancer substances prevent the regressive effects of brain aging.

DEP was described first as the selective inhibitor of MAO-B. It has been known since 1988 that mammals treated with 0.25 mg/kg DEP, which blocks MAO-B activity in the brain, live significantly longer than their saline treated peers. The first longevity study performed with low doses of DEP and BPAP, demonstrating that the enhancer effect is responsible for life extension was published in 2016 **[4].** We presented experimental evidence that the enhancer-sensitive catecholaminergic neurons in rats treated from sexual maturity until death with 0.0001 mg/kg BPAP kept their unchanged learning ability.

We treated rats daily with saline versus 0.0001 mg/kg BPAP and measured in the shuttle box their ability to fix a conditioned avoidance reflex (CAR). Due to aging of the catecholaminergic (primarily dopaminergic) neurons, the rat's learning ability is subject to age-related decline. We found in this study that the young, 3-month-old group of saline-treated rats worked with full capacity in the shuttle box and built on the fifth day of training an average nearly 90% of the possible 100% of CARs. Due to aging 18-month-old saline-treated rats reached on the fifth day of training an average only less than 30% of the possible 100% of CAR.

However, BPAP-treatment counteracted the aging-related decline in learning ability. The group of 18-month-old rats treated from sexual maturity with 0.0001 mg/kg BPAP produced on the fifth day of training an average of more than 90% of the possible 100% of CARs. This is an unprecedented novelty, unquestionably an unparalleled drug effect, proving that the synthetic enhancer substituted the lost natural enhancer, and thus fully prevented the aging-related learning deficit.

BPAP, the selective and most potent synthetic enhancer, is preferentially used as a specific marker to detect hitherto unknown enhancer-sensitive brain regulations. A longevity study performed with low doses of DEP and BPAP detected the operation of an enhancer-sensitive tumor-manifestation-suppressing (TMS) regulation in the rat brain. This was the first discovery of a *previously unknown* enhancer-sensitive brain regulation (Knoll 1993).

Lifelong preventive medication obviously requires unique drug-safeness. Due to their peculiar mechanism of action and safety margin, only the synthetic enhancer substances adhere to this requirement. BPAP exerts in rats its specific enhancer effect in a subcutaneous dose as low as 0.0001 mg/kg, and 20 mg/kg; a 20.000 times higher dose is tolerated without any sign of toxic effect. This is truly an exceptional safety margin and calls for a reasonable trial to test in humans whether synthetic enhancers can counteract brain aging in healthy humans as shown in our recent paper on rats (Knoll and Miklya 2016).

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