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DISCRIMINATION OF NEUROLEPTICS BY MEANS OF THEIR INTERACTION WITH AMFONELIC ACID: AN ATTEMPT TO CHARACTERIZE THE TEST

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Abstract—The non-amphetamine stimulant amfonelic acid (AFA) is known to enhance the effects of haloperidol, trifluoperazine and spiperone but not those of the atypical neuroleptics clozapine, thioridazine, and sulpiride, on the striatal levels of 3,4-dihydroxyphenylacetic acid. Consequently, the interaction between neuroleptics and AFA has been proposed as a test to discriminate typical and atypical neuroleptics.

In this study, these findings were confirmed. Essentially the same results were obtained when the levels of homovanillic acid were measured. However, striatal dopamine levels were decreased similarly by combinations of AFA with typical and atypical neuroleptics.

A comparison of the results with 17 neuroleptics with their reported clinical liability to cause extrapyramidal symptoms supported the idea that the test may discriminate drugs with a better ratio of therapeutic vs side effects. A series of antidepressants and α-noradrenergic antagonists possessing antidopaminergic properties was found to perform like atypical neuroleptics, i.e. their effects on dopamine metabolites were not enhanced by AFA.

Non-amphetamine stimulants like methylphenidate, cocaine or amfonelic acid (AFA) have been found to further enhance the dopamine (DA) disappearance after α-methyl-*p*-tyrosine in haloperidol-treated rats. AFA, in particular, also greatly amplified the increases in the striatal concentrations of the deaminated DA metabolites, homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) induced by haloperidol [1] or spiperone [2]. These effects have been ascribed to a facilitation of the impulse-mediated release, since they only become manifest under conditions of increased impulse flow, and disappear if the latter is inhibited [1]. Although the behavioural stimulant effects of AFA and amphetamine-like compounds are similar, amphetamine and pemoline do not show the same type of interaction with neuroleptics [1, 2].

Recently, McMillen [3] reported that the effects of "atypical" neuroleptics (i.e. clozapine, thioridazine and sulpiride) on striatal DOPAC concentrations were not enhanced by AFA. He suggested that the interaction with the latter could be used to discriminate between "typical" and "atypical" neuroleptics. Since in preliminary experiments we were able to confirm McMillen's results, we were interested in studying a larger number of neuroleptics and other drugs which increase DA metabolism in a neuroleptic-like manner, to obtain information on the significance of this test.

MATERIALS AND METHODS

Haloperidol (Cilag AG, Schaffhausen, Switzerland) and benperidol (Lab. Clin-Comar-Byla, Paris, France) were obtained as injectable solutions. Amfonelic acid was kindly provided by Winthrop Labs. (New York, USA), clozapine 2HClxH_2O and perlapine by Wander AG (Berne, Switzerland); fluperlapine and thioridazine HCl by Sandoz AG (Basel, Switzerland); cis-flupenthixol 2HCl by Lundbeck & Co. (Copenhagen, Denmark); fluphenazine 2HCl by E. Squibb & Sons (Princeton, NJ, USA); metoclopramide HClxH_2O by Kali-Chemie Pharma GmbH (Hannover, FRG); molindone HCl by Endo Labs. (Garden City, NJ, USA); perphenazine injectable solution by Schering Corp. (Kenilworth, NJ, USA); pimozide, pipamperone 2HCl and spiperone by Janssen Pharmaceutica (Beerse, Belgium); sulpiride by Laboratoires Delagrange (Paris, France); trazodone HCl by Angelini S.P.A. (Rome, Italy); trifluoperazine 2HCl by Smith, Kline and French Labs. (Philadelphia, PA, USA); and zetidoline HCl by Gruppo Lepetit (Milan, Italy). Trimipramine maleate and esproquin HCl were synthesized in our Chemistry Department by Dr. H. Schroeter, chlorpromazine HCl by Dr. V. Mychajlyszyn, piperoxan HCl by Dr. A. Storni, and WB 4101 HCl by Dr. Ostermayer. Corynanthine HCl and rauwolscine HCl were purchased from Carl Roth (Karlsruhe, FRG), and yohimbine HCl from E. Merck (Darmstadt, FRG).

Female Tif:RAIf(SPF) rats (Tierfarm Sisseln, Switzerland) weighing 160–220 g received 2.5 mg/kg s.c. amfonelic acid 5 min before the test compounds and were decapitated 90 min later. Striata were dissected from the brain and stored frozen at −20° until analyzed. Pairs of striata were homogenized in 2 ml of the mobile phase for the HPLC separation described below, containing 1000 ng vanillic acid per extract as an internal standard. Cell debris were removed by centrifugation. Fifty to two hundred microlitres of the supernatant were injected into a BAS liquid chromatography system (Bioanalytical Systems, W. Lafayette, IN, USA) fitted with a C_{18}

μBondapak reversed phase column (Waters Ass., Milford, USA), a TL3 electrochemical detector cell, and a LC4 controller. The detector cell contained cp_w carbon paste and the potential was set to +0.85 V. The mobile phase contained 0.1 moles/l citric acid, 0.075 moles/l Na_2HPO_4, 2.5% tetrahydrofuran, and 0.05 mmoles/l sodium octylsulphate and was brought to pH 3.0 with HCl. Column temperature was set between 28 and 40° and flow between 1 and 1.3 ml/min, as required to obtain optimal separation. Five animals were used per group.

In some experiments with the same treatment schedule, the levels of DA were measured in striatal tissue homogenized in acidified *n*-butanol. The amine was extracted after addition of *n*-heptane into 0.2 moles/l HCl [4] and quantitated fluorometrically [5].

Statistical evaluation of presence or absence of additivity of the effects of AFA and the test drugs in the HVA/DOPAC experiments was done as follows. Let A be the mean value of the group treated with AFA, B that of the group treated with the test drug, C that of the control group, and D that of the group receiving both drugs. The difference (C + D) − (A + B) is then a measure of non-additivity of the effects of the two substances. The statistical significance of this non-additivity can be tested by dividing the difference by an estimate of its standard error and comparing this ratio with Student's *t*-distribution. However, this test is based on the assumption of equal variances in all treatment groups. The variance of the difference can be estimated by the sum of the error variances of the four treatment means [6], but in the present case these variances are evidently not equal. The estimate is therefore dominated by the largest variance component, and behaves approximately as a variance estimate with less degrees of freedom than are actually present. The reduced number of degrees of freedom is calculated by a formula given by Welch [7], and this number is used for looking up the critical *t*-value in the table of Student's distribution.

RESULTS

The doses of the test compounds were chosen to be at least twice those which in preceding experiments had been found to increase HVA concentrations twofold with respect to untreated controls (ED_{200}). Thus, these doses can be expected to cause the maximal or almost the maximal increase of the levels of the DA metabolites which can be attained by DA receptor blockade alone.

Table 1 shows the effects of striatal HVA and DOPAC of the test compounds alone, AFA alone, and their combinations, in percent of the control values.

AFA enhanced the effects of a number of neuroleptics, i.e. benperidol, haloperidol, spiperone, flupenthixol, fluperlapine, fluphenazine, metoclopramide, perphenazine, pimozide and trifluoperazine, on both HVA and DOPAC concentrations more than additively. Those of chlorpromazine, pipamperone, zetidoline, sulpiride and thioridazine were only slightly or not at all increased, whereas those of clozapine and perlapine were rather decreased, at least as far as the effects on DOPAC are concerned. AFA did not enhance the effects of any of the other compounds, which are not used clinically as neuroleptics, but increase DA metabolism (although in some cases at relatively high doses) in a neuroleptic-like manner [8–10], on HVA concentrations. On the contrary, they were reduced in the cases of trazodone and piperoxane. The effects on DOPAC were also not changed by AFA, or, in the majority of the cases, even reduced.

Since the enhancement by AFA of haloperidol's effect on DOPAC was not observed after lower doses of the neuroleptic (at which it already increased DOPAC when given alone, however [11]), the above differences in the pattern of the interaction with AFA might have been caused by the use of too low doses of some of the compounds. This is rendered quite unlikely by the criteria by which these doses were chosen (see above). However, to further clarify this point, the relation between the extent of the enhancement of the effects of the test compounds by AFA and the extent of their effect on DA metabolites when given alone (at the same dose) was analyzed. As a measure of the extent of enhancement, the factor $f = ([\text{drug} + \text{AFA}] - [\text{AFA}])/([\text{drug}] - [\text{controls}])$ was calculated for the HVA and the DOPAC data from Table 1. A factor of 1 is expected if the effects of the two treatments are additive; a factor >1 indicates enhancement and one <1 attenuation of the effects of the test drug by AFA. These factors were then plotted against the increases of the DA metabolites caused by the same doses of the test drugs administered alone (Fig. 1). The plots do not suggest the existence of a relation between these parameters; in fact, the distribution of the points was such that a regression analysis did not seem to be warranted. One may therefore conclude that the different performance of the drugs in the AFA interaction test cannot be explained by non-equivalent doses.

A plot of $f_{(HVA)}$ against $f_{(DOPAC)}$ showed a significant linear correlation ($r = 0.909$, $N = 28$; not shown), indicating that measuring either metabolite in the AFA interaction test provides essentially similar effects. Plots of $f_{(HVA)}$ or $f_{(DOPAC)}$ against $ED_{200(HVA)}$ or $ED_{200(DOPAC)}$ (the doses of the test drug which, when given alone, doubled the baseline values of the two DA metabolites; these data were determined in other experiments not reported here), resp., showed some degree of dependence (plots not shown). Calculation of linear regressions gave correlation coefficients of −0.687 (HVA) and −0.726 (DOPAC). However, this merely indicates that there are more compounds which are not potentiated by AFA among those which are less potent to increase DA metabolism.

In separate experiments, the effects on striatal DA concentrations of the combinations of some of the above compounds with AFA were also studied. The results are shown in Table 2. Note that none of the compounds had an effect on DA concentrations when given alone; neither did AFA. However, when both treatments were combined, a strong and highly significant reduction was observed in every case, irrespective of whether the compound's effects on

Table 1. Interactions of AFA with neuroleptics and related compounds with respect to rat striatal DA metabolism

Drug	Dose (mg/kg)		HVA (percent of controls) Controls	AFA	Drug	AFA + drug	DOPAC (percent of controls) Control	AFA	drug	AFA + drug
Haloperidol	1	po	100 ± 7	180 ± 10	333 ± 19	**711 ± 26**	100 ± 7	133 ± 11	319 ± 25	**858 ± 79**
Benperidol	0.1	ip	100 ± 11	208 ± 29	444 ± 59	**1094 ± 85**	100 ± 10	170 ± 23	507 ± 106	**2034 ± 203**
Spiperone	0.3	po	100 ± 4	147 ± 4	212 ± 23	**408 ± 13**	100 ± 3	140 ± 12	238 ± 27	**406 ± 19**
Pipamperone	30	ip	100 ± 8	158 ± 10	276 ± 22	379 ± 25	100 ± 5	121 ± 6	299 ± 35	273 ± 33
Pimozide	1	ip	100 ± 4	171 ± 13	412 ± 29	**597 ± 26**	100 ± 4	135 ± 10	393 ± 31	527 ± 37
Chlorpromazine	30	po	100 ± 7	151 ± 5	254 ± 13	**398 ± 32**	100 ± 5	127 ± 5	199 ± 10	235 ± 23
Perphenazine	10	po	100 ± 12	237 ± 26	349 ± 8	**738 ± 50**	100 ± 5	168 ± 9	342 ± 22	**1034 ± 83**
Trifluoperazine	3	po	100 ± 7	185 ± 8	241 ± 22	**437 ± 25**	100 ± 5	117 ± 4	183 ± 15	**287 ± 25**
Thioridazine	100	po	100 ± 7	185 ± 8	248 ± 11	341 ± 15	100 ± 5	117 ± 4	187 ± 5	**177 ± 4**
Fluphenazine	3	po	100 ± 5	220 ± 12	261 ± 27	**593 ± 53**	100 ± 9	125 ± 7	259 ± 21	**540 ± 73**
Metoclopramide	10	ip	100 ± 7	178 ± 15	382 ± 22	**643 ± 59**	100 ± 5	100 ± 13	218 ± 12	**322 ± 29**
Sulpiride	80	ip	100 ± 6	159 ± 11	193 ± 8	240 ± 26	100 ± 3	123 ± 5	164 ± 6	155 ± 15
Zetidoline	3	ip	100 ± 6	176 ± 15	266 ± 16	400 ± 113	100 ± 9	133 ± 10	273 ± 12	324 ± 89
Clozapine	30	ip	100 ± 2	187 ± 6	463 ± 46	518 ± 37	100 ± 9	137 ± 6	422 ± 35	**261 ± 21**
Clozapine	100	po	100 ± 7	175 ± 15	280 ± 23	273 ± 12	100 ± 6	115 ± 8	215 ± 16	**165 ± 10**
Perlapine	20	po	100 ± 12	237 ± 26	282 ± 13	**310 ± 9**	100 ± 5	168 ± 9	332 ± 9	**221 ± 13**
Fluperlapine	100	ip	100 ± 6	176 ± 15	328 ± 8	591 ± 72	100 ± 9	133 ± 10	312 ± 20	551 ± 95
Flupenthixol	10	po	100 ± 5	220 ± 12	355 ± 20	**788 ± 47**	100 ± 9	125 ± 7	337 ± 17	**873 ± 69**
Molindone	3	po	100 ± 12	237 ± 26	362 ± 24	629 ± 48	100 ± 5	168 ± 9	309 ± 27	735 ± 130
Trimipramine	50	ip	100 ± 6	176 ± 15	205 ± 17	**205 ± 9**	100 ± 9	133 ± 10	218 ± 15	**143 ± 10**
Opipramol	50	ip	100 ± 8	158 ± 10	287 ± 20	361 ± 22	100 ± 5	121 ± 6	241 ± 15	**218 ± 3**
Trazodone	100	ip	100 ± 2	187 ± 6	438 ± 20	**351 ± 46**	100 ± 9	137 ± 6	226 ± 2	**121 ± 2**
Corynanthine	30	ip	100 ± 6	176 ± 15	221 ± 3	270 ± 11	100 ± 9	133 ± 10	214 ± 14	**156 ± 7**
Rauwolscine	10	ip	100 ± 11	208 ± 30	461 ± 92	448 ± 45	100 ± 10	170 ± 23	534 ± 111	309 ± 20
Yohimbine	5	ip	100 ± 2	187 ± 6	333 ± 19	**357 ± 13**	100 ± 9	137 ± 6	269 ± 27	**198 ± 4**
Piperoxane	50	ip	100 ± 5	153 ± 10	330 ± 7	**213 ± 11**	100 ± 6	129 ± 12	252 ± 11	**150 ± 7**
Esproquin	30	ip	100 ± 6	280 ± 15	486 ± 39	588 ± 9	100 ± 9	157 ± 4	351 ± 29	**263 ± 14**
WB 4101	20	ip	100 ± 5	153 ± 10	269 ± 15	308 ± 48	100 ± 6	129 ± 12	228 ± 13	**139 ± 6**

Groups of 5 rats were treated with 2.5 mg/kg s.c. AFA 5 min prior to the administration of the test compound. The animals were decapitated 90 min later, and HVA and DOPAC in the striatum determined by HPLC with electrochemical detection. Data are given as means ± S.E.M. in percent of controls. The number of animals per group was 4–6. The means of the absolute control values were between 524 and 903 ng/g for HVA and between 901 and 1503 ng/g for DOPAC. In the AFA + drug columns, numbers printed in bold indicate a statistically significant deviation from additivity (P at least <0.05; for statistical procedure see Methods).

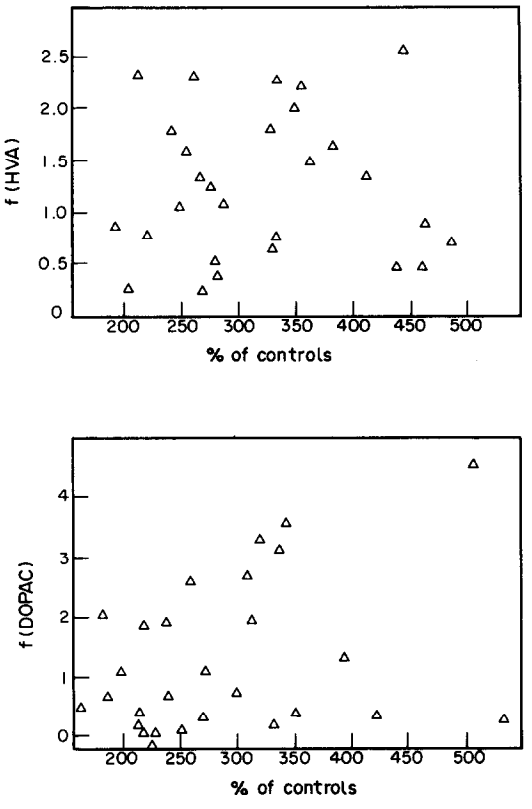

Fig. 1. Relation between the deviation from additivity of the combined effects of AFA and test drugs and the extent of the effects on DA metabolites of the latter alone. The factors $f_{(HVA)}$ (upper panel) and $f_{(DOPAC)}$ (lower panel), calculated as described in the text, were plotted against the effects of the respective test drugs, given alone in the same experiment, on the levels of HVA and DOPAC, respectively, expressed in percent of controls.

HVA or DOPAC had been potentiated by AFA in the previous experiments.

DISCUSSION

The findings of McMillen [3], that AFA enhanced the effects of haloperidol and trifluoperazine on striatal DOPAC levels in a more than additive manner, whereas this was not the case with clozapine, sulpiride or thioridazine, could be fully confirmed. Moreover it was shown that essentially the same results are obtained if HVA levels are considered.

Of a series of other neuroleptics, some were found to be potentiated by AFA, others were not, and the effects of a third group of compounds were even reduced. Using $f_{(HVA)}$ and $f_{(DOPAC)}$, the compounds might be ranked with respect to their interaction with AFA; for the present, it seems more useful, however, to simply categorize them into 3 groups as mentioned above. The result of this is given in Table 3, considering only those compounds which are clinically used as neuroleptics.

The problem of assessing the significance of this categorization can be approached by trying to relate it to similar categorizations derived from comparisons of other animal data, e.g. the ratios between the potencies of neuroleptics to elicit catalepsy and to inhibit amphetamine-induced stereotypies in rats (cat/amph ratio) or similar ratios between potencies to inhibit avoidance of punishment and to block amphetamine effects in dogs (jumping box/amph ratio [12]). Such procedures are widely used in attempts to predict the ratios between antipsychotic effects and neurological side effects in patients. However, a comparison of these predictions (animal data from refs. [12] and [13], and some unpublished data of Dr. Delini-Stula were considered) with clinical ratings of extrapyramidal side effect (EPS) liability (see e.g. the Bobon classification as presented by Kelder [14]) is not particularly encouraging: for instance, chlorpromazine, which has a rather bad prediction from cat/amph and jumping box/amph ratios, seems to cause comparatively less EPS than trifluoperazine, perphenazine or benperidol, for which prediction from animal data is much better.

The agreement between the categorization according to the AFA test and similar grouping based on cat/amph or jumping box/amph ratios is rather poor. Thus, the effects of benperidol, trifluoperazine, or perphenazine, which perform rather well in the behavioural test pairs, are potentiated by AFA. Conversely, those of chlorpromazine and pipamperone, which perform rather badly in the behavioural test pairs, are only moderately or not at all potentiated; but neither are those of clozapine, thioridazine and sulpiride, which are hardly able to cause catalepsy at all.

A comparison of the categorization or neuroleptics according to the AFA test with the clinical rating of

Table 2. Interaction of AFA with DA antagonists: effects on striatal DA concentrations

Drug	Dose (mg/kg)		Controls (ng/g)	AFA (ng/g)	Drug (ng/g)	AFA + drug (ng/g)	% of control.
Haloperidol	1	po	6669 ± 444	6650 ± 467	6103 ± 217	**2874 ± 57**	43 ± 1
Clozapine	100	po			6926 ± 425	**3348 ± 131**	50 ± 2
Sulpiride	100	ip			6389 ± 154	**3571 ± 454**	54 ± 7
Metoclopramide	10	ip	6064 ± 358	5823 ± 161	5452 ± 260	**3012 ± 121**	50 ± 2
Perphenazine	10	po			5738 ± 214	**2775 ± 93**	46 ± 2
Perlapine	20	po			6342 ± 323	**2414 ± 69**	40 ± 1
Piperoxane	50	ip			5338 ± 367	**2770 ± 227**	46 ± 4

The table shows the results of two separate experiments. The treatment schedule was the same as in the HVA/DOPAC experiments shown in Table 1. DA concentrations were determined fluorometrically. Data are means ± S.E.M. (N = 6). Bold figures indicate a significant difference from controls ($P < 0.01$, Dunnett's test). None of the test compounds nor AFA altered DA levels significantly.

Table 3. Categorization of neuroleptics with respect to their interaction with AFA

More than additive	Additive	Less than additive
Flupenthixol	Molindone	Perlapine
Haloperidol	Pipamperone	Clozapine
Perphenazine	Sulpiride	Thioridazine
Benperidol	Zetidoline	
Spiperone		
Trifluoperazine		
Fluphenazine		
Metoclopramide		
Fluperlapine		
Pimozide		
Chlorpromazine		

The neuroleptic compounds investigated in this study were categorized into 3 groups, according to the results presented in Table 1 and the pertinent statistical calculations: at left those which in combination with AFA showed a more than additive effect on the levels of HVA, DOPAC, or both; within the column, there is a rank order from top to bottom according to the extent and level of significance of the synergism. The center column contains the drugs which showed an additive effect in alphabetical order, and the left column contains those which showed a less than additive effect, again ranked according to extent and a statistical significance.

EPS liability (Bobon classification; see [14]) reveals a better agreement. Of the compounds specified in both Kelder's list and in Table 3, those rated clinically to cause moderate to very marked EPS (benperidol, haloperidol, flupenthixol, trifluoperazine) all are markedly potentiated by AFA. Those considered to cause weak, very weak, or no EPS are all either weakly or not at all potentiated or even attenuated by AFA (chlorpromazine, pimozide, pipamperone, sulpiride, clozapine, thioridazine).

Although this rather reasonable agreement might be taken to indicate that absence of synergism with AFA might be predictive for a good ratio between antipsychotic efficacy and EPS, the results with some compounds, of which the clinical effects are less well documented, should also be considered. Perlapine, for instance, which definitely possesses antidopaminergic properties [15, 16], has been reported to lack antipsychotic activity [17] (see also [16]). Since details of these clinical studies have never been published, it is not possible to know whether this failure was due to insufficient doses, as suggested by Wilk and Stanley, and if so, whether it would have caused EPS. The significance of the fact that its effects on DA metabolites was attenuated by AFA can therefore not be assessed. The related new compound fluperlapine, on the other hand, synergized with AFA to a moderate extent. Published clinical data are as yet scarce, but it seems to have antipsychotic effects without causing much EPS [18, 19]. If this characterization persists, it will question the significance of the AFA test.

On the other hand, metoclopramide, which was initially reported to lack antipsychotic effects in patients [20], but proved to be effective and also caused EPS in almost all the subjects in a later study with higher dosages [21], was also potentiated by AFA.

Of the 3 antidepressants and the 6 α_1- or α_2-noradrenergic antagonists with antidopaminergic properties, which were also tested, none synergized with AFA; on the contrary, a clearcut attenuation of their effects was seen in several cases. The significance of this in terms of EPS liability cannot be estimated due to the absence of relevant clinical data. It may perhaps, however, prove to be of interest in mechanistic considerations. The absence of synergism with AFA of clozapine and all the α-noradrenergic antagonists should not be taken to indicate a crucial involvement of α-receptors: as pointed out by McMillen [3], the fact that sulpiride lacks α-noradrenolytic properties argues against this. Moreover, there is no difference between compounds possessing α_1- or α_2-noradrenolytic properties, which should be expected in view of the different location of these receptor types. The results with sulpiride probably also exclude an involvement of histaminic, muscarinic, and serotenergic receptors. The question whether a possible explanation might be that the drugs which do not synergize with AFA do not interfere with presynaptic DA receptors, has been discussed [3]. Blockade of the inhibiting effect of increased synaptic DA concentrations on further release would explain the decrease in DA levels after AFA + haloperidol, since persistently and grossly increased DA release would eventually exhaust the stores. However, the decrease of DA levels also occurred when AFA was combined with those DA antagonists with which there was no synergism with respect to HVA and DOPAC. Two possible explanations may account for this: first, the route of disposal of the DA released from the stores may be different with the two types of combinations. It might be of interest in this context that, in the groups receiving the combined treatment AFA + neuroleptic, the increase of DOPAC was greater or equal to that of HVA with 8 of the 11 compounds classified as "more than additive" in Table 3 (exceptions: chlorpromazine, trifluoperazine, metoclopramide). Conversely, with 6 of the 7 compounds classified as "additive" or "less than additive", the increase in DOPAC was clearly inferior to that of HVA (exception: molindone). The same holds true for all of the other DA antagonists (not clinically used as neuroleptics) listed in Table 1. As a second possibility, the two types of combinations might affect DA synthesis differently. In either case, we are left with the task of explaining the reason, which requires further experiments.

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