Effect of depolarizing $\text{GABA}_\text{A}$-mediated membrane responses on the excitability of Cajal-Retzius cells in the immature rat neocortex

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Running title: Effect of depolarizing GABA responses in Cajal-Retzius cells

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Abstract

In immature neurons activation of ionotropic GABA receptors induces depolarizing membrane responses due to a high intracellular Cl⁻ concentration ([Cl⁻]ᵢ). However, it is difficult to draw conclusions about the functional consequences of subthreshold GABAergic depolarizations, since GABAergic membrane shunting and additional effects on voltage-dependent ion channels or action potential threshold must be considered. In order to systematically investigate factors that determine the GABAergic effect on neuronal excitability we performed whole-cell patch-clamp recordings from Cajal-Retzius cells in immature rat neocortex using [Cl⁻]ᵢ between 10 and 50 mM. The effect of focal GABA application was quantified by measuring various parameters of GABAergic responses including the shift in minimal threshold current (rheobase). The rheobase shift was correlated with other parameters of the GABAergic responses by multiple linear regression analyses with a set of simple mathematical models. Our experiments demonstrate that focal GABA application induces heterogeneous rheobase shifts in Cajal-Retzius cells that could not be predicted reliably from [Cl⁻]ᵢ or the GABAergic membrane depolarization. Implementation of a linear mathematical model, which is taking the GABAergic membrane conductance and the difference between action potential threshold and GABA reversal potential into account, resulted in a close correlation between calculated and experimentally obtained rheobase shifts. Addition of a linear term proportional to the GABAergic membrane depolarization improved the accuracy of correlation. The main advantage of using multiple linear regression with simple models is that direction and strength of GABAergic excitability shifts can be analyzed using only measured parameters of GABAergic responses and with minimal a priori information about cellular parameters.
45 **Keywords:** cortical development, shunting inhibition, intracellular chloride
46 concentration, rheobase
**Introduction**

GABA is the main inhibitory neurotransmitter in the adult nervous system and mediates its effect via ionotropic GABA$_A$ or GABA$_B$ receptors and metabotropic GABA$_B$ receptors (Farrant and Kaila 2007; Emson 2007). Due to the Cl$^-$ conductance associated with ionotropic GABA receptors, their membrane responses depend on the intracellular Cl$^-$ concentration ([Cl$^-$]$_i$), with a small contribution of the outwardly directed bicarbonate gradient (Farrant and Kaila 2007). In most mature neurons the low [Cl$^-$]$_i$ is maintained by the activity of the neuron specific K$^+$-Cl$^-$-cotransporter KCC2 (Rivera et al. 1999; Hubner et al. 2001; Lee et al. 2005). During early postnatal development the low expression of KCC2 and the high activity of a Na$^+$-dependent K$^+$-2Cl$^-$-cotransporter (NKCC1) maintains a high [Cl$^-$]$_i$, which renders GABAergic responses depolarizing in most neuronal cell types (Ben-Ari 2002; Yamada et al. 2004; Wang and Kriegstein 2009). However, even in the adult nervous system a variety of neurons or neuronal compartments show depolarizing GABAergic responses (Staley and Mody 1992; Gulledge and Stuart 2003; Gilbert et al. 2007; Khirug et al. 2008; Tyzio et al. 2008). Changes in the GABA reversal potential ($E_{GABA}$) and/or increased expression of NKCC1 has also been described after ischemic/traumatic insults (van den Pol et al. 1996; Yamada et al. 2001; Nabekura et al. 2002), in tissue-samples of epileptic patients (Huberfeld et al. 2007), and after GABAergic stimulation (Ling and Benardo 1995; Woodin et al. 2003; Kolbaev et al. 2011). Thus the membrane responses mediated by GABA$_A$ receptors are dynamically regulated by the developmental and (patho-)physiological state of individual neurons (Fiumelli and Woodin 2007; Farrant and Kaila 2007).

However, the functional implications of such $E_{GABA}$ changes are not directly evident, since depolarizing GABAergic membrane responses are not excitatory *per-se* and
can also contribute to inhibition. In particular, opening of the GABA\textsubscript{A} receptor associated Cl\textsuperscript{-} conductance has a considerable inhibitory capacity by shunting membrane currents (Eccles et al. 1962; Edwards 1990; Staley and Mody 1992). Accordingly, both excitatory and inhibitory effects were reported in the immature brain (Khazipov et al. 1997; Cherubini et al. 1998; Gao et al. 1998; Khalilov et al. 1999; Lu and Trussell 2001; Howard et al. 2007). The effect of GABA on excitability is obviously directly influenced by the relationship between action potential threshold (E\textsubscript{TH}) and E\textsubscript{GABA} and by the GABAergic membrane conductance (G\textsubscript{GABA}) (Owens and Kriegstein 2002; Ben-Ari 2002; Farrant and Kaila 2007). However, the GABAergic membrane depolarization can also indirectly affect the GABAergic excitability shift, e.g. via activation of voltage-dependent Na\textsuperscript{+} or K\textsuperscript{-} currents and by decreasing E\textsubscript{TH} (Monsivais and Rubel 2001; Valeeva et al. 2010; Rojas et al. 2011). In addition, the different timing between GABAergic depolarization and membrane conductance as well as spatial constraints complicate the prediction of GABAergic effects on neuronal excitability (Gao et al. 1998; Gulledge and Stuart 2003; Morita et al. 2005; Jean-Xavier et al. 2007).

Since to our knowledge it has not systematically been investigated how different parameters of GABAergic responses convey to influence neuronal excitability, we determined a variety of these parameters (E\textsubscript{GABA}, E\textsubscript{TH}, G\textsubscript{GABA}, amplitude and slope of GABAergic depolarizations, rheobase in the absence and presence of GABA) and developed a mathematical model to relate these parameters to the GABAergic excitability shift. These experiments were performed in Cajal-Retzius cells (CRc) from immature rat neocortical slices by means of whole-cell recordings using different [Cl\textsuperscript{-}]\textsubscript{i} concentrations and GABAergic stimulation strengths. Our experiments demonstrate that in CRc the effect of GABA on the rheobase mainly depends on the
product of $G_{\text{GABA}}$ and the difference between $E_{\text{GABA}}$ and $E_{\text{TH}}$. Multiple linear
regression analyses using simple mathematical models revealed that the GABAergic
depolarization itself has a small additional influence on the rheobase shift, while the
rheobase shift did not directly depend on the rate of the GABAergic depolarization.
Materials & Methods

Electrophysiological recordings

Animal handling was performed in accordance with the EU directive 86/609/EEC for the use of animals in research and approved by the local ethical committee (Landesuntersuchungsanstalt RLP, Koblenz, Germany). All efforts were made to minimize the number of animals and their suffering. For electrophysiological experiments tangential slices of the neocortex were prepared from pups of wistar rats between postnatal days 1-4 (P1-P4) as described previously (Kilb and Luhmann 2000). Briefly, brains of postnatal rats deeply anesthetized by isoflurane (Forene, Abbot, Wiesbaden, Germany) were isolated and immediately immersed in ice-cold (<4°C) artificial cerebrospinal fluid (ACSF). After removal of the leptomeninges, thin (<600 µm) tangential slices were prepared and incubated for at least 1 h in ACSF at room temperature before experiments. Electrophysiological setup and procedures were described in detail previously (Kilb et al. 2008). The patch-clamp setup was equipped with a fluorescence microscope (BW51WI, Olympus) with infrared differential interference contrast (IR-DIC) optics (Dodt and Zieglgänsberger 1990). All experiments were performed at 30 ± 1 °C. Whole cell patch-clamp experiments were conducted with a discontinuous voltage-clamp/current-clamp amplifier (SEC05L, NPI, Tamm, Germany), which allows very fast and accurate voltage-clamp recordings (Polder and Swandulla 2001). Pipettes were made from borosilicate tubing (2.0 mm outside diameter, 1.16 mm inside diameter; Science Products, Hofheim, Germany) using a vertical puller (PP-83, Narishige, Tokyo, Japan). Whole-cell patch-clamp recordings were performed with a pipette solution containing (in mM) 1 CaCl₂, 2 MgCl₂, 11 EGTA, 10 HEPES, 2 Na₂-ATP, 0.5 Na-GTP and varying amounts of K-gluconate and KCl, depending on the required Cl⁻ concentration (with [gluconate] +
[Cl\textsuperscript{−}] = 130 mM, pH adjusted to 7.4 with KOH and osmolarity to 300 mOsm with sucrose. For this study Cl\textsuperscript{−} concentrations in the patch pipettes ([Cl\textsuperscript{−}]\textsubscript{p}) of 10, 15, 17.5, 20, 25, 30 and 50 mM were used. Some additional cell-attached experiments were performed using 30 mM [Cl\textsuperscript{−}]\textsubscript{p}. In all of these 13 experiments the membrane under the pipette was subsequently opened and the cells showed suprathreshold GABAergic responses using focal GABA pulses applied under cell-attached conditions. For gramicidin-perforated patch-clamp recordings 10-50 µg/ml Gramicidin D (Sigma, St Louis, MO, USA) was added from a stock solution (1-2 mg/ml in DMSO) to a pipette solution containing 100 mM K-gluconate, 50 mM KCl and 10 mM HEPES (adjusted to 7.4 with KOH and osmolarity to 300 mOsm with sucrose) directly before the experiments. All potentials were corrected for liquid-junction potentials, that were calculated using Clampex 8.1 (Axon Instruments, Burlingame, CA) and amounted to 14.8, 14.4, 14.1, 13.9, 13.4, 13.1 and 10.4 mV for 10, 15, 17.5, 20, 25, 30 and 50 mM [Cl\textsuperscript{−}]\textsubscript{p} solutions, respectively. [Cl\textsuperscript{−}]\textsubscript{i} was estimated from $E_{GABA}$ using the Goldman equation under the assumption of a permeability ratio $P_{HCO_3}/P_{Cl}$ of 0.2, an intracellular HCO\textsubscript{3}\textsuperscript{−} concentration of 15 mM and an extracellular HCO\textsubscript{3}\textsuperscript{−} concentration of 26 mM (Farrant and Kaila 2007).

To minimize any interference with spontaneously occurring synaptic inputs, all experiments were performed on Cajal-Retzius cells (CRc), which are known to receive only sparse synaptic input (Kilb and Luhmann 2001; Radnikow et al. 2002; Soda et al. 2003; Kirmse and Kirischuk 2006). Furthermore, CRc reveal a relatively homogeneous [Cl\textsuperscript{−}]\textsubscript{i} (Mienville 1998; Pozas et al. 2008). CRc were easily identified in tangential slices by DIC-videomicroscopy according to their typical morphological properties: an ovoid soma with one thick tapered dendrite and long horizontal axonal projections. Cells were only analyzed if they showed the typical electrophysiological
behavior of CRc including broad repetitive action potentials and a prominent voltage
sag due to the activation of H-currents (Zhou and Hablitz 1996; Mienville 1998; Kilb
and Luhmann 2000). In all recordings, ~0.5% biocytin (Sigma, Taufkirchen,
Germany) was added to the pipette solutions for later morphological identification of
the recorded cells. After recording slices were fixed in 4% paraformaldehyde and the
biocytin-labeled cells were stained with Cy-3 conjugated streptavidin (Dianova,
Hamburg, Germany) as described previously in detail (Achilles et al. 2007).

Resting membrane potential (RMP) was measured directly after establishing the
whole-cell configuration. The measured resting membrane potential was corrected
for voltage shunts via the seal contact according to the methods given by Tyzio et al.
(2003). This correction could be performed in 65 of the 80 cells, in which the seal-
resistance was precisely determined before whole-cell configuration was achieved.
The standard membrane potential was held close to the measured RMP to minimize
constant injection currents that would complicate the analysis of GABAergic effects.
Action potential threshold (E_{TH}) was defined as the intersection between tangents on
the depolarization shortly before the AP onset and the rising phase on the AP (see
Fig. 4A). The membrane resistance (R_{INPUT}) was determined from the slope
conductance of the current-voltage relationship. For the construction of current-
voltage relationships the peak currents/voltages induced by a series of
hyperpolarizing current or voltage steps were used. For activation of GABA_A
receptors, GABA was applied focally for (nominally) 2-5 ms to the soma of a CRc at
>20 µm distance via an application pipette (tip opening: 1-1.5 µm) using a pressure
application system (LHDA0533115H, Lee, Westbrook, CT, USA). The effect of focal
GABA application was quantified by the maximal absolute amplitude of GABAergic
membrane potential responses (E_{PEAK}), the reversal potential of GABAergic currents (E_{GABA}), and the maximal GABAergic conductance (G_{GABA^{MAX}}).

**Solutions and drugs**

ACSF used for slice preparation and electrophysiological recordings consisted of (in mM) 126 NaCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 1 MgCl₂, 2 CaCl₂, 2.5 KCl, 10 glucose, and was equilibrated with 95% O₂/5% CO₂ (pH = 7.4, osmolarity = 300-310 mOsm).

The following stock solutions were used: 100 mM γ-amino butyric acid (GABA) and 3 mM gabazine (GBZ = SR-95531) in distilled water. Most of the chemicals were obtained from Sigma while glucose, NaH₂PO₄, MgCl₂ and CaCl₂ were from Merck (Darmstadt, Germany).

**Analysis of the data using mathematical models**

In order to study the relationship between the experimentally observed parameters (G_{GABA}, E_{GABA}, E_{PEAK}, E_{TH}, dl), we derived a general expression for the difference between membrane currents in the absence and presence of GABA and then linearized it using Taylor expansion (please see appendix 1 for details):

\[ dI_{TH} = AV_{D}^{MAX} + B \left( \frac{dV_{D}}{dt} \right)^{MAX} + D\Delta E_{TH} + G_{GABA}^{*} (E_{TH}^{*} - E_{GABA}) + b; \]  

(1)

(with V_{D}^{MAX} = maximal GABA evoked depolarization (= E_{PEAK} - V_{M}), (dV_{D}/dt)^{MAX} = maximal slope of GABAergic depolarization, E_{TH}^{*} = voltage threshold in the presence of GABA, ΔE_{TH} = (E_{TH}^{*} - E_{TH}), A, B and D are appropriate partial derivatives of I_{ION} function and b is a constant). The partial derivatives of I_{ION} were determined empirically, using a multiple linear regression with further analysis of uniqueness of
appropriate dependencies. These parameters A, B and D define the contribution of certain GABAergic membrane effects to $dI_{TH}$.

In CRc the experimental observation that $E_{TH} = E_{TH}^*$ simplifies the final expression to:

$$dI_{TH} = A V_D^{MAX} + B \left( \frac{dV_D}{dt} \right)^{MAX} + G_{GABA}^*(E_{TH} - E_{GABA}) + b;$$  \hspace{1cm} (2)

In addition to this complete model we considered a few nested, simplified models where one or two of the parameters A and B were nullified, so that:

Model 1: $$dI_p = b + \alpha G_{GABA}^{MAX} (E_{TH} - E_{GABA}); A = 0; B = 0;$$ \hspace{1cm} (3)

Model 2: $$dI_p = b + A V_D^{MAX} + \alpha G_{GABA}^{MAX} (E_{TH} - E_{GABA}); B = 0$$ \hspace{1cm} (4)

Model 3: $$dI_p = b + B \left( \frac{dV_D}{dt} \right)^{MAX} + \alpha G_{GABA}^{MAX} (E_{TH} - E_{GABA}); A = 0;$$ \hspace{1cm} (5)

Model 4 is described by eq 2.

The parameters of these four models were estimated using appropriate linear or multiple linear regression. A comparison of the goodness-of-fit of the data points to one of the models was made by F test. The equation for the F ratio: $$\frac{(SS1-SS2)/(df1-df2))/(SS2/df2)}$$ was derived from the sum of squares (SS) of the residuals from each model and the respective degrees of freedom (df), i.e., the number of data points minus the number of parameters. Since all these models predict absolute value of $dI$ on the basis of individually measured parameters ($E_{TH}$, $E_{GABA}$, $G_{GABA}^{MAX}$) we did not normalize $dI$ for cellular membrane capacitance when we fitted our data with the models. A model was considered to fit the data points significantly better if $p<0.05$. Appropriate statistical tests were calculated using GraphPad Prism (Version 5.00 for Windows; GraphPad Software, San Diego, CA, USA
Results

Properties of the investigated Cajal-Retzius cells

The average RMP of CRc recorded in the present study was -64 ± 0.6 mV (n=80), which after consideration of shunting effects via the seal contact (Tyzio et al. 2003) corresponds to an undisturbed RMP of -77 ± 1.5 mV (n=65). Injection of positive currents induced repetitive action potentials with an amplitude of 66 ± 1.5 mV (n=80) and a half width of 13±0.6 ms. The AP threshold (E_{TH}) showed a remarkable cell-to-cell variability from -58 to -31 mV with a mean value of -47 ± 0.7 mV (n=80). The current-voltage plot of peak currents revealed a linear current-voltage relation between -100 and -60 mV and an average R_{INPUT} around RMP of 1.3 ± 0.03 GΩ (n=80), corresponding to a membrane conductance (G_M) of 0.8 ± 0.03 nS.

Effects of focal GABA applications on Cajal-Retzius cells

Focal application of GABA to the soma of CRc induced membrane currents that were completely blocked by bath application of 3 μM gabazine (n=6, Fig. 1A), indicating that the responses were exclusively mediated by GABA_A receptors. Under gramicidin-perforated patch-clamp conditions E_{GABA} was -33 ± 1.2 mV (n=19), which corresponds to a [Cl⁻] of ~34 mM. According to this high [Cl⁻], focal GABA application evoked excitatory responses (dI <0, mean -3 ± 0.6 pA) in 6 of the 7 investigated cells under gramicidin-perforated patch-clamp conditions. Similar results were obtained from cell-attached recordings, where focal GABA applications provoked action potentials in 9 out of 13 CRc.
For a detailed analysis of the GABAergic effects of the excitability of CRc we performed whole-cell experiments using $[\text{Cl}^-]_p$ between 10 and 50 mM. The effect of focal GABA application was quantified by the maximal amplitude of GABAergic membrane potential responses ($E_{\text{PEAK}}$), the reversal potential of GABAergic currents ($E_{\text{GABA}}$), and the maximal GABAergic conductance ($G_{\text{GABA}}^{\text{MAX}}$). $E_{\text{PEAK}}$ was estimated from current-clamp recordings (Fig. 1B, upper traces), with $V_M$ held close to RMP between -60 and -67 mV. $E_{\text{GABA}}$ and $G_{\text{GABA}}^{\text{MAX}}$ were determined from intersection and slope of a linear fit in the current-voltage relationship of GABAergic current responses under voltage-clamp conditions (Fig. 1B, C). By changing the position of application pipette and the duration of GABA pulses we were able to vary $G_{\text{GABA}}^{\text{MAX}}$ (between 0.1 and 7.3 nS) and $E_{\text{PEAK}}$ (from -69 to -28 mV). To allow a comparison of different cells, $G_{\text{GABA}}^{\text{MAX}}$ was related to $G_M$ to obtain the relative membrane resistance ($R_{\text{REL}} = G_M/[G_M + G_{\text{GABA}}^{\text{MAX}}]$). As a result of this approach we obtained 124 experimental points from 80 cells. In 104 out of 124 cases the $(dV/dt)^{\text{MAX}}$ value was reliably measured and was in the range between 20 to 870 with a mean value 220 ± 14 mV/s (n=104).

Influence of different parameters of GABAergic responses on the excitability shift

To investigate how these GABAergic parameters influence the excitability of CRc, we determined the rheobase (i.e. the minimal injection current required to evoke an AP) in the absence ($I_{\text{TH}}$) and presence of GABA ($I_{\text{TH}}^*$) and calculated the GABAergic rheobase shift ($dI=I_{\text{TH}}^*-I_{\text{TH}}$) for each set of $[\text{Cl}^-]_p$ and $G_{\text{GABA}}^{\text{MAX}}$ (Fig. 2A). In each of these experiments $E_{\text{PEAK}}$ was aligned with the time point of the first AP by adjustment of the latency between current injection and GABA pulse (Fig. 2B). The rheobase
under control conditions varied from 0.06 to 1.6 pA/pF with a mean value of 0.48 ± 0.04 pA/pF (n=80).

At low [Cl\textsubscript{P}] (10 mM) simultaneous GABA application increased the rheobase in most experiments, indicating inhibitory responses, while at high [Cl\textsubscript{P}] (30 and 50 mM) in most experiments excitatory responses were observed (Fig. 2C, D). In contrast, at [Cl\textsubscript{P}] between 15 and 25 mM both, excitatory and inhibitory responses could be observed (Fig. 2D). Although these results show the expected trend that higher [Cl\textsubscript{P}] promote excitation and lower [Cl\textsubscript{P}] inhibition, the effect of GABA application varied considerably at identical [Cl\textsubscript{P}], indicating that [Cl\textsubscript{P}] is not sufficient to predict the GABAergic effect on the excitability.

Next we assessed, to which extend the GABAergic effect on excitability depends on the GABAergic membrane depolarization. Analyses of the relationship between \(V_D^{\text{MAX}}\) and rheobase shift (\(dl/C_M\)) revealed a significant \(r^2=0.42, p<0.001, n=105\) correlation between \(dl/C_M\) and \(V_D\) (Fig. 3A), however with a large scatter. Therefore we correlated \(dl\) with the difference between \(E_{GABA}\) and \(E_{TH}\) (Fig. 3B). This analysis also revealed a significant \(r^2=0.42, p<0.001\) correlation between \(dl/C_M\) and \((E_{TH}-E_{GABA})\). The linear regression line crossed the axes near to their origin (intercept at 1.5 mV and -0.02 pA/pF). Based on this correlation we were able to predict the sign of the GABAergic rheobase shift in 99 out of 124 cases. In 18 of the remaining cases GABA had no effect on the rheobase, while only in 7 cases the direction of the experimentally observed rheobase shift was opposite to the predicted one (black squares in Fig. 3B). This observation indicates that the difference between \(E_{TH}\) and \(E_{GABA}\) is a major determinant of the GABAergic rheobase shift.
Effect of GABAergic depolarization on AP threshold

Because our results indicate that the effect of GABA depends significantly on the difference between $E_{TH}$ and $E_{GABA}$, but previous studies suggested that GABAergic depolarizations can shift $E_{TH}$ due to shunting effects of GABAergic conductances, inactivation of voltage-gated Na$^+$ channels and/or activation of K$^+$ channels (Edwards 1990; Staley et al. 1995; Rojas et al. 2011), we next had to analyze to which extent $E_{TH}$ is affected by activation of GABA$\_A$ receptors. Comparison between the first AP evoked by current injection in the absence and presence of GABA application revealed that $E_{TH}$ was not significantly affected by the GABA pulse ($p=0.6$, $n=80$ experiments, Wilcoxon matched pairs test; Fig. 4A, B). Even for large GABAergic conductances ($R_{REL}<0.5$) no significant ($p=0.58$) effect of the synchronous GABA application on $E_{TH}$ was observed (Fig. 4C). In addition, we investigated the effect of GABAergic depolarization on $I_{Na}$ and $E_{TH}$ by means of depolarizing voltage ramps (Fig. 4D). A voltage ramp with a rate of 200 mV/s induced a significantly ($p=0.005$) smaller $I_{Na}$ ($7.9 \pm 2.2$ pA/pF, $n=10$) than fast voltage ramps ($10.4 \pm 2.5$ pA/pF at 800 mV/s), but $E_{TH}$ was not significantly ($p=0.57$) different between both conditions (-39.3 ± 2.3 mV vs. -40.6 ± 2.0 mV). At slow voltage ramps with a rate of 40 mV/s $I_{Na}$ was even smaller ($5.8 \pm 1.8$ pA/pF), but $E_{TH}$ was still not significantly ($p=0.33$) affected (-39.7 ± 1.3 mV, $n=10$). In summary, these results demonstrate that in CRc $E_{TH}$ was stable during the GABA application.

Correlation of the GABAergic excitation shift with determined membrane parameters

In order to analyze the functional relationship between the experimentally determined parameters of GABAergic responses ($E_{TH}$, $E_{GABA}$, $G_{GABA}^{MAX}$, $V_{D}^{MAX}$ and $(dV_{D}/dt)^{MAX}$)
and the rheobase shift $dI$, we considered four mathematical models (for details see Materials and Methods). First we investigated the relationship between $dI$ and $G_{GABA}^{MAX}(E_{TH}-E_{GABA})$, a parameter directly derived from model 1 (Fig. 5). This plot reveals a substantially better fit ($r^2 = 0.79$) of $dI$ than the previous correlations, suggesting that $dI$ is closely correlated with $G_{GABA}^{MAX}(E_{TH}-E_{GABA})$.

Accordingly, linear regression between $dI$ and the parameters $G_{GABA}^{MAX}(E_{TH}-E_{GABA})$, $V_D^{MAX}$, and $(dV_D/dt)^{MAX}$ demonstrated that 80% of $dI$ variation (quantified by $r^2$) can be explained by $G_{GABA}^{MAX}(E_{TH}-E_{GABA})$, while $V_D^{MAX}$ account for 51% and $(dV_D/dt)^{MAX}$ for only 6% of the overall variability in $dI$. Based on these results we next analyzed how $V_D^{MAX}$ and $(dV_D/dt)^{MAX}$ influence $dI$ using multiple linear regression with the more complex models 2, 3 and 4 (Fig. 6 A, B). The parameters delivered by this analysis (Table 1) showed that the fit of $dI$ becomes more accurate with the models 2 and 4 (which take $V_D^{MAX}$ into account), while model 3 (which considers only $[dV_D/dt]^{MAX}$) did not considerably increase the accuracy of the fit (see Fig. 6B). Furthermore, the goodness of fit obtained with these models (considered as nested models in the configuration 1-2-4 and 1-3-4) was analyzed with an F-test. This analysis showed that, as expected, the more complex models 2, 3 and 4 fit the data significantly better than model 1 (in all three cases $p<0.001$). While the most complex model 4 obtained significantly better results than model 3 ($p<0.001$), it had no advantage over model 2 ($p=0.26$), again suggesting that for CRc only the parameter $V_D^{MAX}$ is a relevant additional factor that influences $dI$.

In particular in the domain where $G_{GABA}^{MAX}(E_{TH}-E_{GABA})$ is small ($-4pA<G_{GABA}^{MAX}(E_{TH}-E_{GABA})<4pA \ [with \ -2mV<(E_{TH}-E_{GABA})<2 \ mV]$) model 2 obtains a substantially better fit of $dI$ (Fig. 6C) than the simple model 1 (which does not consider $V_D^{MAX}$). In this subset of experimental data points a significant correlation between $dI$ and $V_D^{MAX} (r^2$
= 0.28, p=0.006) is obtained, whereas the correlation between $dI$ and $(dV_D/dt)^{\text{MAX}}$ is not significant ($r^2 = 0.14, p=0.06$).

In summary, these results show that in CRc $dI$ can be sufficiently described by a linear mathematical model under consideration of $G_{\text{GABA}}^{\text{MAX}}(E_{\text{TH}}-E_{\text{GABA}})$ and $V_D^{\text{MAX}}$. Introduction of an additional term depending on $(dV_D/dt)^{\text{MAX}}$ does not improve the quality of the fit.
In the present study we systematically investigated the factors that determine the effect of a brief GABA application on the excitability of CRc in the early postnatal rat neocortex. The effect of GABA was determined by a set of parameters, including the GABAergic rheobase shift ($d_I$), that allow a detailed quantification of GABAergic response. This study provides direct experimental evidence that in CRc the effect of GABA on $d_I$ is proportional to the difference between $E_{GABA}$ and $E_{TH}$ and to $G_{GABA}$. Using a set of mathematical models for multiple linear regression analysis, which are based directly on experimentally determined parameters of GABAergic responses, we were able to disclose that the GABAergic depolarization itself has a small additional influence on $d_I$, while the rate of the GABAergic depolarization has no effect. In addition, we revealed that an activation of GABA$_A$ receptors does not affect the action potential threshold of CRc.

CRc provides a convenient experimental basis for the measurement of GABAergic rheobase shifts, since CRc constitute a homogeneous neuronal population that can be easily selected by morphological and electrophysiological parameters, generate reliable APs at early phases of postnatal development and display a rather low level of spontaneous synaptic activity (Luhmann et al. 2003; Soriano and Del Rio 2005). In addition, the observation that the specific GABA$_A$ receptor antagonist gabazine completely abolished the effect of focally applied GABA clearly indicates that our experimental approach involved only the activation of GABA$_A$ receptors, in accordance with previous reports (Mienville 1998; Kilb and Luhmann 2001; Radnikow et al. 2002; Chan and Yeh 2003; Dvorzhak et al. 2008, but see Lopez-Bendito et al. 2002).
Our experiments demonstrate that in a rather wide range of \([\text{Cl}^-]\) and GABAergic depolarizations \((V_D^{\text{MAX}})\) both positive and negative \(dI\) were obtained, suggesting that the GABAergic effect on excitability in individual experiments can not be reliably predicted from these parameters. This heterogeneity is mainly caused by the rather wide range of AP thresholds \((E_{\text{TH}})\) observed in CRc. Therefore a direct relation of \(dI\) to the GABAergic driving force at \(E_{\text{TH}}\) \((E_{\text{TH}}-E_{\text{GABA}});\) Owens and Kriegstein 2002; Ben-Ari 2002; Farrant and Kaila 2007) resulted, as expected, in a considerably better correlation. In particular, the sign of \(dI\) coincides with the sign of \((E_{\text{TH}}-E_{\text{GABA}})\) in 77% of the experiments, while explicit contradictions were obtained in only 6.4% of the experiments. On the other hand, the amplitude distribution of \(dI\) could not be addressed in this analysis, as \(G_{\text{GABA}}\) was not considered.

A simple linear mathematical expression (model 1), which considers only the term \(G_{\text{GABA}}(E_{\text{TH}}-E_{\text{GABA}})\), can generally be used to analyze direction and amplitudes of \(dI\).

For a better description of the dependency between different parameters of GABAergic responses and \(dI\) we used slightly more complex mathematical models for multiple regression analysis. In these models (model 2-4) additional terms proportional to \(V_D^{\text{MAX}}\) and/or \((dV_D/dt)^{\text{MAX}}\) were included. These additional terms represent effects on neuronal activity that are indirectly caused by the activation of GABA\(_A\) receptors, but did not directly depend on current fluxes via this receptor. Our experiments revealed that the correlation between \(dI\) and the experimental GABAergic parameters was considerably improved when a term proportional to \(V_D^{\text{MAX}}\) was included to the mathematical expression (model 2 and 4). In particular in the domain where \(E_{\text{GABA}}\) is close to \(E_{\text{TH}}\) (and thus \(G_{\text{GABA}}[E_{\text{TH}}-E_{\text{GABA}}]\) is small) it provides an obvious better explanation for the obtained relationship between GABAergic membrane responses and \(dI\). Of course \(V_D^{\text{MAX}}\) depends on the term...
\[ G_{GABA}(E_{TH}-E_{GABA}), \] as these factors determine the GABAergic depolarization.

Therefore we estimated the direct relationship between \( dI \) and \( V_D^{MAX} \), using partial correlation coefficient between both values at fixed \( G_{GABA}^{MAX}(E_{TH}-E_{GABA}) \), and revealed that a significant correlation remains under this restriction \((r=-0.54, p<0.001)\). Thus \( dI \) is most probably also directly influenced by \( V_D^{MAX} \), independent of the correlation between \( V_D^{MAX} \) and \( G_{GABA}^{MAX}(E_{TH}-E_{GABA}) \).

The introduction of a term proportional to \( (dV_d/dt)^{MAX} \) in models 3 or 4 does not considerably improve the goodness of fit, suggesting that the rate of GABAergic depolarization does not influence \( dI \). This suggestion is in accordance with the observation that \( (dV_d/dt)^{MAX} \) accounts for only 6\% of the variability in \( dI \) and it is probably directly related to the observation that in CRc \( E_{TH} \) was independent from the slope of depolarizing voltage ramps \( (dV/dt) \) in a range between 40 and 800 mV/s.

The observed independency between \( E_{TH} \) and \( dV/dt \) in CRc is in striking contrast to other neurons, where \( dV/dt \) considerably influences \( E_{TH} \) (Bradley and Somjen 1961).

On the other hand, the amplitude of \( I_{Na} \) showed, as expected, a clear dependency on the slope of slowly depolarizing voltage ramps. We propose that the high input resistance of CRc will make even reduced \( I_{Na} \) sufficient to trigger the entry in the Hodgkin cycle, thus resulting in a constant \( E_{TH} \).

Under conditions that resemble the \([Cl^-]\) of CRc (between 28 mM and 50 mM, Mienville 1998; Kilb et al. 2002; Achilles et al. 2007) we observed a stable negative shift in \( dI \). Accordingly GABA promotes a reliable excitatory action in CRc under gramicidin-perforated patch-clamp and cell-attached conditions (see also Mienville 1998; Achilles et al. 2007). The high \([Cl^-]\) of immature neurons is maintained by the activity of a \( Na^+ \)-dependent \( K^+\)-\( Cl^- \) cotransporter (NKCC-1, Farrant and Kaila 2007), therefore inhibition of this transporter with bumetanide substantially reduces \([Cl^-]\).
(Yamada et al. 2004; Dzhala et al. 2005). In CRc bumetanide reduces $E_{GABA}$ to a passive distribution (corresponding to a $[\text{Cl}^-]$ of $\sim 16$ mM, (Achilles et al. 2007), which according to our and previous results (Achilles et al. 2007), attenuated the excitatory effect of GABA.

The coefficient of proportionality $A$ has a dimension of conductance. In CRc it has a negative value, which indicates that the GABAergic depolarization additionally facilitates AP generation in CRc. Although a direct correlation of this parameter to distinct electrophysiological processes is not possible, this parameter most probably reflects an interaction between GABAergic membrane responses and voltage-dependent conductances. In the present study we did not perform additional experiments to disclose the nature of this factor since its contribution to $dl$ indicates that the underlying voltage-dependent currents are most probably too small for a direct investigation.

The analysis method developed in this study can also be adapted to other cell types. In contrast to more elaborated neuronal models, which require a set of a priori information or assumptions, the approach using multiple regression analyses of linear mathematical models exploits only experimentally derived parameters. Multiple linear analyses using the global expression (see appendix, formula 1) allows to quantify the additional influence of particular GABAergic membrane effects, like the GABAergic depolarization or a GABAergic shift in $E_{\text{TH}}$, on neuronal excitability. Even analyses with a simple model like model 2 can be used to unravel whether the experimentally observed GABAergic rheobase shift differs from the theoretically expected direct dependency between $G_{\text{GABA}}(E_{\text{TH}}-E_{\text{GABA}})$ and $dl$. For example, it was recently shown by Valeeva et al. (Valeeva et al. 2010) that in CA3 hippocampal cells even subthreshold GABAergic depolarizations can induce AP, which was mediated
by the activation of persistent Na\(^+\) channels. This complex relation between \(dI\) and GABAergic parameters could not be estimated from the relation between \(E_{\text{TH}}\) and \(E_{\text{GABA}}\). In this case our approach would predict a major additional contribution of \(V_{\text{D}}^{\text{MAX}}\) to the excitability (i.e. a considerably large negative factor A). In contrast, in neurons from the auditory brainstem GABA mediates an inhibition, despite the fact that \(E_{\text{GABA}}\) is positive to \(E_{\text{TH}}\) (Monsivais and Rubel 2001). This observation would lead to a positive factor A if the simple model 2 would be used, while an analysis with the global expression (1) could uncover to which extent a GABAergic shift in \(E_{\text{TH}}\) or additional voltage-dependent conductances contribute to this effect. However, as already stated, our approach is not suited to uncover the electrophysiological properties of these factors.

While our experimental approach focussed on simultaneous GABAergic effects, a series of elegant experiments already demonstrated that the GABAergic effect on excitability depends on the delay between excitatory and GABAergic inputs as well as on the spatial relation between both inputs (Gao et al. 1998; Gulledge and Stuart 2003; Morita et al. 2005; Jean-Xavier et al. 2007). Therefore our experimental conditions substantially simplified the complexity of GABAergic effects on the excitability. On the other hand, tonic GABAergic activation plays a major role for the regulation of neuronal excitability (Farrant and Nusser 2005), in particular in the immature nervous system (Owens et al. 1999; Demarque et al. 2002; Sipila et al. 2007). Under this condition, a simultaneous GABAergic influence on all excitatory inputs must be considered, which makes our proposed approach relevant.

In summary, the present study shows that a multiple linear regression with simple mathematical models can be used to estimate whether membrane responses caused either primarily by the activation of GABA\(_A\) receptors or secondarily by an influence of
the GABAergic membrane depolarization on ion currents or $E_{TH}$ contribute to the
GABAergic excitability shift. Using this method, we demonstrate in CRc that the
GABAergic effect on the neuronal excitability is mainly determined by the properties
of GABAergic currents ($G_{GABA}[E_{TH}-E_{GABA}]$) and that the GABAergic depolarization has
a minor additional influence.
Appendix 1:

The derivation of the expression is based on a basic equation, which describes currents injected into the cell under assumption of an isopotential cellular membrane:

\[ I_P = C_M \frac{dV_M}{dt} + I_{ION}(V_M, \frac{dV_M}{dt}, E_{TH}, ...) \]; \hspace{1cm} (1)

(With \( I_P \) = current injected through the pipette, \( C_M \) = membrane capacitance, \( V_M \) = membrane potential, \( I_{ION} \) = current through channels expressed on the surface of the plasma membrane). The term \( I_{ION} \) is a non-linear function, which is a sum of all currents passing through different channels except the GABA \(_A\) receptor associated anion channel. Using this equation, the rheobase \( I_{TH} \) is described by the expression:

\[ I_{TH} = C_M \frac{dV_M}{dt} \bigg|_{V_M = V_{TH}} + I_{ION}(V_M, \frac{dV_M}{dt}, E_{TH}, ...) \bigg|_{V_M = V_{TH}} \]; \hspace{1cm} (2)

Under conditions when activated GABA \(_A\) receptors contribute to membrane currents, a separate term for the GABAergic ion conductance is added to the equation:

\[ I_{TH}^* = C_M \frac{dV_M}{dt} \bigg|_{V_M = V_{TH}} + I_{ION}^*(V_D^{MAX}, \left(\frac{dV_D}{dt}\right)^{MAX}, E_{TH}^*, E_{GABA}^*) \bigg|_{V_M = V_{TH}} + G_{GABA} \big|_{V_M = V_{TH}} (E_{TH}^* - E_{GABA}) \]; \hspace{1cm} (3)

The function \( I_{ION}^*() \) represents a disturbed variant of the membrane conductances \( I_{ION} \) in the presence of GABA. The disturbance is assumed to be a function of experimentally measured parameters of GABA response with \( V_D^{MAX} \) = maximal depolarization from RMP and \( (dV_D/dt)^{MAX} \) = maximal slope of depolarization. The voltage dependence of the GABA conductance is not considered here since in CRc no substantial deviation of experimental I-V from linearity in the relevant voltage domain was obtained (data not shown).
Next we consider quasi threshold values for \( I_{TH} \) or \( I'_{TH} \) (maximal depolarization infinitesimal close to the action potential threshold \( (E_{TH} \) or \( E'_{TH} \)) without generation of action potential). Under rheobase conditions \( (E_{TH} \) at infinitely long depolarization) \( dV/dt \) equals to zero. In addition, due to the alignment of current injection and GABA pulse \( t'_{TH} \) is the same as \( t_{TH} \). Thus the expression for \( I'_{TH} - I_{TH} \) can be written:

\[
I'_{TH} - I_{TH} = I'_{ION}(V_M, \frac{dV_M}{dt}, E'_{TH}, \ldots) - I_{ION}(V_M, \frac{dV_M}{dt}, E_{TH}, \ldots) + G_{GABA} \mid_{t_{TH}} (E'_{TH} - E_{GABA});
\]

Functions \( I_{ION} \) and \( I'_{ION} \) describe the same set of ion channels. However in the second case ion currents are influenced by the activation of GABA\(_A\) receptors and associated depolarization. Thus \( I'_{ION} \) can be considered as a function of GABAergic disturbance of \( V_M, dV_M/dt \) and \( E_{TH} \) resulting in:

\[
I_{TH} = I_{ION}(0,0,0,\ldots);
\]

\[
I'_{TH} = I'_{ION}(\Delta V_M, \Delta \frac{dV_M}{dt}, \Delta E_{TH}, \ldots) + G_{GABA} \mid_{t_{TH}} (E'_{TH} - E_{GABA});
\]

with \( \Delta V_M, \Delta (V_M/dt) \) and \( \Delta E_{TH} = \) GABAergic influence on maximal GABA evoked depolarization \( (V_D^{\text{MAX}}) \), maximal slope of GABAergic depolarization \( ((dV_D/dt)^\text{MAX}) \) and difference in \( E_{TH} \), respectively.

Using the Taylor (Maclaurin) expansion for linear terms:

\[
f(x, y) - f(0,0) = \frac{\partial f}{\partial x} x + \frac{\partial f}{\partial y} y + o();
\]

we obtain:

\[
\Delta I_{TH} = \frac{\partial I_{ION}}{\partial (V_D^{\text{MAX}})} V_D^{\text{MAX}} + \frac{\partial I_{ION}}{\partial (\frac{dV_D}{dt})} \left( \frac{dV_D}{dt} \right)^{\text{MAX}} + \frac{\partial I_{ION}}{\partial (E_{TH})} (E_{TH}) + G_{GABA} (E_{TH} - E_{GABA}) + o(V_D^{\text{MAX}}, \frac{dV_D}{dt}, \ldots);
\]

After substitution of
\[ A = \frac{\partial I_{\text{ION}}}{\partial (V_D^{\text{MAX}})}; \quad B = \frac{\partial I_{\text{RON}}}{\partial (\frac{dV_D}{dt})}; \quad D = \frac{\partial I_{\text{ION}}}{\partial (\Delta E_{\text{TH}})}; \quad b = o(V_D^{\text{MAX}}, \frac{dV_D}{dt},...); \]

we receive:

\[ \Delta I_{\text{TH}} = A V_D^{\text{MAX}} + B \left( \frac{dV_D}{dt} \right)^{\text{MAX}} + D \Delta E_{\text{TH}} + G_{\text{GABA}}^* (E_{\text{TH}}^* - E_{\text{GABA}}) + b; \]

In CRc the experimental observation that \( E_{\text{TH}} = E_{\text{TH}}^* \) simplifies the final expression to

\[ \Delta I_{\text{TH}} = A V_D^{\text{MAX}} + B \left( \frac{dV_D}{dt} \right)^{\text{MAX}} + G_{\text{GABA}}^* (E_{\text{TH}} - E_{\text{GABA}}) + b; \]
Acknowledgements

This work was supported by a grant of the Deutsche Forschungsgemeinschaft to WK (DFG Ki 835/2). The authors thank B. Krumm for excellent technical assistance and Dr. Robin White for critically reading the manuscript. All authors have no conflict of interest to disclose.
References:


Figure legends:

Fig. 1: Properties of GABAergic membrane responses. (A) Representative current traces obtained in voltage-clamp mode at holding potentials of -50 mV (upper trace) and -70 mV (lower traces) upon focal application of 100 µM GABA (arrowhead) with 10 mM [Cl\(^{-}\)]_P. Note the reversal of the current between -50 and -70 mV and the block of GABAergic currents by 3 µM gabazine. (B) Effect of [Cl\(^{-}\)]_P on GABAergic responses under current-clamp (upper traces) and voltage-clamp conditions (lower traces). At 30 mM [Cl\(^{-}\)]_P voltage responses are either supra- or subthreshold (see upper traces). (C) Current-voltage relationship of the voltage-clamp recordings shown in B. The linear fit of the datapoints was used to determine \( E_{GABA} \) and \( G_{GABA}^{MAX} \). Note that GABAergic currents reversed close to the calculated reversal potential (arrows).

Fig. 2. Determination of GABAergic effects on excitability. (A) Schematic current and recorded voltage traces demonstrating the definition of \( dI \). The left traces represent the minimal injection current required to trigger an AP (rheobase). The right traces show membrane responses to focal GABA application (arrowhead) without (gray trace) and with minimal suprathreshold current (black trace). An increase in the rheobase in the presence of GABA (\( dI >0 \)) represents an inhibitory action, while a decrease (\( dI <0 \)) represents an excitatory action. (B) Procedure of alignment used in the experiments. The delay between current injection and GABA application (arrowhead) was calculated from the differences between the latency of AP after suprathreshold current injection (\( t_{AP} \), upper trace) and the time to peak depolarization upon GABA applications (\( t_{DEP} \), middle traces). (C) Typical recordings illustrating the protocol used for the determination of \( dI \) at two different [Cl\(^{-}\)]_P. Note that synchronous GABA application increases the rheobase at 10 mM [Cl\(^{-}\)]_P, while it was decreased at
30 mM \([\text{Cl}^-]_p\). (D) Pie chart diagrams summarizing the results of all experiments with different \([\text{Cl}^-]_p\) (10-50 mM). Note that at \([\text{Cl}^-]_p\) between 15 and 25 mM both, inhibitory and excitatory actions are observed. Numbers of experiments and cells are given below the diagrams.

Fig. 3 Dependency between rheobase shift and different parameters of GABAergic responses. (A) Dependency between rheobase shift \((dI/C_M)\) and GABAergic depolarization \((E_{\text{PEAK}}-V_M)\). The solid line represents a linear regression between both parameters. Note that the regression line crosses the abscissa at \(~10\ \text{mV}\). (B) Dependency between \(dI/C_M\) and \((E_{\text{GABA}}-E_{\text{TH}})\). The solid line represents a linear regression between both parameters. Note that in this graph only 7 datapoints (marked in black) are placed outside the expected quadrant.

Fig. 4: Effect of GABA application on AP threshold. (A) Original registration illustrating \(E_{\text{TH}}\) of an AP induced by current injection in the absence (left traces) and the presence (right traces) of a focal GABA pulse (arrowhead). \(E_{\text{TH}}\) is determined from the intersection of linear fits of the voltage changes before and after AP induction (dotted lines). (B) Pair wise comparison of AP threshold in the absence \((E_{\text{TH}}^{\text{CTRL}})\) and presence \((E_{\text{TH}}^{\text{GABA}})\) of a focal GABA pulse reveals that GABA has no systematic effect of \(E_{\text{TH}}\). The line represents a \(y=x\) function. (C) Similar plot as in B but only for strong \((R_{\text{REL}}<0.5)\) GABA pulses. Note the absence of a systematic GABAergic effect even in these experiments. (D) Representative voltage-clamp recordings illustrating the effect of voltage ramps with different slopes (800, 200 and 40 mV/s) on \(I_{\text{Na}}\). Note the reduction in \(I_{\text{Na}}\) of the first AP (arrow) at slower voltage-ramps.
Fig. 5: Fit of the GABAergic rheobase shift by a simple linear model. The graph illustrates the relationship between $dI$ and the parameter $G_{\text{GABA}}(E_{\text{TH}}-E_{\text{GABA}})$. The line represents the optimal fit using model 1 (see text for details).

Fig. 6: Fit of the GABAergic rheobase shift by different models. (A1) 3D graph illustrating the dependency between $dI$, $G_{\text{GABA}}(E_{\text{TH}}-E_{\text{Cl}})$ and $V_{D_{\text{MAX}}}$. (A2-4) For visualization of the fit by model 2 three groups of the dataset, as separated by the vertical planes in graph A1, were displayed separately. Solid lines represent intersections of the fit calculated with model 2 with the vertical planes, while the dotted line represents the fit of the data with model 1. Note the obvious divergence between both fits. (B1) 3D graph illustrating the dependency between $dI$, $G_{\text{GABA}}(E_{\text{TH}}-E_{\text{Cl}})$ and $dV_{D}/dt$. (B2-4) Fit of three groups of the dataset using model 3 (solid lines) and model 1 (dotted lines). Note the small divergence between the fits by both models. (C) Dependency of $dI$ on the GABAergic depolarization ($V_{D_{\text{MAX}}}$) in a subset of datapoints with $-4\text{pA}<G_{\text{GABA}}^{\text{MAX}}(E_{\text{TH}}-E_{\text{GABA}})<4\text{pA}$ and $-2\text{mV}<(E_{\text{TH}}-E_{\text{GABA}})<2\text{ mV}$. The solid line represents the prediction with model 2, while the dotted line represents the averaged results obtained by model 1 in this domain. Note the obvious dependency between $dI$ and $V_{D_{\text{MAX}}}$ that is resolved by model 2.
Table 1: Data table presenting the results of the fits of experimental data with the 4 different models. The rows $\alpha$, A, B, b represent parameters used in the models, for each parameter the value which gives the best result for least-square fitting (value) and the 95% confidence interval (95% CI) are given. The row $r^2$ lists the squared Pearson’s coefficient of correlation for the fit with each model. For details see Materials & Methods and Results.
Fig 1

A. 

-50 mV  

100 µM GABA

-70 mV

10 pA

3 µM gabazine

0.5 s

100 µM GABA

100 µM GABA

100 µM GABA

B. 

10 mM [Cl]_P

30 mM [Cl]_P

C. 

V_M, (mV)

I_{GABA}, (pA)

10 mM [Cl]_P

30 mM [Cl]_P
Fig 2
Fig 3
Fig 4
Fig 5
Fig 6
Table 1: Summary of parameters for fit of data in model 1 to 4

<table>
<thead>
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<th>Parameters</th>
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</table>

Table 1: Data table presenting the results of the fits of experimental data with the 4 different models. The rows α, A, B, b represent parameters used in the models, for each parameter the value which gives the best result for least-square fitting (value) and the 95% confidence interval (95% CI) are given. The row r² lists the squared Pearson's coefficient of correlation for the fit with each model. For details see Results and Materials & Methods.