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RESEARCH PAPER

Modulatory effects of neuropsychopharmaca on intracellular pH of hippocampal neurones *in vitro*

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Background and purpose: The intracellular pH (pHi) of neurones is tightly regulated by, for example, membrane-bound acid-exchangers and loaders. Nevertheless, excessive bioelectric activity lowers steady-state pHi. In turn, even a moderate acidification can inhibit neuronal activity, a process believed to be part of a negative feedback loop controlling neuronal excitation. As moclobemide, an antidepressant, and also some antiepileptic drugs can reduce neuronal pHi in hippocampus slices *in vitro*, we screened a panel of currently used neuropsychopharmaca for comparable effects.
Experimental approach: BCECF-AM loaded hippocampal slices were superfused with 16 different neuroleptics, antidepressants and antiepileptics under bicarbonate-buffered conditions. Changes in steady-state pHi of CA3 neurones were measured fluorometrically.
Key results: The antipsychotics haloperidol, clozapine, ziprasidone, and the antidepressants amitriptyline, doxepin, trimipramine, citalopram, mirtazapine, as well as the anticonvulsive drug tiagabine reversibly reduced the steady-state pHi by up to 0.35 pH-units in concentrations of 5–50 μM. In contrast, venlafaxine, the anticonvulsants carbamazepine, clonazepam, gabapentin, lamotrigine, zonisamide, and the mood stabilizer lithium had no effect on neuronal pHi.
Conclusion and implications: These data substantiate the view that clinically relevant concentrations of neuroleptics and antidepressants can mediate changes in neuronal pHi, which may contribute to their pharmacological mode of action. Effects on pHi should be taken into account when therapeutic or even harmful effects of these drugs are evaluated.
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Keywords: antidepressants; antipsychotics; anticonvulsants; lithium; intracellular pH; pH regulation

Abbreviations: AE, anion exchanger; BCECF-AM, 2′,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymetyl ester; CA3, cornu ammonum region 3; CAT, catecholamine transporter; MAO, monoamine oxidase; MCT, monocarboxylate transporter; NCBE, Na^+-dependent Cl^-/HCO_3^- -exchanger; NBC, Na^+/HCO_3^- exchanger; NHE, sodium proton exchanger; NPP, neuropsychopharmaca; pHi, intracellular pH; SERT, 5-HT transporter

Introduction

Cellular functions are largely influenced by the concentration of intracellular free H^+, which is mainly determined by the interplay of cell metabolism and acid extrusion. In neurones, several lines of evidence point to a pivotal role of intracellular pH (pHi) for inter- and intracellular signalling as well as for cellular and synaptic plasticity (reviewed by Chesler, 2003). It is now widely accepted that neuronal activity modulates pHi and that pHi changes in turn can influence neuronal activity, for example, via altered membrane channel conductivity (Chesler and Kaila, 1992; Takahashi and Copenhagen, 1996; Bonnet *et al.*, 2000a, Xiong *et al.*, 2000; Schuchmann *et al.*, 2006). Membrane-bound antiporters are important as well and, for example, vesicular Ca^{2+}/H^+-exchange and Na^+/H^+-exchange are known to influence neurotransmission (Goncalves *et al.*, 1999; Jang *et al.*, 2006). Catecholamine neurotransmitters can directly activate the neuronal sodium-proton exchange (Smith *et al.*, 1998). Monoamine oxidase (MAO) activity itself, necessary for the degradation of catecholamines and other biogenic amines, is intimately linked to mitochondrial function and ATP production (Cohen and Kesler, 1999) and it has been shown that MAO-A inhibitors themselves, such as moclobemide, clorgyline and pargyline, are able to reduce pHi of CA3 neurones (Bonnet *et al.*, 2000b). With regard to other neuroactive clinical drugs, some anticonvulsants such as levetiracetam (Leniger *et al.*, 2004a),

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topiramate (Leniger *et al.*, 2004b), valproate (Bonnet *et al.*, 2002) and sulthiame (Leniger *et al.*, 2002) have been demonstrated to influence pHi of hippocampal CA3 neurones. These substances evoked reversible acidifications, which were, at least in part, based on an interference with neuronal pHi-regulation and/or carbonic anhydrase inhibition.

We previously hypothesized that a modestly lowered neuronal pHi might contribute to the therapeutic potential of some neuropsychopharmaca (NPP) (Bonnet and Wiemann, 1999b; Bonnet *et al.*, 2000b), whereas a higher acidification is regarded as being cytotoxic (Siesjö *et al.*, 1993; Ding *et al.*, 2000). In the present investigation, we extended our studies on the pHi modulating effects of clinical drugs to currently used NPP and screened relevant concentrations of antipsychotics, antidepressants and additional anticonvulsants. Neuronal pHi changes were studied in a well-established *ex vivo* model system, namely the 2′,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymetyl ester (BCECF-AM) loaded hippocampal slice, which allows for the detection of changes in the steady state pHi upon drug treatment in adult hippocampal neuronal somata (Bonnet and Wiemann, 1999a; Hentschke *et al.*, 2006).

Drug concentrations in the extracellular brain fluids of rodents and humans, that are equivalent to therapeutic plasma concentrations, were found to be in the low micromolar range (0.5–10 μM) (Glotzbach and Preskorn, 1982; Walker *et al.*, 2000; Wang *et al.*, 2004; Rambeck *et al.*, 2006). Nevertheless, in the present study, initial experiments were conducted with 50 μM. This concentration was chosen to obtain faster tissue penetration and because BCECF-AM loaded slice preparations bleach over time, restricting the duration of experiments (Bevensee *et al.*, 1995). Moreover, effects on pHi are easier to detect if they occur abruptly. Concentrations more closely resembling the therapeutic range (5–10 μM) were then tested in a second step. With this approach, we show that a considerable number of NPP can modify neuronal pHi, at least in concentrations equivalent to the upper therapeutic range.

Methods

Transverse hippocampal slices (400–500 μm thick) were prepared from brains of adult guinea-pigs (300–400 g) as described previously (Leniger *et al.*, 2004a). Brain slices were pre-incubated for 2 h in 28°C warm saline equilibrated with 5% CO_2 in O_2. This saline contained in mM: NaCl 124, KCl 3, $CaCl_2$ 0.75, $MgSO_4$ 1.3, KH_2PO_4 1.25, $NaHCO_3$ 26 and glucose 10. After pre-incubation, slices were transferred to a perspex recording chamber (vol. 4 mL) which was mounted on the stage of an upright microscope (Olympus Bx50Wi, Olympus, Hamburg, Germany) equipped with water immersion objectives. In this chamber, the submerged slices were continuously superfused with 32°C warm saline at a rate of 4.5 mL·min^{-1}. The composition of this control solution was the same as during the pre-incubation period except for the calcium concentration, which was raised to 1.75 mM. The following drugs were added to the superfusate: amitriptyline hydrochloride (pKa: 9.4), doxepin hydrochloride (pKa: 10.4), trimipramine hydrogenmaleonate (pKa: 9.4), citalopram hydrobromide (pKa: 9.5), mirtazapine (pKa: 7,7), venlafaxine hydrochloride (pKa: 9.4), carbamazepine (pKa: 7.0), tiagabine hydrochloride (pKa: 9.4 and 13.3), zonisamide (pKa: 10.2), gabapentin (pKa: 4.5 and 9.5), lamotrigine (pKa: 5.7), clonazepam (pKa: 1.5 and 10.5), ziprasidone hydrochloride (pKa: 6.5), haloperidol (pKa: 8.3), clozapine (pKa: 7.5) and lithium chloride. None of the additives changed the pH of the bicarbonate buffered superfusate, which was continuously controlled to be 7.35–7.4 and kept constant throughout each experiment.

Analysis of pHi changes

Hippocampal slices were stained with 0.5–1 μM BCECF-AM for 3–5 min in the pre-incubation saline. Slices were superfused with saline for at least 30 min without illumination to allow the pHi to stabilize. Then superficially located neurones of the stratum pyramidale of the CA3 region were identified by stained apical dendrites (Bonnet *et al.*, 1998; Bonnet and Wiemann, 1999a) and examined under a 40× or 60× water immersion objective. Up to three neighbouring neurones were recorded simultaneously. In some optical recordings, pHi transients of neuronal somata were also compared with adjacent regions of interest placed, for example, in the stratum radiatum to better reveal specific pHi responses (see Figure 1B). Slices were intermittently excited at 440 and 490 nm (duration of illumination: 0.2 s at each wavelength) using a computer operated filter wheel (Sutter Instruments, Novato, CA, USA) equipped with a 100 W halogen lamp. Fluorescence images were captured (0.05 Hz) by an intensified

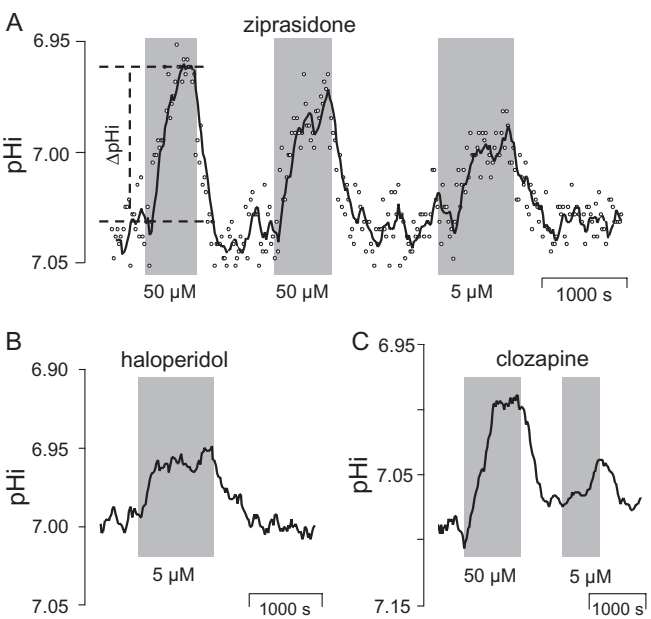

Figure 1 Effects of typical and atypical neuroleptics on the steady-state intracellular pH (pHi) of BCECF-laden hippocampal CA3 neurones. Application periods of drugs at indicated concentrations are shown as shaded areas. (A) Ziprasidone, (B) haloperidol, (C) clozapine. All curves represent sliding averages from eight data points measured in single neuronal somata. Open circles in (A) show original data points measured at 20 s intervals. Definition of ΔpHi (see Tables 1–3 for group data) is demonstrated in (A).

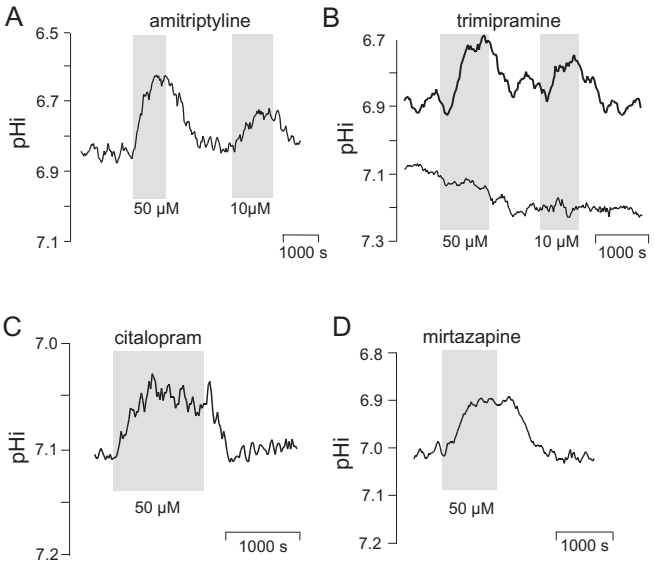

Figure 2 Effects of antidepressants on steady state intracellular pH (pHi) of BCECF-laden hippocampal CA3 neurones. Application periods of drugs at indicated concentrations are shown as shaded areas. (A) Amitriptyline, (B) trimipamine, (C) citalopram, (D) mirtazapine. Curves represent sliding averages from four to eight data points measured in one pyramidal soma. In (B), pHi deflections within near-by regions (150–250 μm^2) of the stratum radiatum are additionally shown.

CCD camera (PTI, Surbiton Surrey, England). Background fluorescence was taken from unstained slices using the same camera and microscope settings. To ratio background subtracted images, a CARAT system (Dr O. Ahrens, Bargteheide, Germany) was used. At the end of an experiment, the ratio 440/490 was calibrated by a standard curve; this was obtained by the *in vitro* calibration method adapted to an upright microscope (Bonnet and Wiemann, 1999a; Hentschke *et al.*, 2006). Background light was equally included into the standard curve and, thus, eliminated from the measurement.

To achieve optical recordings of single CA3 neurones (located in the stratum pyramidale) for up to 3 h, lowest possible intensity of excitation light combined with a near-to-maximum gain of the camera was found to be essential. A fairly stable BCECF fluorescence signal (excitation: 440 nm, emission >520 nm) with a loss of intensity <1% per min was taken as a criterion for neurones to be in good condition (Bevensee *et al.*, 1995). Only neurones fulfilling this criterion were subjected to further drug treatment. None of the drugs changed the background fluorescence parameters when tested at their final concentrations. pHi signals were collected from a region of interest positioned over neuronal somata. Spontaneous baseline ΔpHi-deflections were <0.03 pH-units. Sliding averages of 4–8 values are shown in Figures 1–3 to eliminate noise and demonstrate pHi shifts more clearly.

Evaluation criteria and definition of 'pHi activity'
To ensure that the changes in steady-state pHi were mediated by the drug, we used the following three mandatory criteria:

Timing. Changes in pHi must be closely related in time to the onset of drug application.

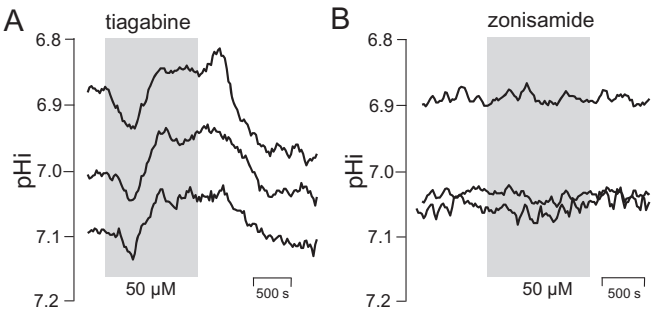

Figure 3 Effects of antiepileptics on steady state intracellular pH of BCECF-laden hippocampal CA3 neurones. Application periods of drugs at indicated concentrations are shown as shaded areas. (A) Tiagabine, (B) zonisamide. Curves represent sliding averages from eight data points measured in three pyramidal somata. Note that neurones respond to tiagabine but not to zonisamide.

Reversibility. Changes in pHi must be at least partly reversible upon washout.

Magnitude. Amplitudes of pHi changes must clearly exceed the noise level (ΔpHi > 0.03 pH-units). Calculations of ΔpHi were uniformly carried out on sliding averages of eight values (see Figure 1 for illustration). As drug-mediated pHi deflection may be shaped by ongoing pHi regulation (i.e. by progressive pHi recovery, overshooting pHi deflection upon washout), we calculated ΔpHi between the mean pHi immediately before drug application and the maximum pHi deflection during drug application.

Drugs were designated as being 'pHi active' if at least 2/3 of the experiments fulfilled the said criteria. In the case of 'pHi-activity' of 50 µM drug concentration (except for lithium), a lower concentration (5 or 10 µM) was additionally tested (except for doxepin). In some experiments, both concentrations were successively applied to the same neurone (see e.g. Figures 1 and 2).

Statistics
Group values are given as mean ± standard deviation (SD). Effects of drug application were tested for significance by Student's *t*-test for paired samples and *P* values are indicated in Tables 1–3. Differences between concentration groups (5 or 10 vs. 50 µM) were analysed by independent sample *t*-tests and confirmed by ANOVA. Significance was assumed for $P \leq 0.05$.

Drugs
All the drugs were obtained from Sigma (Hamburg, Germany) unless stated otherwise. Mirtazapine was obtained from Organon (Oberschleissheim, Germany); tiagabine hydrochloride from Chemos (Regenstauf, Germany); gabapentin from Parke-Davis (Freiburg, Germany); clozapine from Novartis (Neuss, Germany) and BCECF-AM from Molecular Probes (Leiden, the Netherlands).

Results

This study was carried out on 161 pyramidal neurones of the CA3 region in 121 hippocampal slices. The mean intracellular

Table 1 Effects of antipsychotics on pHi of CA3 neurones

Drug	*Slices*	*Neurones*	*Responses*	*pHi*[1]	*pHi (treatment)*	*ΔpHi*[2] *(range)*	*ΔpHi*[2] *(mean ± SD)*	*pH-active*[3]
All	17	22	26 of 27	7.05 ± 0.07	6.98 ± 0.08	0.04–0.18	0.07 ± 0.03	
Haloperidol	6	8	5 μM: 3 of 3	7.02 ± 0.08	6.95 ± 0.08**[4]	0.06–0.07	0.06 ± 0.01	Y
			50 μM: 6 of 6	7.03 ± 0.05	6.95 ± 0.07**	0.05–0.15	0.09 ± 0.04	Y
Clozapine	5	7	5 μM: 3 of 3	7.07 ± 0.06	7.00 ± 0.06*	0.04–0.10	0.06 ± 0.09	Y
			50 μM: 6 of 6	7.06 ± 0.08	6.98 ± 0.09**	0.05–0.18	0.09 ± 0.05	Y
Ziprasidone	6	7	5 μM: 5 of 5	7.04 ± 0.05	6.99 ± 0.05**	0.04–0.06	0.05 ± 0.01	Y
			50 μM: 3 of 4	7.07 ± 0.09	7.03 ± 0.12*	0.06–0.07	0.06 ± 0.01	Y

[1]Steady pHi prior to treatment (control condition).
[2]Responding cells only (ΔpHi > 0.03 pH units).
[3]Y = Yes, N = No.
[4]Asterisks indicate significant differences from control. *P < 0.05; **P < 0.01.
pHi, intracellular pH; SD, standard deviation.

Table 2 Effects of antidepressants on pHi of CA3 neurones

Drug	*Slices*	*Neurones*	*Responses*	*pHi*[1]	*pHi (treatment)*	*ΔpHi*[2] *(range)*	*ΔpHi*[2] *(mean ± SD)*	*pHi-active*[3]
All	46	68	55 of 77	7.01 ± 0.16	6.92 ± 0.16	0.05–0.35	0.12 ± 0.08	
Amitriptyline	9	10	10 μM: 3 of 4	6.92 ± 0.09	6.80 ± 0.18*[4]	0.07–0.2	0.15 ± 0.07	Y
			50 μM: 5 of 7	6.94 ± 0.15	6.88 ± 0.16*	0.05–0.2	0.07 ± 0.02	Y
Doxepin	4	4	50 μM: 4 of 4	6.96 ± 0.07	6.88 ± 0.04*	0.06–0.12	0.09 ± 0.04	Y
Trimipramine	10	20	10 μM: 9 of 12	6.95 ± 0.07	6.86 ± 0.15**	0.05–0.25	0.19 ± 0.10	Y
			50 μM: 7 of 10	7.02 ± 0.20	6.91 ± 0.19**	0.06–0.35	0.15 ± 0.07	Y
Citalopram	8	11	10 μM: 3 of 3	7.23 ± 0.03	7.16 ± 0.02**	0.06–0.08	0.07 ± 0.01	Y
			50 μM: 7 of 10	7.04 ± 0.10	6.97 ± 0.06**	0.05–0.13	0.08 ± 0.04	Y
Mirtazapine	10	16	10 μM: 10 of 14	7.07 ± 0.15	6.96 ± 0.16**	0.05–0.20	0.12 ± 0.05	Y
			50 μM: 5 of 6	6.96 ± 0.18	6.78 ± 0.18*	0.05–0.30	0.16 ± 0.13	Y
Venlafaxine	5	7	50 μM: 2 of 7	6.99 ± 0.18	6.98 ± 0.18	0.04–0.05	0.05 ± 0.01	N

[1]Steady pHi prior to treatment (control condition).
[2]Responding cells only (ΔpHi > 0.03 pH units).
[3]Y = Yes, N = No.
[4]Asterisks indicate significant differences from control. *P < 0.05; **P < 0.01.
pHi, intracellular pH; SD, standard deviation.

Table 3 Effects of anticonvulsants on pHi of CA3 neurones

Drug	*Slices*	*Neurones*	*Responses*	*pHi*[1]	*pHi (treatment)* *ΔpHi*[2] *(range)*	*ΔpHi*[2]	*ΔpHi*[2] *(mean ± SD)*	*pH-active*[3]
All	47	58	18 of 68	6.94 ± 0.14	6.92 ± 0.14	0.04–0.10	0.07 ± 0.02	
Carbamazepine	6	6	50 μM: 1of 6	6.95 ± 0.14	6.93 ± 0.15	0.10	0.10	N
Clonazepam	6	6	50 μM: 0 of 6	7.01 ± 0.09	7.01 ± 0.09	<0.03	<0.03	N
Gabapentin	6	6	50 μM: 0 of 6	6.97 ± 0.10	6.97 ± 0.10	<0.03	<0.03	N
Lamotrigine	6	6	50 μM: 0 of 6	6.95 ± 0.08	6.95 ± 0.08	<0.03	<0.03	N
Tiagabine[4]	8	10	10 μM: 3 of 3	7.03 ± 0.15	6.97 ± 0.16*[5]	0.05–0.07	0.06 ± 0.01	Y
			50 μM: 11 of 11	6.95 ± 0.12	6.87 ± 0.12**	0.04–0.10	0.07 ± 0.02	Y
zonisamide	15	24	50 μM: 3 of 30	6.91 ± 0.17	6.90 ± 0.16	0.04–0.10	0.08 ± 0.04	N

[1]Steady pHi prior to treatment (control condition).
[2]Responding cells only (ΔpHi > 0.03 pH units).
[3]Y = Yes, N = No.
[4]Only acidic deflections.
[5]Asterisks indicate significant differences from control. *P < 0.05; **P < 0.01.
pHi, intracellular pH; SD, standard deviation.

steady state pHi of the whole collective was 6.98 ± 0.14 (mean ± SD) under control conditions.

Antipsychotics

Effects of the atypical neuroleptics ziprasidone and clozapine and of the typical neuroleptic haloperidol on steady state pHi were evaluated from a total of 27 optical recordings (see Table 1 for details). Mean steady state pHi (±SD) in this series of experiments was 7.05 ± 0.07 prior to drug application. At concentrations of either 5 or 50 μM, all drugs evoked significant decreases of pHi within 5–10 min (Figure 1). The largest pHi deflections were seen for clozapine (0.18 pH units) and haloperidol (0.15 pH units). There were no significant

differences between mean values of the concentration groups (5 and 50 μM) for either haloperidol, clozapine or ziprasidone (*P* values: 0.23, 0.41, and 0.98 respectively). Within the limits of this study, we obtained no evidence that the pHi responses were dependent on the respective starting pHi. All pHi deflections evoked by antipsychotics were reversible. Alkalotic overshoots, possibly pointing to ongoing or disturbed pHi regulation (Figure 1), were not observed in any of the experiments. On the whole, application of 5 or 50 μM of each tested antipsychotic decreased pHi in 26 of the 27 optical recordings (96%).

Antidepressants

Effects of older (amitriptyline, doxepin, trimipramine) and more modern antidepressants (citalopram, mirtazapine, venlafaxine) on pHi were tested in 68 neurones of 46 slices. The mean steady state pH in this series of experiments was 7.01 ± 0.16. The results are summarized in Table 2.

The tricyclics amitriptyline, doxepin, trimipramine (applied to 34 neurones) reversibly reduced the steady state pHi of CA3 neurones in the majority of experiments (28 out of 37). There were no significant differences between mean values of the concentration groups (10 and 50 μM) of either amitriptyline and trimipramine (*P* values: 0.26 and 0.46 respectively).

Citalopram, a selective 5-hydroxytryptamine (5-HT)-reuptake inhibitor, was effective in 10 out of 13 recordings. Mean values of the concentration groups (10 and 50 μM) were also not different from each other ($P = 0.33$).

In the group in which the effects of combined 5-HT and noradrenaline enhancers were investigated, the results were less uniform. Thus, mirtazapine elicited acidification in 15 out of 20 experiments, whereas venlafaxine was classified as to be not pHi active because small responses were seen in only 2 out of 7 experiments. In the case of mirtazapine, no difference was seen between mean values of the concentration groups (10 and 50 μM, $P = 0.43$).

Thus, with the exception of venlafaxine, all the antidepressants tested significantly lowered the steady state pHi in a reversible manner (Figure 2)

Anticonvulsants

In the third part of the study, we investigated the older anticonvulsants carbamazepine and clonazepam as well as the newer drugs gabapentin, lamotrigine, tiagabine and zonisamide in 58 neurones from 47 slices. Significant changes in pHi were observed only upon application of the GABA-transport inhibitor tiagabine (Table 3). With 10 and 50 μM tiagabine, an acidification of 0.06 ± 0.01 and 0.07 ± 0.02 pH units occurred, respectively, after at least 10 min (Table 3, Figure 3). The difference between the two concentrations was not significant ($P = 0.16$). However, in 6 out of 10 neurones, acidification was preceded by a transient alkalosis (ΔpHi 0.053 ± 0.008, $P < 0.001$), as shown in Figure 3.

Lithium

The mood stabilizer lithium (1.2 mM) failed to evoke pHi changes in 13 CA3-neurones taken from 11 different slices. Mean starting pH_i was 7.02 ± 0.14 in these experiments and remained unaltered even when exposure times were extended to more than 40 min.

Discussion

General aspects and limitations of the study

The main finding of this investigation was that 9 out of 16 NPP influenced steady state pHi of hippocampal CA3 neurones *in vitro* and were, therefore, classified as 'pHi active'. All the antipsychotics tested (haloperidol, clozapine, ziprasidone) and most of the antidepressants (amitriptyline, doxepin, trimipramine, citalopram, mirtazapine) were among the drugs that lowered pHi by up to 0.35 units. The majority of anticonvulsants, that is, carbamazepine, clonazepam, gabapentin, lamotrigine, zonisamide had no effect and only tiagabine elicited a biphasic response. Lithium which interferes with sodium proton exchange (NHE) and lowers pHi in cultured cells within several days of treatment (Wall *et al*., 1988; Bitran *et al*., 1990; Kobaysashi *et al*., 2000) had no acute effect on steady state pHi of hippocampal CA3 neurones.

When screening NPP, a low (5–10 μM) and a high concentration (50 μM) of each drug evoked largely similar, that is, not significantly different effects. This suggests that drug binding sites responsible for pHi effects may be largely saturated even at low micromolar concentrations. With respect to amplitude and slope of the pHi deflection, a concentration-dependent acidification was observed for single neurones subjected to repetitive treatments with amitriptyline, trimipramine and clozapine (see Figures 1A, C and 2A, B). A more detailed description of dose-response effects awaits further studies on well-defined *in vitro* systems. Concentrations between 5–10 μM, at which effects on pHi were initially observed, represent the upper range of therapeutic concentrations in the brain (Glotzbach and Preskorn, 1982; Walker *et al*., 2000; Wang *et al*., 2004; Rambeck *et al*., 2006). This and the fact that a lowering of pHi by less than 0.1 pH unit can reduce neuronal excitability or frequency of epileptic discharges (Bonnet *et al*., 1998; 2000a; Xiong *et al*., 2000) suggest that our findings may be clinically relevant.

The mechanisms underlying the shift in the steady-state pHi upon application of NPP were beyond the scope of this investigation. Nevertheless, the possibility that drugs directly acidified the cells because their pKa values (see Methods section) were mostly basic or near neutral was excluded. The finding that the basic drugs (pKa >7.0) failed to increased pHi is most likely due to the high buffering capacity of the bicarbonate buffered superfusate and the comparatively low concentration of the drugs. For example, a 200-fold higher concentration of ammonium chloride (pKa: 9.2) is needed to induce a clear alkalinization under equivalent experimental conditions (Bonnet and Wiemann, 1999a). In line with this, 50 μM gabapentin, lamotrigine and clonazepam, all of which showed pKa values below 7.0, elicited no acidification. Therefore, the possibility that all these drugs have a direct effect on the steady-state pHi can be ruled out.

It may appear surprising that nine drugs elicited acidification whereas a sustained increase in the pHi was not observed. In principal, acidification can arise from increased excitatory

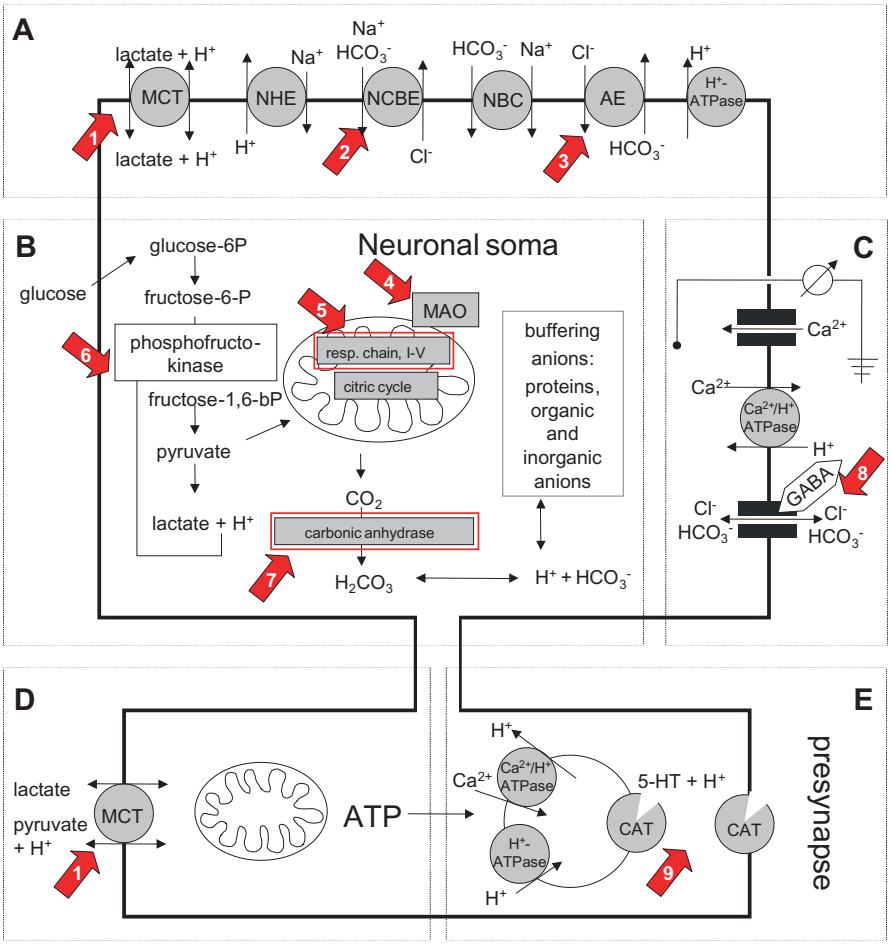

Figure 4 Schematic overview of major components and systems that are relevant to neuronal intracellular pH regulation. (A) Main acid extruders comprise monocarboxylate transporters (MCT), Na^+/H^+ exchangers (NHE), Na^+-dependent Cl^-/HCO_3^- -exchanger (NCBE), Na^+/HCO_3^- -exchanger (NBC), and H^+-ATPases. Anion exchangers (AE), e.g. Cl^-/HCO_3^- exchanger, act as acid loaders. (B) Intracellular protons mainly result from lactic acid production (glycolysis) and H_2CO_3 formation from metabolic CO_2 production (citrate cycles) involving the mitochondria. Carbonic anhydrases are important for the level of cellular bicarbonate and, thus, for intracellular buffering capacity. (C) Membrane potential indirectly drives transmembane fluxes of H^+ and bicarbonate. (D) Presynaptic mitochondria are preferentially fueled by lactate and pyruvate to provide ATP, for example, for neurotransmitter re-uptake by vesicular and transmembraneous transporters. (E) The pH gradient across the synaptic vesicle membrane is a driving force for transmitter re-uptake mediated by catecholamine-transporters (CAT), which comprise 5-HT transporters (SERT) dopamine transporters, noradrenaline transporters, GABA-transporters and excitatory acid transporters. As an example, the effect of SERT is shown. Arrows numbered 1–9 point to known targets of the following neuropsychopharmaca (see also Discussion): (1) valproate (Rumbach *et al.*, 1986); (2) levetiracetam (Leniger *et al.*, 2004a); (3) topiramate (Leniger *et al.*, 2004b); (4) moclobemide (Bonnet *et al.*, 2000b); (5) fluoxetine, haloperidol, valproate (Rumbach *et al.*, 1986; Wallace and Starkow, 2000); (6) valproate (Benavides *et al.*, 1982); (7) topiramate, sulthiame (Woodbury and Kemp, 1989; Leniger *et al.*, 2002); (8) tiagabine, topiramate, valproate (Kaila, 1994; Lueckermann *et al.*, 1997; Bonnet *et al.*, 2002); (9) 5-HT re-uptake inhibitors, tricyclics, mirtazapine, venlafaxine (Cao *et al.*, 1997).

postsynaptic potentials and action potential firing (Chesler and Kaila, 1992). However, most substances are known to dampen neuronal activity and are, therefore, more likely to diminish neuronal activity, making this possibility less likely. Acidification may also result from increased production of lactic acid, for example, via impaired mitochondrial function, and this mechanism cannot be excluded at present (see examples below). Finally, inhibition of the activity of any acid extrusion system will acidify neurones, because the proton motive force is inwardly directed at a membrane potential of –70 mV. Recent studies on major pHi regulating membrane transporters underline the importance of unimpaired acid extrusion for neuronal excitability. The functional knockout of NHE1, AE3 and NCBE in animal models led to the development of altered neuronal activity and/or changed susceptibility to epileptogenic drugs, even though the steady-state pHi was not significantly changed (Cox *et al.*, 1997; Gu *et al.*, 2001; Hentschke *et al.*, 2006; Jacobs *et al.*, 2008). These studies imply that pharmacological inhibition of a single pHi regulating system may lead to complex neurological changes in the organism, which may, however, only be revealed under particular conditions such as over-excitation. The molecules, membrane transporters and channels currently known to be involved in the regulation of neuronal pHi are summarized in Figure 4 (the abbreviations used conform to Alexander *et al.*, 2008). Many of these components are targeted by neuroactive compounds, and in most cases, these interactions led to neuronal acidification. Whether or not the NPP studied here

interfere with these targets should be addressed in future studies.

Sustained alkalinization of neurones was not observed after NPP treatment; for this to occur, an exaggerated extrusion of acid needs to take place, as described in the literature. For example, alkalinization of cultured hippocampal neurones occurs after stimulation of sodium proton transport via β-adrenoceptors (Smith *et al.*, 1998). However, no drug used in the present study binds to or stimulates adrenoceptors. Also, stilbene derivatives can induce an alkalinization in hippocampal neurones, provided that the chloride-bicarbonate-exchange is operating as an acid loader, as has been observed for a subgroup of cultured neurones with a pHi >7.2 (Brett *et al.*, 2002). As the steady-state pHi of neurones in slices was below 7.0, an inhibition of acid loading transporters would have been without effect on pHi. With respect to the drugs tested here, tiagabine was the only substance to induce a transient alkalinization in some neurones and even this was followed by acidification. Tiagabine inhibits GABA re-uptake and increases synaptic GABA, which allows accelerated fluxes of bicarbonate through opened GABA channels (Kaila, 1994; Bonnet and Bingmann, 1995; c.f. Figure 4C). As long as the membrane potential is more negative than the bicarbonate equilibrium potential, an efflux of bicarbonate can take place and this will result in a lowered pHi (Lueckermann *et al.*, 1997). A transient increase in pHi upon addition of tiagabine is therefore unlikely to be due to a direct effect of GABAergic transmission, but may reflect an inhibitory action on neuronal activity, or the involvement of interneurones. Thus, only a few mechanism(s) can lead to the alkalinization of neurones and none of them seems to play role in the effects of NPP.

Effects of antidepressants and antipsychotics on pHi

Synaptic processes are dependent on pH in many ways (see Figure 4B–E). Activation of GABA or glutamate-receptors is followed by a rapid and transient fall in the pHi due to bicarbonate- or proton-fluxes through ion channels or due to stimulation of the re-uptake machinery (Chesler and Kaila, 1992; Hartley and Dubinsky, 1993; Amato *et al.*, 1994). H^+-induced fluxes were also observed in several other ion-coupled transporters including the 5-HT and dopamine transporters (Cao *et al.*, 1997; Figure 4E). Much of the signalling involved in the actions of catecholamines is influenced by protons. For example, the release of dopamine from synaptosomes is affected by a drop in pHi (Trudeau *et al.*, 1999; Cannizzaro *et al.*, 2003). Also, the pH gradient spanning the synaptic vesicle membrane provides a major driving force for catecholamine transport (Toll and Howard, 1978; Moriyama and Futai, 1990). Hence, a lowered steady-state pHi secondary to the effects of pH active antidepressants or antipsychotics might be expected to influence the aforementioned synaptic processes with consequent alterations in brain function.

An increased intracellular H+ concentration may also be linked to mitochondrial function and energy metabolism. The mitochondrial membrane contains an NHE subtype (LeBlanc *et al.*, 1988; Orlowski and Grinstein, 1997; Numata *et al.*, 1998), which may help to control the pH of the mitochondrial matrix (Greenbaum and Wilson, 1991; Skulachev, 1999). Presumably by this means, NHE may exert some influence on the permeability of the mitochondrial transition pore (Friberg and Wieloch, 2002). Mitochondrial NHE may also be linked to catecholamine degrading enzymes such as MAO. Interestingly, MAO inhibitors, such as the antidepressant moclobemide, lowered pHi in hippocampal neurones (Bonnet *et al.*, 2000b). Furthermore, antidepressants seem to act as modulators of the membrane permeability transition pore (Marcocci *et al.*, 2002). Also at the level of mitochondria, the antipsychotic haloperidol and the antidepressant fluoxetine were shown to uncouple oxidative phosphorylation (Wallace and Starkow, 2000). This impairment of mitochondrial function may lead to a marked increase in lactic acid formation and, consequently, to metabolic acidification. Clinically, this situation may culminate in a metabolic disaster, known for instance as the malignant neuroleptic syndrome or as the 5-HT syndrome. These examples demonstrate that at least some antidepressants and antipsychotics are able to influence the basic metabolism of cells and outline a field for future research needed to understand the effects of NPP on pHi.

Effects of anticonvulsants on pHi

Epileptiform discharges are sensitive to a moderate drop in pHi (Bonnet *et al.*, 1998; 2000a; Bonnet and Wiemann, 1999a; Xiong *et al.*, 2000). On the other hand, respiratory alkalosis favours febril epileptic seizures (Schuchmann *et al.*, 2006). In the light of these studies, the modest intracellular acidification previously found upon application of valproate, acetazolamide, sulthiame, topiramate or levetiracetam (Bonnet *et al.*, 2002; Leniger *et al.*, 2002; 2004a,b) probably contributes to the anticonvulsive potency of these drugs. Initial studies on pHi regulation of human cortical brain slices suggest that at least some of these results can be transferred to the human brain (Wiemann *et al.*, 2006). Acidification may also help to limit excitotoxicity and energy expenditure in vulnerable neurones (Tombaugh, 1994). In the present investigation, only tiagabine but neither carbamazepine, clonazepam, gabapentin, lamotrigine nor zonisamide changed pHi in CA3 neurones. With regard to zonisamide, the results from the present study confirm previous findings obtained at the single cell level in neuronal tissue (Thöne *et al.*, 2008). A preliminary conclusion from all these experiments is that about half of the anticonvulsants lower steady state pHi and that this effect probably contributes to the suppression of epileptiform activity both *in vitro* and *in vivo*.

pHi and neuroimaging

A few ^{31}P-MRS studies on patients suffering from bipolar or epileptic disorders have provided evidence of dysfunctions in the pHi-regulation of brain cells (Hugg *et al.*, 1992; Garcia *et al.*, 1994; van der Grond *et al.*, 1998; Kato *et al.*, 1998; Hamakawa *et al.*, 2004). These findings support the idea that some antiepileptics and antidepressants may have a therapeutic impact via pHi-changes. A moderate decrease in the pH_i could lower aberrant intracellular signalling (Vignes *et al.*, 1996), for example, and this would have a favourable effect in critical regions of the limbic forebrain and temporal lobes, where hyperexcitability is evident in temporal lobe seizures

(Koehling *et al.*, 1998) and may occur in affective disorders (Kato *et al.*, 1998). Also, panic disorders may be caused by defects in the regulation of brain pH (Cowley and Arana, 1990; Shioiri *et al.*, 1996; Maddock, 2001). It should be stressed that marked acidification might induce vulnerable neurones to disintegrate or undergo apoptosis (Siesjö *et al.*, 1993; Ding *et al.*, 2000). In some parts of the brain, this might facilitate neurogenesis, which is thought to contribute to the therapeutic effects of some NPP and electroconvulsive therapy (Madsen *et al.*, 2000; Malberg *et al.*, 2000; Duman, 2004). Aberrant pHi regulation in human brain is a developing topic comprising aspects of plasticity, memory and learning (Wemmie *et al.*, 2002). The ability of NPP to acutely influence steady state pHi may, therefore, mean that chronic administration of a drug could initiate slow persistent changes in the brain.

Conclusion

Evidence is provided that many NPP are able to acutely and reversibly lower neuronal steady state pHi in hippocampal CA3 neurones. It appears reasonable to suggest that these moderate acidifications will influence neuronal cell functions, such as excitability and intra- as well as intercellular signalling. Therefore, the pHi activity of NPP should be taken into consideration when therapeutic or even toxic effects of these drugs are evaluated.

Conflicts of interest

The authors declare that, except for income received from their primary employer, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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