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The novel GABA adamantane derivative (AdGABA): design, synthesis, and activity relationship with gabapentin

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Abstract—A facile preparation of 2-aminomethyl-2-tricyclo[3.3.1.1^{1,7}]decaneacetic acid hydrochloride **5** (AdGABA) is described. The synthesis of AdGABA involves the hydrogenation of 2-cyano-2-tricyclo[3.3.1.1^{1,7}]decaneacetic acid **11**, which was synthesized by two different synthetic routes. AdGABA was found to antagonize the pentylenetetrazole (PTZ) and semicarbazide (SCZ) induced tonic convulsions and exhibits analgesic activity in the hot plate test on mice. Although its mechanism of action is quite similar to that proposed previously for gabapentin (interaction with the $\alpha_2\delta$ subunit of the voltage gated Ca^{2+} channels), further studies were undertaken in order to clarify the precise mechanism of the anticonvulsant and analgesic effects of AdGABA on a molecular level.

1. Introduction

Voltage-gated Ca^{2+} (Ca_V) channels mediate Ca^{2+} entry into cells initiating thereby a variety of physiological processes including gene transcription, muscle contraction, and neurotransmitter release. Ca^{2+} currents through these channels are functionally diverse and have been classified into six different types (named T, L, N, P, Q, and R). Ca_V channels are protein complexes consisting of a channel-forming voltage-sensing (α_1) subunit and three auxiliary subunits ($\alpha_2\delta$, β, and γ).[1] Interestingly, the $\alpha_2\delta$ auxiliary subunit has shown to support pharmacological interactions with therapeutic agents for the treatment of neurological disorders.[2] In particular, 1-(aminomethyl)cyclohexaneacetic acid gabapentin (Neurontin®) (GBP) (**1**) was originally designed as a lipophilic γ-aminobutyric acid (GABA) analogue,[3] but has subsequently been shown to interact neither with any of the enzymes on the GABA metabolic pathway nor directly with the $GABA_A$ or $GABA_B$ receptors.[4] However, it is able to efficiently cross the blood–brain barrier (BBB) using an *L*-system amino acid transporter[5] Gabapentin has been introduced as an anticonvulsant agent which is useful as add-on therapy in the treatment of epileptic seizures,[6] while it has also been shown to be a potential agent for the treatment of neurogenic pain.[7] GBP shows few, if any, toxic side effects at clinically relevant doses.[8]

Recent studies have shown that GBP binds with high affinity to a novel binding site on the $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits of Ca_V channels.[9] Therefore, it has been postulated that GBP reduces Ca^{2+} current by modulating the α_1 subunit expression and/or function through its association with $\alpha_2\delta$.[9c,10]

In the current report, we describe the synthesis and pharmacological profile of a novel GABA adamantane derivative (AdGABA), which displays a close structure–activity relationship with GBP. Since GBP has been shown to reduce the functional expression of

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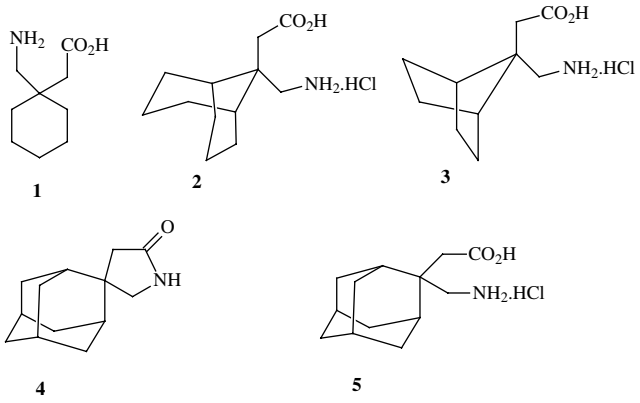
neuronal N-type ($Ca_V2.2$) channels,[11] we have examined the consequences of the AdGABA treatment on the membrane neuronal recombinant $Ca_V2.2$ channels. Our results show an alteration in the $Ca_V2.2$ channel functional expression after chronic treatment with AdGABA presumably caused by a direct interaction of the drug on the $\alpha_2\delta$ subunit. Consequently, AdGABA may bind to the same site as GBP.

Recently, a number of alkyl-substituted GBP analogues and heterocyclic Gabapentin analogues incorporating oxygen, sulfur, or nitrogen at the 4-position have been synthesized. Likewise, the bridged compounds **2** and **3** have also been synthesized by hydrolysis of the lactam precursors (reflux in 6 N HCl).[12]



It was noticed that hydrolysis of the lactam precursor of the bicyclic compound **3** gave two compounds, the desired amino acid hydrochloride **3** and unreacted lactam in a 1:1 ratio. Conversely, in the case of compound **2** the hydrolysis of the lactam precursor required more vigorous conditions (lactam–amino acid, 9:1), suggesting that there was an equilibrium under these conditions.[12a] Thus, it is clear that as the bulk of the lipophilic moiety increases, the equilibrium is shifted toward the lactam. This is even more obvious in the case of the adamantane derivative **5**, where no amino acid was detected, indicating that the equilibrium lay completely in favor of the lactam. Furthermore, when analogue **5** was heated to reflux in hydrochloric acid (6 N) for 5 h, complete γ-lactamization occurred as it was deduced by the elucidation of the product obtained.

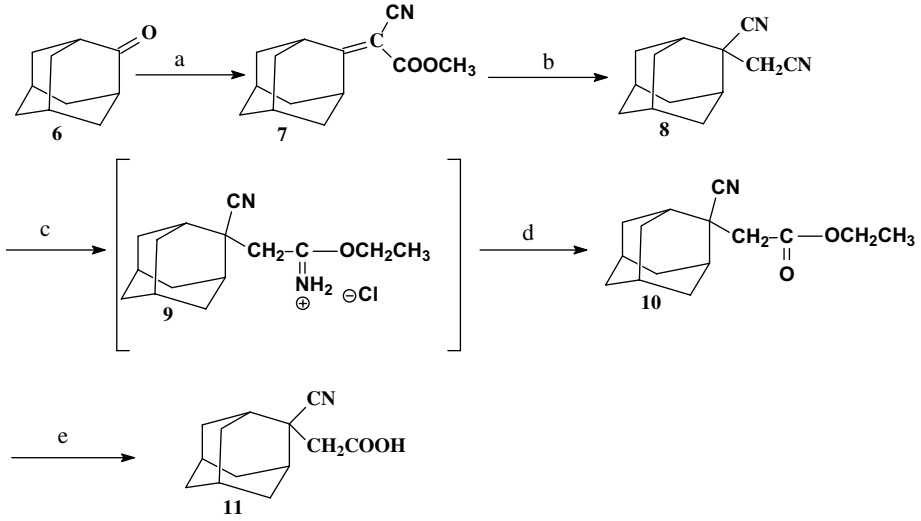
The two bridged compounds **2** and **3** were evaluated for their binding to the Ca_V channel $\alpha_2\delta$ subunit and showed to have good affinity (3-fold stronger than GBP);[12a] we considered that it would be interesting to synthesize amino acid **5**, which is the tricyclic analogue of derivative **2** and evaluate its pharmacological profile.

2. Results and discussion

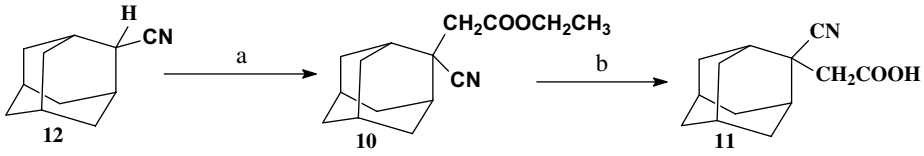
2.1. Chemistry

The key intermediate for the synthesis of GABA-adamantane (AdGABA) **5** is the cyano acid **11**, which we synthesized by the following two different methodologies (**A** and **B**). Route **A** utilizes as a starting material adamantanone **6** (Scheme 1). Ketone is condensed with methyl 2-cyanoacetate to form the conjugated cyanoester **7**.[13] This, in turn, is treated with KCN followed by hydrolysis of the acid and decarboxylation to give the dinitrile **8**.[13] Heating of the dinitrile **8** in a saturated ethanolic solution of gaseous HCl for 8 h did not lead to the desired product **10**. However, when the dinitrile **8** was left in this solution at room temperature for 20 days, it was converted to imino ether hydrochloride **9**, which was hydrolyzed, under mild conditions, to the cyanoester **10**. Saponification of this ester produced the desired cyano acid **11**.

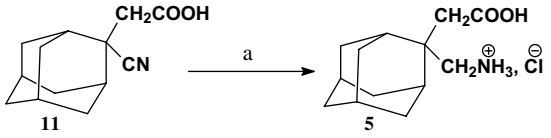
In the synthetic plan **B**, Scheme 2, lithiation at the C-2 of 2-cyanoadamantane **12**[14] by means of LDA and reaction of the resulting carbanion with ethyl bromoacetate gave cyanoester **10**; saponification of the latter afforded the



Scheme 1. Reagents and conditions: (a) NC–CH_2–$COOCH_3$, CH_3COONH_4, CH_3COOH, toluene, 6 h, reflux; (b) KCN, H_2O, EtOH, reflux, 25 h; (c) EtOH–HCl, 20 days, 20 °C; (d) H_2O, HCl, ether, 24 h, 20 °C; (e) EtOH, NaOH, H_2O, 20 °C, 48 h and then HCl.



Scheme 2. Reagents and conditions: (a) LDA, −80 °C, THF, $BrCH_2COOCH_2CH_3$, 24 h, 20 °C; (b) EtOH, NaOH, H_2O, 20 °C, 48 h and then HCl.



Scheme 3. Reagents and conditions: (a) EtOH, HCl, PtO_2, H_2, 50 psi, 25 °C, 5 h.

desired cyanoacid **11**. Synthetic route **B** is faster than route **A**, but it gives the desired product in a lower overall yield. Furthermore, it requires the preparation of the starting material (nitrile **12**). The target compound (amino acid hydrochloride **5**) was obtained by hydrogenating cyanoacid **11** with PtO_2 in the presence of HCl (Scheme 3).

2.2. Stability studies

The fact that heating lactam **4** in HCl (6 N) solution did not produce the desired amino acid hydrochloride **5**,[12a] might be attributed to the facile γ-lactamization of the amino acid. This observation prompted us to undertake a series of stability studies of AdGABA in aqueous media (pH = 7.4 to 3.5). The relative intensity of this ion had no statistically significant variation during all measurements (0–24 h), while the lactam peak is totally absent and hence one can argue that AdGABA does not disintegrate under these conditions.

2.3. Pharmacological evaluation

2.3.1. Electrophysiological studies. As mentioned in the introduction, previous work has shown that the $\alpha_2\delta$ subunit of Ca_V channels is the GBP receptor,[9c,15] and that a down-regulation in the functional expression of native N-type Ca_V channels ($Ca_V2.2$ channels) after chronic treatment (3 days) might play a role in the therapeutic actions of the drug.[10,11] Therefore, in order to investigate the effects of AdGABA at a cellular and molecular level, we tested its actions on $Ca_V2.2$ channels, which were heterologously expressed in HEK-293 cells.

When applied acutely AdGABA had no effect on the recombinant expressed channels. Ba^{2+} currents (I_{Ba}), through the $Ca_V2.2$ channels, were virtually identical before and 5 min after drug application. In contrast, significant current reduction was observed after applications of Cd^{2+}, a well-known blocker of Ca_V channels (not shown). Given that acute (5 min) GBP treatment has, conversely to chronic,[10,11] shown not to affect significantly $Ca_V2.2$ channel activity, we set to characterize the long-term actions of AdGABA on these channels. To this end, HEK-293 cells heterologously expressing $Ca_V2.2$ channels were cultured 24–72 h in the presence of 30 μM AdGABA prior to being subjected to the whole-cell configuration of the patch-clamp technique. In contrast to what was found after acute treatment, a significant inhibition of $Ca_V2.2$ channel activity was observed upon chronic treatment with the drug. As depicted in Figure 1A, we found that AdGABA inhibited the functional expression of recombinant channels in a time-dependent fashion. 24–72 h after the start of the treatment, the number of cells in the culture expressing currents was reduced by ∼30% and the current density by ∼48% and ∼61% after 48 and 72 h

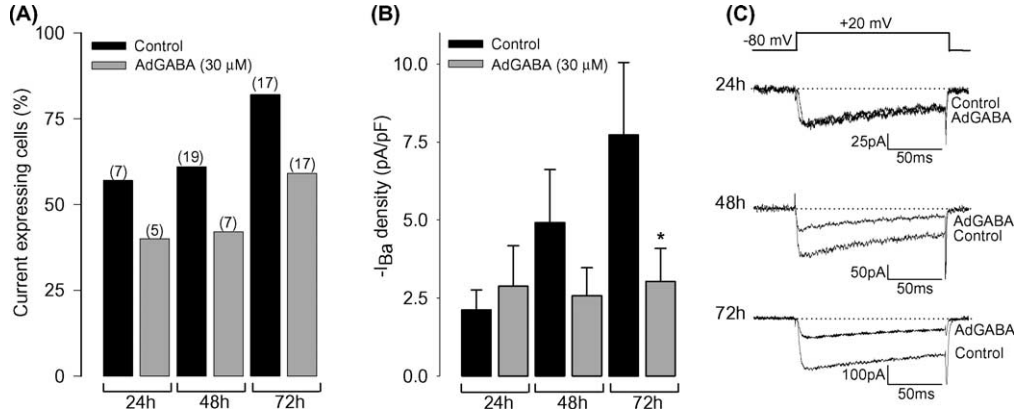


Figure 1. Chronic treatment with AdGABA reduces neuronal recombinant Ca_V current density. (A) Percentage of recombinant $Ca_V2.2/\alpha_2\delta$-1 channels expressing cells in the control condition and after chronic treatment with AdGABA as indicated beneath each bar. The number of recorded cells is shown in parenthesis. (B) Mean + SEM whole cell Ba^{2+} current density of Ca_V expressing HEK-293 cells 24, 48 and 72 h post-treatment with AdGABA. Currents were obtained at +20 mV from a HP of −80 mV. Asterisk indicates significant difference from control ($P < 0.05$, Student's *t*-test). (C) Representative Ba^{2+} currents through Ca_V channels elicited by a voltage step from −80 to +20 mV in HEK-293 cells transiently transfected with $Ca_V2.2$, $\alpha_2\delta$-1, and GFP, in absence and presence of AdGABA (30 μM).

of AdGABA treatment, respectively (Fig. 1B). The traces shown in Figure 1C illustrate representative whole-cell Ba^{2+} currents through $Ca_V2.2$ channels elicited by 140 ms depolarizing pulses to +20 mV from a holding potential (HP) of −80 mV in HEK-293 cells kept in a culture in the presence and absence of AdGABA. The application of the drug treatment stimulated important effects on channel activity with AdGABA mediating strong inhibition after 48 and 72 h.

Interestingly, functional expression decrease of $Ca_V2.2$ channels was not accompanied by any significant changes in the voltage dependence of current activation (Fig. 2A) or steady-state inactivation (Fig. 2B). This suggests that AdGABA, in a similar manner to GBP, does not affect the function of the channels but instead induces a significant reduction in the number of functional channels in the plasma membrane.[11]

In order to investigate the mechanisms underlying AdGABA inhibitory effects on amplitude we analyzed the peak currents through $Ca_V2.2$ channels in various combinations of auxiliary subunits in the presence and absence of the drug (Fig. 2C). It was found that the current amplitude of $Ca_V2.2$ channels was not significantly modified by AdGABA when the $\alpha_2\delta$-1 subunit was absent. However, when this auxiliary subunit was co-expressed, the current amplitude decreased from the control value of -128 ± 29 pA to -56 ± 12 pA in the chronically treated cells (Fig. 2C). These results strongly suggest that the inhibitory effect of AdGABA on $Ca_V2.2$ channel current amplitude is dependent on the presence of the $\alpha_2\delta$ subunit.

2.3.2. Radioligand binding experiments. Having shown that the GBP-like actions of AdGABA on recombinant Ca_V channels were dependent on the presence of the $\alpha_2\delta$ auxiliary subunit, we tested whether or not the drug was binding to $\alpha_2\delta$. To this end, we performed a radioligand binding assay using 4,5-[^{3}H]Leucine. It is well known that certain *L*-amino acids, particularly those with uncharged aliphatic side chains, potently and competitively displace ^{3}H-GBP binding from brain membranes.[5] Notably, ^{3}H-Leu has proved to be remarkably stable and specific for the $\alpha_2\delta$ Ca_V channel subunit.[16] As radiolabeled AdGABA is not available, we used ^{3}H-Leu as an alternative agent for measuring $\alpha_2\delta$ subunits. Competition analysis of radioligand binding to rat brain membranes showed that AdGABA was as potent as GBP in displacing ^{3}H-Leu from the $\alpha_2\delta$ subunit (Fig. 3). Hence, AdGABA (30 μM) strongly competes (∼60%) for the ^{3}H-Leu binding sites, and this competition is similar to that observed for GBP (10 μM) which displaces ∼74% ^{3}H-Leu specific binding. Isoleucine (Iso), another neutral amino acid was used as a positive control (Fig. 3) since it, as well as leucine, competitively displaces ^{3}H-GBP binding from crude brain membrane fractions.[9c] Thus, it is conceivable that AdGABA, like GBP, may have a binding site in this protein.

2.4. In vivo experiments

2.4.1. Animal models for anticonvulsant potency. AdGABA was tested in two animal models of epilepsy (a) the pentylenetetrazole (PTZ)-induced clonic and tonic convulsions[17] and (b) the semicarbazide (SCZ)-induced tonic seizure,[12a] in mice. Our results show that no significant effects were induced below 200 mg/kg of AdGABA in the in vivo tests. In the PTZ epilepsy test, AdGABA (200 mg/kg, ip) augmented the latency of the first clonic convulsions (Lc) by 138% (81.0 ± 11.8 s, for the AdGABA group versus 34.0 ± 3.2 s for controls: $p < 0.01$)

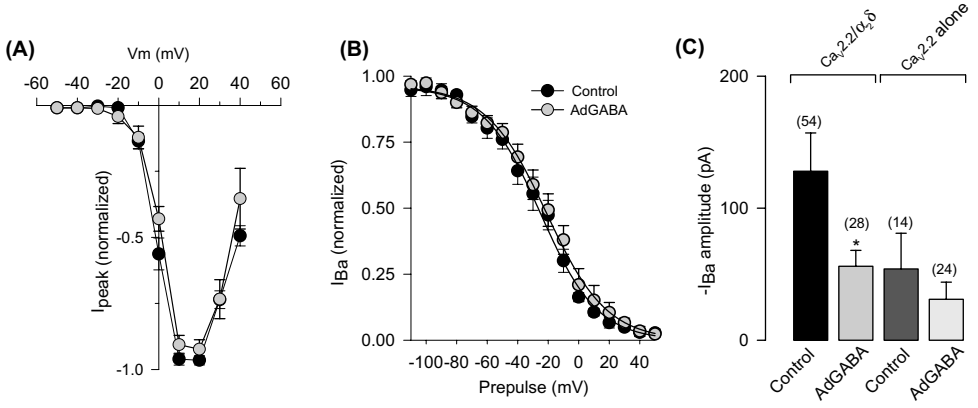


Figure 2. AdGABA does not affect the voltage dependence of Ca_V channels, and requires the $\alpha_2\delta$ subunit for reducing functional channel expression. (A) Current–voltage relationships for transfected cells kept in the culture in control conditions or treated 72 h with 30 μM AdGABA. Mean + SEM from 8 to 11 experiments for each group are shown. (B) Voltage dependence of current inactivation. Ba^{2+} currents were measured in $Ca_V2.2/\alpha_2\delta$-1 expressing HEK-293 cells during depolarizations to +20 mV, preceded by 1 s inactivating pulses (prepulses) of various amplitudes applied from a HP of −80 mV, in the absence and presence of AdGABA. Steady-state inactivation relationships were fitted with a Boltzmann equation of the form: $I = I_{max}\{1 + \exp[(V_{1/2} - V_m)/k]\}$, where I_{max} represents the maximal current amplitude, $V_{1/2}$ the potential for half-maximal inactivation of I_{max}, and k is a slope factor. The resulting kinetic parameters for control and AdGABA treated cells, respectively, were as follows: k (mV) 18.7, 19.7; $V_{1/2}$ (mV) 25, 21.1. Mean + SEM from 8 to 11 experiments for each group are shown. (C) Representative control $Ca_V2.2$ current amplitude and $Ca_V2.2$ co-expressed with $\alpha_2\delta$-1, in absence and presence of AdGABA (72 h; 30 μM). The number of recorded cells is shown in parenthesis and the asterisk denotes significant difference from the respective control ($P < 0.05$, Student's *t*-test).

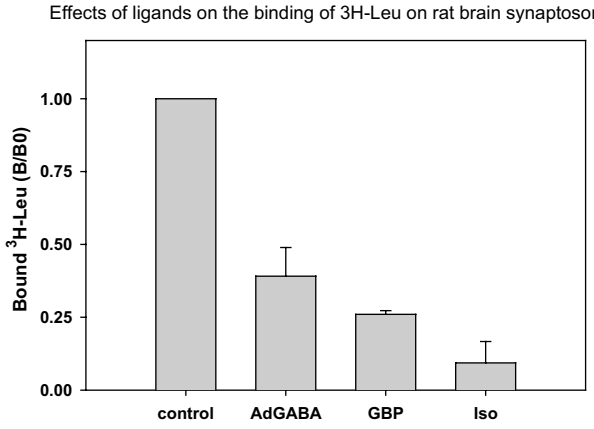


Figure 3. Inhibition of [^{3}H]-Leucine binding to synaptosomal membranes. Rat brain synaptosomal membranes were incubated with ^{3}H-Leucine in the absence (total binding) or presence of different ligands (AdGABA 30 μM, GBP 10 μM, Iso 1 mM). Specific binding is defined by subtraction of the total binding from the non specific binding (in the presence of an excess of unlabeled-Leucine, 1 mM). B0 is the binding (B) in the absence of competitor. Each point represents the mean of at least three separate experiments.

and the latency of the hindpaw extension (Lht) by 234% (381.0 ± 68.2 s, for the AdGABA treated group versus 114.0 ± 17.5 s for controls: $p < 0.01$). Low protection (14%) against the PTZ-induced tonic extension and lethality (0% survival) was noticed at the concentration of 200 mg/kg. In addition, 80–100% protection against PTZ-induced tonic extension was recorded when 400 and 800 mg/kg (ip) of AdGABA was used ($p < 0.01$): $ED50_{AdGABA}$ = 321 mg/kg (ip), while the increase of Lc for the above doses is similar to those obtained at 200 mg/kg (144% and 112%, respectively). However, survival against the PTZ-induced lethality, 57% at 400 mg/kg, was decreased to 28% at 800 mg/kg of AdGABA, as at the latter dose the drug induced sedation and toxic respiratory depression in mice, causing death in the days following the administration ($LD_{50\,(AdGABA)}$ = 850 mg/kg, ip). In the SCZ epilepsy test, doses of AdGABA of 200 and 400 mg/kg (ip) increased the latency by 30% and 45%, respectively; the latency to the tonic extension of forepaws (Lft): 73.7 ± 1.8 min and 82.5 ± 2.7 min for the above doses of AdGABA versus 56.9 ± 3.5 min for controls ($p < 0.01$). These findings suggest that AdGABA is less active than both GBP and its 3-methyl derivative (3-MeGBP).[12a]

2.4.2. Hot plate test of pain sensibility. GBP is known to exhibit antinociceptive effects related to neuropathic pain.[18] In order to study whether AdGABA is also effective on pain sensibility we used the hot plate test. Similar to what was found in the epilepsy tests, no significant changes of the latency of the first forepaws licking (Llf) were observed below 200 mg/kg. Increases of Llf by 93% and 221% were obtained after treatment with 200 and 400 mg/kg, respectively (Lfl: 65.6 ± 7.5 and 108.9 ± 7.7 s for the above mentioned AdGABA doses versus 33.9 ± 4.4 s for controls: $p < 0.01$). This trend was not, however, repeated even after three hours of administration.

2.4.3. Forced swim test for the investigation of antidepressive action. It is firmly established that several antiepileptic drugs, especially Na^+ and/or Ca^{2+} channel inhibitors, possess antidepressive properties.[19] Thus, it was interesting to investigate the putative anti-immobility action of AdGABA in the forced swim test (FST) in mice,[20] which is considered a reliable index of antidepressive action.[20,21] The results from this series of experiments indicate that AdGABA does not exhibit a noteworthy anti-immobility effect in the FST. Moreover, immobility was significantly increased at 250 mg/kg (220.0 ± 2.0 s) compared to the controls (207.0 ± 4.0 s; $p < 0.05$). This increase is probably related to the sedative action of AdGABA at 250 mg/kg.

3. Conclusion

The results presented herein suggest that 2-aminomethyl-2-tricyclo[3.3.1.1^{1,7}]decaneacetic acid hydrochloride **5** is a new γ-aminobutyric acid (GABA) adamantane derivative (AdGABA) endowed with anticonvulsive and antinociceptive actions. These potentially therapeutic properties of AdGABA might be associated with its capability to bind to the neuronal Ca^{2+} channel $\alpha_2\delta$ auxiliary subunit (Fig. 3). In analogy to what has been observed for GBP,[10,11] which is a structural analogue of the neurotransmitter GABA, our results imply that AdGABA significantly affects the functional expression of neuronal recombinant N-type Ca^{2+} channels after chronic exposure (Figs. 1 and 2).

Voltage gated Ca^{2+} (Ca_V) channels are multisubunit complexes that permit the influx of Ca^{2+} ions into cells after changes in the plasma membrane potential. Neuronal channels consist of an α_1 pore protein that conducts current and three auxiliary subunits, $\alpha_2\delta$, β and γ. The $\alpha_2\delta$ subunit invariably increases the current density of Ca_V channels by increasing the amount of the functional channel at the cell surface.[2] As mentioned earlier, GBP has been found to bind specifically to $\alpha_2\delta$,[9c] and given that Ca_V channels are involved in controlling the electrical excitability of neurons, it has been postulated that this drug reduces Ca^{2+} current by modulating α_1 indirectly through its association with $\alpha_2\delta$.[2] In an analogous way AdGABA, through its interaction with $\alpha_2\delta$, might produce reduced Ca^{2+} influx with a subsequent modification of neurotransmitter release which could account for its therapeutic properties.

On the other hand, albeit the fact that the pharmacological evaluation of AdGABA demonstrated anticonvulsive and antinociceptive properties, these properties were detectable only at high (sedative) doses. Indeed, AdGABA was less potent than GBP, though they both seem to be acting via the same mechanism. The reason for this discrepancy is not known; however, one potential explanation is that AdGABA and GBP might be binding in vivo with different affinities to different members of the $\alpha_2\delta$ family. However, differences in the pharmacokinetic profile of the two compounds could also explain the higher anticonvulsant activity of GBP versus

AdGABA, as GBP crosses the BBB using an *L*-system amino acid transporter[5] while AdGABA, in accordance with the in vivo activity, could cross the BBB by unspecific hydrophobic process. In addition, these drugs could have diverse therapeutic actions if more than one of the $\alpha_2\delta$ subunit types binds the same compound. In this regard, it is known that $\alpha_2\delta$-1 and $\alpha_2\delta$-2, but not $\alpha_2\delta$-3, are capable of binding GBP and that $\alpha_2\delta$-1 has a higher affinity for the drug than $\alpha_2\delta$-2.[22] Characterizing the binding affinity of AdGABA to the different isoforms of the $\alpha_2\delta$ is an interesting topic for future studies.

Alternatively, from the results obtained in the in vivo assays (after acute administration of AdGABA), an implication of a GABAergic component in the mechanism of action of AdGABA cannot be ruled out. Indeed, AdGABA antagonism of the PTZ and SCZ tonic convulsions is suggestive of a GABAergic mechanism and a GABA(A) receptor activation was recently reported for GBP.[23] In line with this, it is worth mentioning that in the hot plate acute pain test, AdGABA exhibited a more Tiagabine-like profile than GBP-like.[18a] Tiagabine is a GABA analogue that elevates synaptic GABA levels by inhibiting the GABA uptake transporter (GAT1) and preventing the uptake of GABA into neurons and glia.[24] Likewise, AdGABA-induced increase of immobility in the FST could be in more accordance with a Tiagabine-induced reduction of potency against depression than with GBP's weak antidepressive profile.[12a,19,25] Further studies will be needed to elucidate the precise mechanism of action of AdGABA at a molecular level.

4. Experimental

4.1. Chemistry

Melting points were determined using a Büchi capillary apparatus and are uncorrected. IR spectra were recorded on a Perkin–Elmer 833 photometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC200 and MSL 400 spectrometers, respectively, using $CDCl_3$ as a solvent and TMS as an internal standard. Carbon multiplicities were established by DEPT experiments. The 2D NMR techniques (HMQC and COSY) were used for the elucidation of the structures of some of the new derivatives. Microanalyses were carried out by the Service Central de Microanalyse (CNRS) France, and the results obtained had a maximum deviation of ±0.4% from the theoretical value.

4.2. 2-Cyano-2-tricyclo[3.3.1.1^{1,7}]decaneacetic acid (11)

4.2.1. Method A. Dinitrile **8** (11 g, 55 mmol) was dissolved in a chilled absolute ethanol solution (100 mL) which was saturated with gaseous HCl. The flask was stoppered and the solution left to stand at room temperature for 20 days. Dry ether was added and the precipitated iminoether hydrochloride **9** was filtered off and washed with dry ether: yield 12.7 g (82% from the dinitrile **8**); mp 233 °C (EtOH–Et_2O).

The hydrochloride salt **9** (10.5 g) was dissolved in a mixture of water (50 mL), ether (40 mL) and HCl 36% (1 mL). The resulting mixture was stirred for 24 h at 20 °C, the organic phase separated and the aqueous layer was extracted with ether. The combined ether extracts were washed with water and dried (Na_2SO_4). The solvent was removed in vacuo to give 8.9 g (quantitative yield) of cyanoester **10** as an oil: IR(Nujol) ν(CN) 2233 cm^{-1}, ν(C=O) 1741 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm) 1.23 (t, 3H, CH_2CH_3), 1.63–1.70 (br t, 4H, 6, 10-H), 1.77–1.91 (complex m, 6H, 4e, 5, 7, 8, 9e-H), 2.01 (br s, 2H, 1, 3-H), 2.26 (br d, 2H, 4a, 9a-H), 2.78 (s, 2H, $CH_2COOCH_2CH_3$), 4.14 (q, 2H, $COOCH_2CH_3$); ^{13}C NMR ($CDCl_3$, 50 MHz), δ (ppm) 14.0 (CH_3), 26.3 (7-C), 26.5 (5-C), 30.7 (8, 10-C), 33.4 (1, 3-C), 34.8 (4, 9-C), 37.6 (6-C), 39.4 ($CH_2COOCH_2CH_3$), 42.1 (2-C), 60.7 ($COOCH_2CH_3$) 122.9 (C≡N), 169.3 (C=O). Anal. ($C_{15}H_{21}NO_2$) C, H.

A solution of cyanoester **10** (9.37 g, 37.9 mmol) in ethanol (50 mL) was added to a solution of NaOH (3.03 g, 75 mmol) in water (20 mL), the flask was stoppered and left standing at room temperature for 48 h. After evaporation of the solvent the precipitated salt was dissolved in warm water. The solution was chilled in an ice bath and acidified with concd. HCl. The precipitated solid was filtered off, washed with water, and dried: yield 7.75 g (93% from the cyanoester **10**); mp 148 °C (Et_2O–*n*-pentane); IR (Nujol) ν(CN) 2234 cm^{-1}, ν(C=O) 1707 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm) 1.67–1.72 (br d, 4H, 8, 10-H), 1.80–1.94 (complex m, 6H, 4e, 5, 6, 7, 9e-H), 2.14 (br d, 2H, 1, 3-H), 2.29 (br d, 2H, 4a, 9a-H), 2.88 (s, 2H, CH_2COOH), 10.41 (br s, *COOH*); ^{13}C NMR ($CDCl_3$, 50 MHz), δ (ppm) 26.3 (7-C), 26.5 (5-C), 30.8 (8, 10-C), 33.4 (1, 3-C), 34.9 (4, 9-C), 37.7 (CH_2COOH), 39.2 (6-C), 42 (2-C), 123 (C≡N), 175.7 (C=O). Anal. ($C_{13}H_{17}NO_2$) C, H.

4.2.2. Method B. A solution of nitrile **12** (8 g, 49.6 mmol) in dry THF (25 mL) was added dropwise to a solution of LDA; the latter was prepared by adding a dry THF solution of freshly distilled diisopropylamine (7 g, 70 mmol) to a solution of *n*-BuLi (24 mL, 2.5 M or 60 mmol) in hexane and stirring at −80 °C under an argon atmosphere for 30 min. After stirring the mixture for 1.5 h and raising the temperature to −70 °C, a solution of freshly distilled ethyl bromoacetate (34.7 g, 207 mmol) in dry THF (15 mL) was added and the mixture was stirred overnight to slowly reach room temperature. The solution was then poured into crashed ice, extracted with ether; the organic phase was washed with water and brine, dried (Na_2SO_4) and evaporated under reduced pressure. The crude oil was purified by *vacuum* distillation ($bp_{0.01}$ = 130 °C) to afford 3.75 g of ester **10** as a viscous oil. This product was saponified in the presence of a NaOH (4 g, 100 mmol) EtOH–H_2O solution during 48 h at room temperature. After evaporation of the solvent, water was added and the mixture was extracted three times with ether. The aqueous layer was acidified with concd. HCl under cooling (0 °C). Evaporation of the solvent in vacuo gave the title compound, acid **11**, which was filtered off, washed with water, and dried: yield 1.5 g (13.8%); mp 146 °C (Et_2O–*n*-pentane).

4.2.3. 2-Aminomethyl-2-tricyclo[3.3.1.1^{1,7}]decaneacetic acid hydrochloride (5). A solution of cyanoacid **11** (6.5 g, 29 mmol) in absolute ethanol (50 mL) and HCl (37%, 5 mL) was hydrogenated in the presence of PtO_2 (1 g) catalyst under a pressure of 50 psi, at 25 °C, for 5 h. A white solid was formed, which was dissolved in methanol. The suspension was filtered to remove the catalyst and the filtrate was evaporated until a small volume remained. Dry ether was then added and the mixture was chilled. The amino acid hydrochloride **5** solid formed, was filtered, and washed with dry ether: yield 6.1 g (80% from the **11**); mp 212 °C (MeOH–Et_2O), IR (Nujol) ν(C=O) 1717 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm) 1.65–1.73 (complex m, 8H, 1, 3, 6, 8, 10-H), 1.86 (br s, 2H, 5, 7-H), 2.01–2.15 (m, 4H, 4, 9-H), 2.72 (s, 2H, CH_2COOH), 3.35 (s, 2H, CH_2NH_2), 7.97 (br s, NH_2), 12.51 (br s, COOH); ^{13}C NMR ($CDCl_3$, 50 MHz), δ (ppm) 28.7 (7-C), 28.8 (5-C), 33.4 (4, 8, 9, 10-C), 33.5 (1, 3-C), 38.4 (CH$_2$COOH), 40.2 (6-C), 41.1 (2-C), 45 (CH$_2$NH$_2$), 175.6 (C=O). Anal. ($C_{13}H_{22}NCl$) C, H.

4.3. Stability studies

In order to examine the stability of AdGABA in physiologically simulated intestinal fluids a stock solution of AdGABA (50 μg/mL) in a HEPES/NaOH buffer (0.05 M, pH = 7.4) was prepared; (HEPES: *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]). The solution was stirred continuously at 37 °C in a Selecta water bath for 24 h. At designated times, 100 μL of the buffer was mixed with 600 μL of MeOH (HPLC grade) and 300 μL of CH_3COOH in a way that the final solution had a pH of ~3.5. Aliquots of this solution were transferred into 1.5 mL Eppendorf tubes and analyzed using a Finnigan AQA LC/MS system equipped with electrospray ionization (ESI) source, a quadrupole mass analyzer and a suitable interface (Xcalibur Data System).

All samples were transferred from AQA reservoir to the ESI probe via a fused silica capillary by pressurizing the reservoir. A pressure of approximately 0.408 atm was needed to produce the desired infusion flow rates of (10 μL/min). The probe heater temperature was set at 150 °C and probe and cone voltages were held at 3000 and 25 V, in a positive ion mode, respectively. Data were acquired across the mass range 160–230 *m*/*z* and the ion attributed to protonated AdGABA was detected at *m*/*z* 224.2.

4.4. Biological evaluation

4.4.1. Recombinant $Ca_V2.2$ channel expression and electrophysiology. Human embryonic kidney (HEK-293) cells were grown in DMEM-high glucose supplemented with 10% equine serum, 2 mM L-glutamine, 110 mg/L sodium pyruvate and 50 μg/mL gentamycin, at 37 °C in a 5% CO_2–95% air humidified atmosphere. Transfections were performed using the Lipofectamine Plus reagent (Gibco BRL, Grand Island, NY) with 1 μg plasmid cDNA encoding the rabbit brain N-type Ca^{2+} channel $Ca_V2.2$ pore-forming subunit (formerly α_{1B}; GenBank accession number D14157)[26] alone or in combination with 1 μg plasmid cDNA encoding the rat brain Ca_V channel $\alpha_2\delta$-1 accessory subunit (GenBank accession number M86621).[27] For electrophysiology, 0.1 μg of a plasmid cDNA encoding the green fluorescent protein (pGreen Lantern-1; Gibco BRL) was also added to the DNA transfection mixture to select cells that expressed Ca_V channels. AdGABA was dissolved in sterile distilled water (~ 2.9 mM stock solution) and diluted to the desired concentration (30 μM) in the culture medium, which was replenished every 12 h.

Two days after transfection, cells expressing the green fluorescent protein (GFP) were subjected to the whole-cell mode of the patch clamp technique.[28] In brief, currents were recorded with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) and acquired on line using a Digidata 1320A interface with pClamp8 software (Axon). After establishing the whole-cell mode, capacitive transients were canceled with the amplifier. Currents were obtained from a holding potential (HP) of −80 mV applying test pulses every 20 s. Current signals were filtered at 2 kHz (internal 4 pole Bessel filter) and digitized at 5.71 kHz. The bath solution contained (in mM) 10 $BaCl_2$, 125 TEA-Cl, 10 HEPES and 15 glucose (pH 7.3). The internal solution consisted of (in mM) 110 CsCl, 5 $MgCl_2$, 10 EGTA, 10 HEPES, 4 Na-ATP (pH 7.3).

4.4.2. Membrane preparation from rat cerebral cortex and radioligand binding assays. Cerebral cortexes from rats were dissected and homogenized in a glass/Teflon apparatus in 0.32 M sucrose, 10 mM HEPES buffer adjusted to pH 7.4 with NaOH. After 10 min of centrifugation at 800 × *g*, the supernatant was recovered and spun at 27,000 × *g* for 20 min. Pellet was washed with normal 10 mM HEPES buffer, pH 7.4. Protein content of synaptosomal membranes was assayed by the Lowry method.

4,5-[^{3}H]Leucine (63 Ci/mmol, code TRK 170) was from Amersham International Biosciences, UK. L-Leucine and L-isoleucine were from ICN Biomedicals Inc, Ohio-USA. Binding of [^{3}H]leucine to rat cortical brain synaptosome preparations was carried out at 25 °C in 10 mM Hepes/KOH, pH 7.4, for 45 min. Assay tubes contained protein in 200 μL of 10 mM Hepes/KOH buffer, 25 μL of [^{3}H]leucine (final assay concentration 20 nM), and other additions as required to a final volume of 250 μL. Separation of bound and free ligand was effected by 5 min centrifugation at 12,000 rpm. Pellets were washed three times with cold 50 mM Tris/HCl, pH 7.4, and counted in a scintillation counter. Nonspecific binding was defined as that obtained in the presence of 1 mM unlabeled L-leucine and subtracted from the total binding to yield the specific component. At 20 nM [^{3}H]leucine, nonspecific binding accounted for ~15%. Competition binding data were transformed and analyzed using SigmaPlot version 5.0 (SPSS Software Inc.). Membrane protein concentrations were chosen so that ~8% of the radioligand was bound in the course of the experiment. Under these conditions, the

total ligand concentration is considered to be an acceptable approximation of the free ligand concentration.

4.4.3. Animal models for anticonvulsant potency. Balb-C adult male mice (23–28 g), from Athens Pasteur Institute, were used throughout the following tests in groups of at least six mice. PTZ (130 mg/kg, ip) and SCZ (750 mg/kg, ip) were injected 1 h after the AdGABA administration (10–800 mg/kg, ip, of hydrochloride). Control mice received saline and PTZ or SCZ. In PTZ-induced convulsions it was noted the latency to the first clonic convulsions (Lc) and the hindpaw extension (Lht)—the latter being, few seconds after, was followed by death of controls.[17] In SCZ-induced tonic convulsions, the latency to the tonic extension of forepaws (Lft) was noted.[12a] Analysis of variance or Mann-Whitney (MW) non-parametric test were used for statistics.

4.4.4. Hot plate test of pain sensibility. For the hot plate test, mice were placed on a metal plate (Ugo Basile apparatus) maintained at 52 °C. The latency of the first forepaws licking (Llf) was measured as the response to pain.[18b] Ad-GABA (10–400 mg/kg, ip, of hydrochloride) was administered one hour prior to the test.

4.4.5. Forced swim test in the investigation of antidepressive action. To investigate the putative anti-immobility action of AdGABA in the forced swim test (FST) animals received AdGABA (10–250 mg/kg, ip, of hydrochloride) and, 1 h later, they were forced to swim in a glass cylinder (9 cm diameter) containing water (at 25 °C ± 1) of 6 cm depth. Mice swam for 6 min, but only in the last 4 min some anti-immobility activity (time during which mice tried to escape) was noticed.

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