E-Books

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Chapter 3

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

(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 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

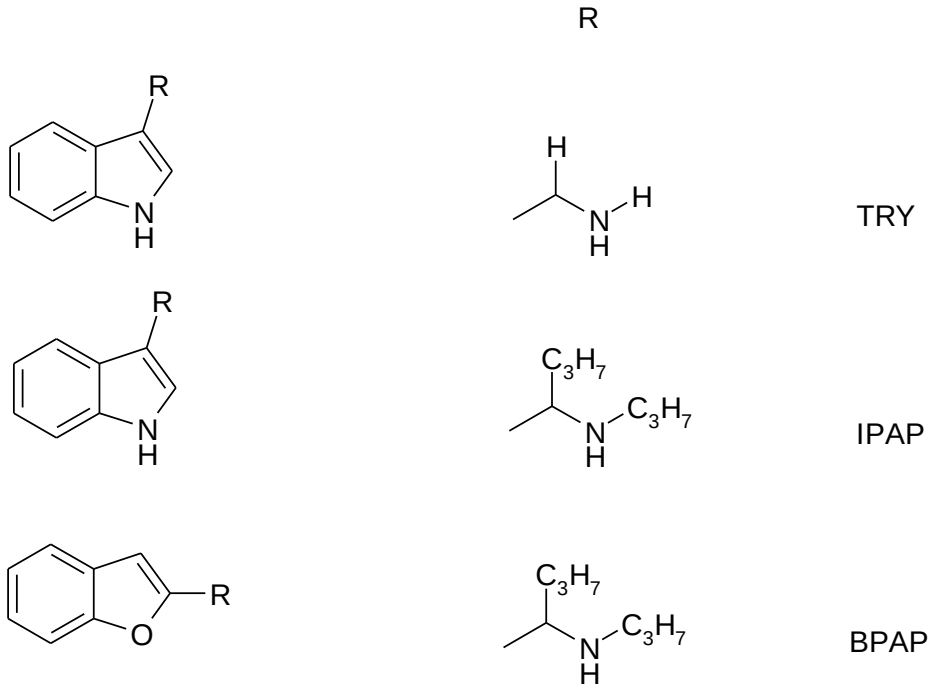

Figure 3.1 Schematic chemical structure of tryptamine (TRY), the natural enhancer substance of the serotonergic neurons; IPAP, the simplest TRY-derived synthetic enhancer substance; and BPAP, most potent TRY-derived synthetic enhancer substance (Knoll et. al. 1999)

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

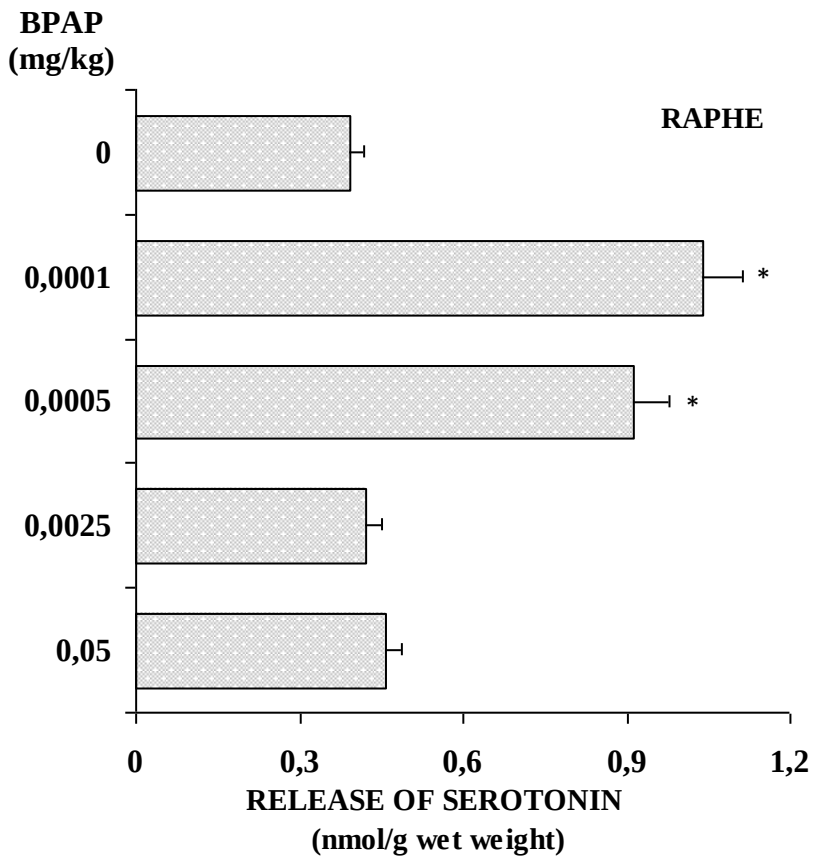

Figure 3.2 Illustration of the significant enhancement of the outflow of serotonin (SE) from the raphe of rats isolated 30 min after the subcutaneous administration of a single dose of BPAP. The amount of SE released from

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 Fig. 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 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 et al. 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
		d o p a m i n e			*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
		d o p a m i n e			*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 et al. 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, Fig.1). 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 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 et al. 1999) and precisely analyzed on isolated locus coeruleus of rats (Knoll et al. 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 et al. 1999, 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 et al. 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 et al. 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

A B

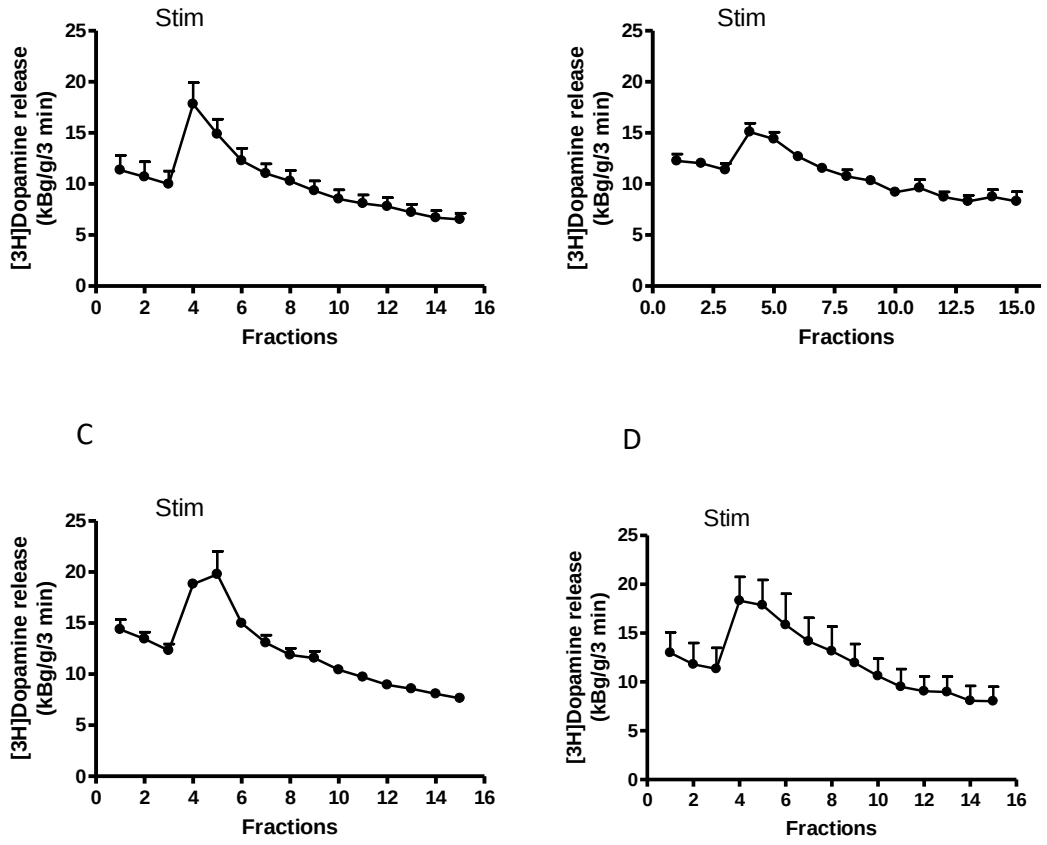

Figure 3.3 BPAP reverses TBZ-induced decrease of [^{3}H]-dopamine release in rat striatum.

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

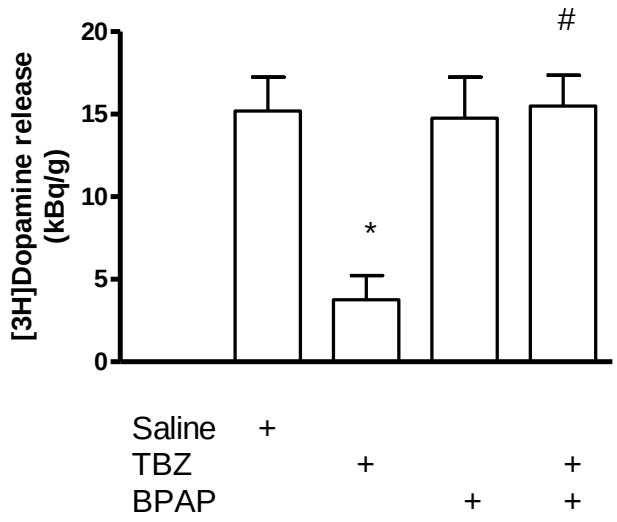

Figure 3.4 Electrical stimulation-induced [^{3}H]-dopamine release from rat striatal slices obtained from saline, TBZ (1 mg/kg sc.), BPAP (0.0001 mg/kg), and TBZ (1 mg/kg) + BPAP (0.0001 mg/kg) pretreated rats. Rats were injected with drugs sc. TBZ pretreatment reduced [^{3}H]-dopamine release, (one-way 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 (Fig. 3.3, Fig. 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 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 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 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 et al. 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 et al. 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 et al. 1989; second, Knoll et al. 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 et al. 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 et al. 1989; Knoll et al. 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 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 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 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 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 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 et al. 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.

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