Joseph Knoll: Enhancer Sensitive Brain Regulations and Synthetic Enhancers (Selegiline, BPAP) Which Counteract 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**

Fig. 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 (Fig. 10.1B). Both in the lower, 0.0001 mg/kg dose ("specific" enhancer effect) (Fig. 10.1C) and in the higher, 0.05 mg/kg dose ("non-specific" enhancer effect) (Fig. 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).

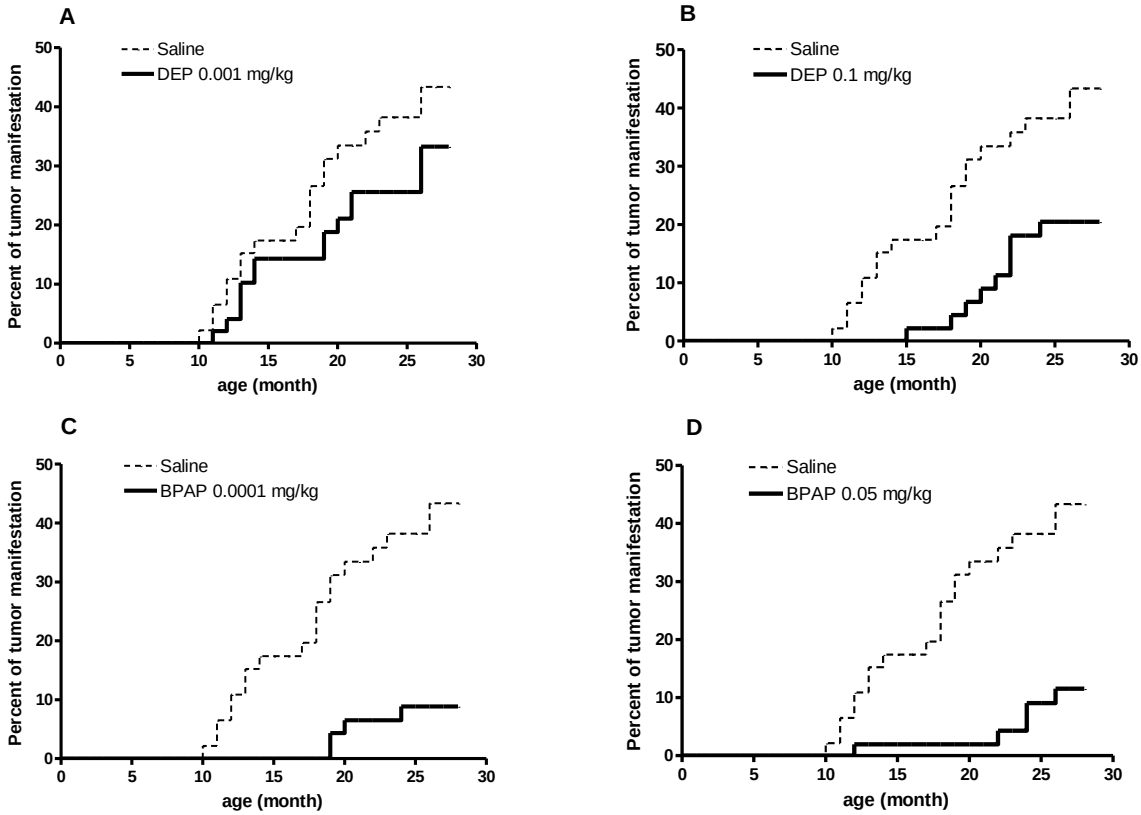

*Figure 10.1 Suppression of tumor manifestation due to low dose treatment with enhancer substances. Treatment with saline versus DEP (A, B) and BPAP (C, D) in doses selected in the shuttle box test for the longevity study (see Methods). Statistics: Kaplan-Meier test, A: DEP 0.001 mg/kg P=0.1155 (ns),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

Fig. 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.

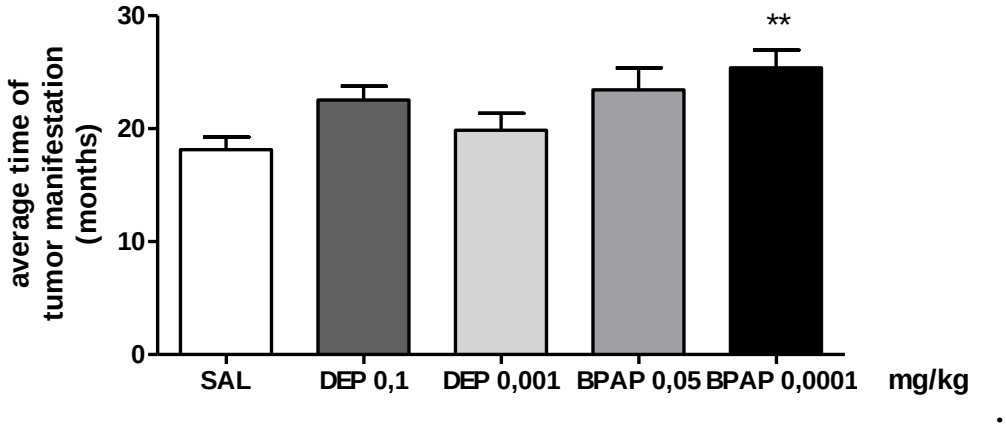

*Figure 10.2 Enhancer-treatment-induced delayed tumor manifestation. One-way ANOVA followed by Neuman-Keuls post hoc test: Sal v. 0.0001 mg/kg BPAP **P<0.01.*

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.

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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; Palhagen, 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.

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