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Superpotent [Dmt[1]]Dermorphin Tetrapeptides Containing the 4-Aminotetrahydro-2-benzazepin-3-one Scaffold with Mixed μ/δ Opioid Receptor Agonistic Properties

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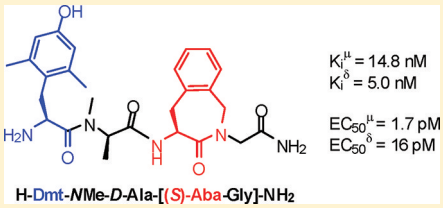
Supporting Information

ABSTRACT: Novel dermorphin tetrapeptides are described in which Tyr[1] is replaced by Dmt[1], where D-Ala[2] and Gly[4] are N-methylated, and where Phe[3]-Gly[4] residue is substituted by the constrained Aba[3]-Gly[4] peptidomimetic. Most of these peptidic ligands displayed binding affinities in the nanomolar range for both μ- and δ-opioid receptors but no detectable affinity for the κ-opioid receptor. Measurements of cAMP accumulation, phosphorylation of extracellular signal-regulated kinase (ERK1/2) in HEK293 cells stably expressing each of these receptors individually, and functional screening in primary neuronal cultures confirmed the potent agonistic properties of these peptides. The most potent ligand H-Dmt-*N*Me-D-Ala-Aba-Gly-NH_2 (BVD03) displayed mixed μ/δ opioid agonist properties with picomolar functional potencies. Functional electrophysiological in vitro assays using primary cortical and spinal cord networks showed that this analogue possessed electrophysiological similarity toward gabapentin and sufentanil, which makes it an interesting candidate for further study as an analgesic for neuropathic pain.



INTRODUCTION

Opioid drugs exert their biological effects through their binding to the three types of opioid receptor subtypes μ, δ, and κ (μ-OR, δ-OR, and κ-OR), which belong to the superfamily of G-protein-coupled receptors.[1] The binding of these ligands to the opioid receptors, however, also results in other effects like tolerance, dependence, respiratory depression, and inhibition of gastrointestinal motility, which often limit their use. Therefore, the design and discovery of new analgesics with a different pharmacological profile and with diminished adverse side effects are a major challenge for medicinal chemists.

An emerging approach is the use of mixed μ/δ opioid ligands, e.g., μ agonist/δ agonist and μ agonist/δ antagonist.[2−7] Various studies proved the existence of physical and functional interactions between the μ and δ receptors.[8−14] Studies have shown that the μ-OR−δ-OR heterodimer affects ligand binding and receptor signaling and that this heterodimer could depict a functional unit distinct from the μ-OR and δ-OR.[10,15,16] Indeed, activation of this heterodimer will result in different downstream effects such as inhibition of adenylyl cyclase activity, phosphorylation of extracellular signal-regulated kinase 1/2 (ERK), activation of K^+ currents, and inhibition of Ca^{2+} channels compared to the activation of isolated μ-OR and δ-OR.[17] The existence of these intermodulary effects stimulated the development of mixed μ/δ opioid ligands with low propensity to induce tolerance and physical dependence.[7,18,19]

Dermorphin (H-Tyr[1]-D-Ala[2]-Phe[3]-Gly[4]-Tyr[5]-Pro[6]-Ser[7]-NH_2), an opioid heptapeptide isolated from the skin of the South American frog *Phyllomedusa sauvagei*, is a selective μ-opioid agonist that produces intense opioid analgesia.[20,21] Besides its higher antinociceptive efficacy and potency compared to morphine, dermorphin is also less likely to produce tolerance, dependence, and opiate side effects than morphine.[22] SAR studies showed that the N-terminal tetrapeptide (H-Tyr[1]-D-Ala[2]-Phe[3]-Gly[4]-NH_2) is the minimal sequence required for opiate-like activity in vivo. Despite the reduced potency of the tetrapeptide compared to the parent heptapeptide, the μ-selectivity was retained. It was also shown that amidation of the C-terminus increased the μ-receptor affinity and potency.[22] The amine and phenolic group of Tyr[1] and the aromatic ring of Phe[3] in a specific relative orientation are key requirements for receptor recognition of these peptides.[23] Inversion of the configuration at residue 2 to the L-Ala[2] isomer produced a 100-fold decrease in μ-receptor affinity and GPI activity. Therefore,

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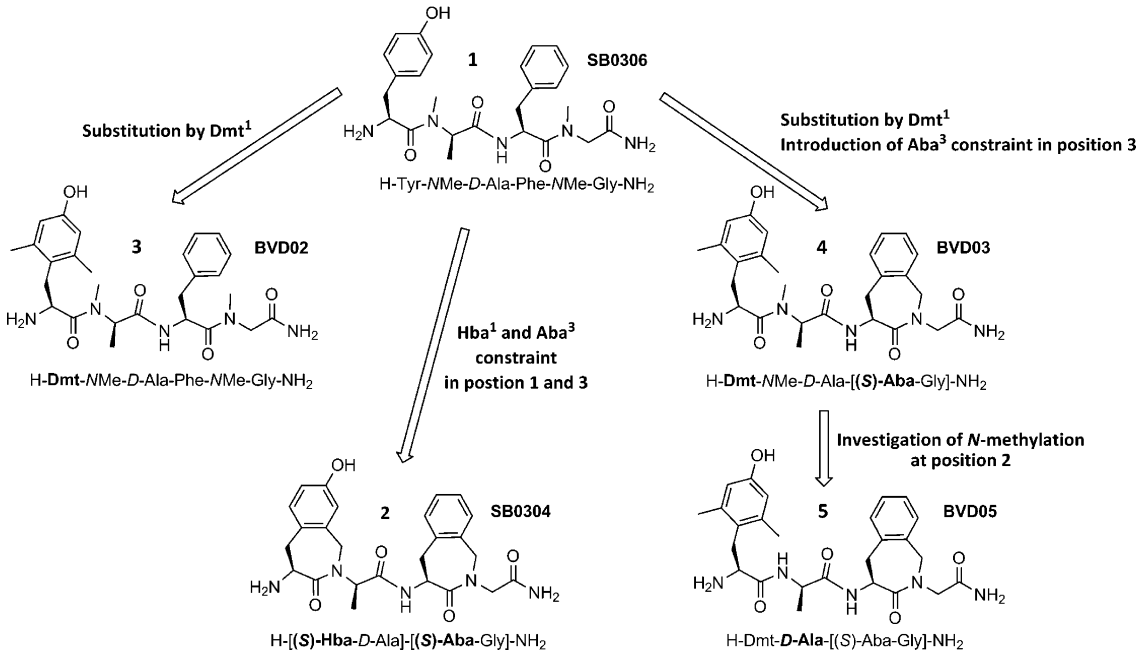

Figure 1. Design of N-methylated and constrained [Dmt[1]]dermorphin tetrapeptide analogues.

the second amino acid residue must play a significant role in orienting these functional groups.

The use of opioid peptides as therapeutics has severe limitations due to the rapid degradation in vivo by peptidases and the poor bioavailability that is mainly due to their inability to pass the blood–brain barrier (BBB). These problems can be circumvented by the introduction of (i) unnatural amino acids, (ii) N-methylation of amide bonds, and (iii) conformational constraints in the native peptide chain.[24−27] Although it was reported that the dermorphin tetrapeptides were highly stable against enzymatic degradation, their oral bioavailability remained low. Several studies have shown that methylation at the fourth position increased lipophilicity and resulted in compounds such as H-Tyr[1]-D-Arg[2]-Phe[3]-*N*Me-Gly[4]-OH or N^{α}-amidino-Tyr[1]-D-Arg[2]-Phe[3]-*N*Me-βAla[4]-OH with improved antinociceptive activity after peripheral administration.[28,29] In contrast, methylation of the third amide bond resulted in a detrimental loss in μ- and δ-receptor affinity.[30,31] Our group reported a highly active, double N-methylated dermorphin tetrapeptide H-Tyr[1]-*N*Me-D-Ala[2]-Phe[3]-*N*Me-Gly[4]-NH_2 **1** (SB0306) with a strong analgesic effect after iv administration.[32] This peptide was equipotent to the constrained dermorphin tetrapeptide H-Hba[1]-D-Ala[2]-Aba-Gly[4]-NH_2 **2** (SB0304)).[32,33] In this analogue the Tyr[1] and Phe[3] residues were conformationally constrained by using the 4-amino-tetrahydro-2-benzazepin-3-on (Aba) scaffold (Figure 1).

In this manuscript we report the preparation of two dermorphin tetrapeptide analogues **3** and **4** that were designed from two reference peptides by substituting the Tyr[1] residue in **1** with 2′,6′-dimethyltyrosine (Dmt[1]) or the Hba[1]-D-Ala residue in **2** with Dmt-*N*Me-D-Ala in order to enhance bioactivity at the μ- and δ-ORs. To determine receptor affinity, specificity, and agonist efficacy, these opioid tetrapeptides were evaluated using a variety of biochemical approaches in intact HEK293 cells stably expressing μ-, δ-, or κ-ORs or in primary neuronal cell cultures. On the basis of the hypothesis that opioid-mediated ERK1/2 activation plays an important role in pain regulation and analgesia[34] and ERK1/2 signaling is involved in the rewarding effects of drugs of abuse and adaptive responses (tolerance),[35,36] the μ-OR and δ-OR mediated ERK1/2 phosphorylation was also examined.

The electrophysiological properties of these peptides can be evaluated using their ability for induction of activity changes in primary cortical and spinal cord networks grown on microelectrode array neurochips. Such network cultures can remain spontaneously active and pharmacologically responsive for more than a year.[37−40] We have previously demonstrated the interculture repeatability of the networks and that the relevant receptors in the tissue of origin are also expressed in culture.[37,41] The spontaneous neuronal activity is specific to the brain-region culture and responds in a substance-specific manner to the compounds applied.[39,42] This enables the pattern recognition and similarity analysis of the activity profiles of those substances.[42,43] Thereby the effect of new drug candidates on the CNS and possible neurotoxic effects can be predicted early by studying changes in a functional network; those possess the temporal resolution of cell-assembly cooperation. Thus, it is possible to clarify the mode of action and considerably accelerate the preclinical development of CNS drugs by using this non-animal testing model.

RESULTS

Chemistry. Two new dermorphin tetrapeptide analogues **3** and **4** were designed from the reference peptides **1** and **2**.[32,33] The Tyr[1] residue in **1** or the Hba residue in **2** was substituted by 2′,6′-dimethyltyrosine (Dmt[1]) (Figure 1), which is known for its enhancement of the μ and δ receptor interactions and significant increase in opioid agonist potency.[44,45] The conformationally constrained Aba[3] residue, present in **2**, was retained in **4**. The effect of N-methylation of the second residue (Figure 1) on the receptor affinity and biological activity was investigated by comparing **2** to **5** (Figure 1).

The peptides **1**, **2**, and **5** were used as references for the novel analogues and were prepared as previously described.[32,33,46] Analogue **3** was prepared using the Fmoc-protected amino acids on Rink amide resin as solid support. For

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Table 1. Receptor Binding of Dermorphin Tetrapeptide Analogues[a]

		K_i [b] (nM)				IC_{50} [c] (nM)	
compd	sequence	μ-OR	δ-OR	κ-OR	K_i^{δ}/K_i^{μ}	μ-OR	δ-OR
1	H-Tyr^1-*N*Me-D-Ala^2-Phe^3-*N*Me-Gly^4-NH_2	1100 ± 160	>10^4	>10^6	N/D	28.1 ± 6.132	7.1 ± 5.9[32]
3	H-Dmt^1-*N*Me-D-Ala^2-Phe^3-*N*Me-Gly^4-NH_2	60 ± 3	130 ± 5	>10^6	2.2	0.30 ± 0.01	4.90 ± 0.24
4	H-Dmt^1-*N*Me-D-Ala^2-Aba^3-Gly^4-NH_2	14.8 ± 2.0	5 ± 0.4	>10^6	0.34	0.95 ± 0.06	0.95 ± 0.07
5	H-Dmt^1-D-Ala^2-Aba^3-Gly^4-NH_2	170 ± 3	18 ± 3	>10^6	0.106	ND	ND
2	H-Hba^1-D-Ala^2-Aba^3-Gly^4-NH_2	2700 ± 300	160 ± 45	>10^6	0.059	20.8 ± 3.6[32]	17.8 ± 5.0[32]

[a]ND: not determined. [b]Displacement of [^{3}H]diprenorphine binding in HEK293 cells stably expressing μ-, δ-, or κ-ORs, [c]Displacement of [^{3}H]naloxone for μ-OR and [^{3}H]deltorphin II for δ-OR from rat brain binding sites.[32]

Table 2. Functional Activity of Dermorphin Tetrapeptide Analogues

		μ-OR[a]		δ-OR[b]		
compd	sequence	EC_{50} (nM)	E_{max}	EC_{50} (nM)	E_{max}	$EC_{50}^{\delta}/EC_{50}^{\mu}$
1	H-Tyr^1-*N*Me-D-Ala^2-Phe^3-*N*Me-Gly^4-NH_2	3.28 ± 0.25	77 ± 3.4	960 ± 220	74 ± 0.5	293
3	H-Dmt^1-*N*Me-D-Ala^2-Phe^3-*N*Me-Gly^4-NH_2	0.0790 ± 0.0065	71 ± 3.25	4400 ± 500	72 ± 3	55700
4	H-Dmt^1-*N*Me-D-Ala^2-Aba^3-Gly^4-NH_2	0.00174 ± 0.00034	72 ± 2.3	0.016 ± 0.009	61 ± 3.9	9.20
5	H-Dmt^1-D-Ala^2-Aba^3-Gly^4-NH_2	0.0832 ± 0.0085	78 ± 1.55	0.17 ± 0.005	66 ± 1	2.04
2	H-Hba^1-D-Ala^2-Aba^3-Gly^4-NH_2	5.17 ± 0.31	78 ± 0.3	N/D	79 ± 1.7	
DAMGO	H-Tyr^1-*D*-Ala^2-Gly^3-*N*Me-Phe^4-Gly^5-ol	3.18 ± 0.30	73 ± 0.3			
DPDPE	H-Tyr^1-c(D-Pen^2-Gly^3-Phe^4-D-Pen^5)-OH			0.14 ± 0.03	69 ± 0.7	

[a]Agonistic properties of peptides on forskolin-stimulated cAMP accumulation by μ- opioid receptor. The inhibition of cAMP accumulation was measured as described in the Experimental Section. [b]Agonistic properties of peptides on forskolin-stimulated cAMP accumulation by δ-opioid receptor. The inhibition of cAMP accumulation was measured as described in the Experimental Section.

the synthesis of **4** the Boc-Aba-Gly building block was prepared using a published procedure using acyliminium chemistry.[33] The Aba-constrained dermorphin tetrapeptide **2** was then synthesized on 4-methylbenzhydrylamine (MBHA) resin. In both cases Fmoc-Dmt-OH was used in the synthesis, and the Fmoc protection was removed prior to cleavage of the peptides from the resin. The peptides were cleaved from the Rink amide or MBHA resin by using respectively trifluoroacetic acid or HF_{liq}. Subsequent purification by preparative high pressure liquid chromatography (HPLC) yielded the pure peptides (>99%).

Structure–Affinity/Activity Study. Opioid binding affinities of the new ligands (**1**–**5**) for the μ-OR, δ-OR, or κ-OR were determined by radioligand competition binding assays using [^{3}H]diprenorphine in membrane preparations from HEK293 cells stably expressing each of the respective receptor subtype tested. Since the binding affinities for the reference compounds **1** and **2** have previously been determined in rat brain homogenates, using [^{3}H]naloxone and [^{3}H]deltorphin II,[32] the affinities of the new analogues **3** and **4** were also determined in these conditions. The functional activity data of all dermorphin tetrapeptide analogues were obtained by measuring the inhibition of forskolin-stimulated cAMP accumulation in HEK293 cells stably expressing the μ- or the δ-opioid receptor and MAP kinase phosphorylation mediated by these receptor ligands.

As shown in Table 1, the K_i values determined in the HEK293 cells expressing the human μ- and rat δ-opioid receptors using [^{3}H]diprenorphine are much larger than the IC_{50} values determined in rat brain using different radioligands. This is not only due to the fact that the rat brain expresses a different population of (all three) receptors but also because [^{3}H]diprenorphine recognizes both cell surface and internalized receptors, resulting in much higher B_{max} and lower K_d values.[47] Nevertheless, the general trend in potency of these ligands tested in the individual receptors expressed in HEK293 is similar to that detected in rat brain membranes.

As indicated in Table 1, the analogue **4** displayed a high affinity for the μ- and δ-opioid receptor but had no affinity for the κ-opioid receptor. As expected, incorporation of Dmt^1 in lieu of Tyr^1 resulted in analogues with enhanced μ- and δ-opioid receptor affinity, with low nanomolar affinities, and with functional potencies in the picomolar range for the μ-OR and δ-OR with higher potency for the μ-opioid receptor (Table 2). Changing **1** to **3** resulted in a somewhat higher increase in δ-affinity than in μ-affinity, resulting in a compound that is almost nonselective (K_i^{δ}/K_i^{μ} = 2.2) when tested in HEK293 cells and moderately selective ($IC_{50}{}^{\delta}/IC_{50}{}^{\mu}$ = 16) in rat brain membranes. It is worth noting that (remarkably) in the functional assays on adenylyl cyclase, compound **3** showed a 41 times higher potency versus **1** at the μ-receptor but a 5 times decreased potency at the δ-receptor. On the other hand, compound **3** phosphorylated ERK1,2 at much higher levels for the δ-OR than that detected for the μ-opioid receptor. These apparent differences in effector potency could be due to the conformation the receptor adopts upon binding to each ligand, and the population of the Gα, RGS proteins, or other signaling intermediates[1,14] is complexed. It is already evident that ligand-specific receptor states differentially modulate heptahelical receptor signaling.[48,49] The incorporation of the Phe^3 side chain in **3** into an azepinone ring resulted in analogue **4**, with mainly an improved δ-affinity. This is in agreement with our previous findings in dermorphin and its N-terminal tetrapeptide that the Aba^3 residue enhances selectivity toward the δ-OR,[33,50] but in this case also improved μ-OR affinity. However in the functional assays performed measuring cAMP accumulation, the potency of **4** toward the μ-OR was further enhanced compared to that of **3**, resulting in an extremely potent compound. However, remarkably enough this analogue also displayed a picomolar potency for the δ-OR.

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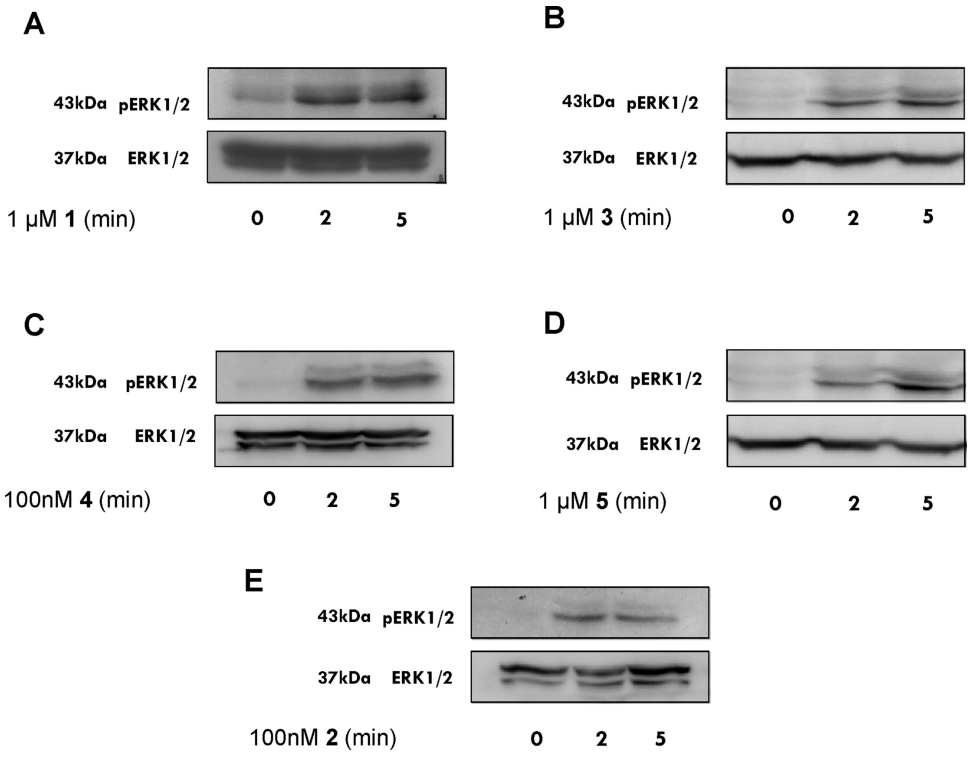

Figure 2. Effect of **1** (A), **3** (B), **4** (C), **5** (D), and **2** (E) on μ-opioid-receptor-mediated ERK phosphorylation. Stably transformed HEK293 cells expressing the μ-opioid receptor were challenged with the appropriate ligands for the indicated time, and cell lysates were resolved in SDS−PAGE (10%) as described by Morou and Georgoussi.[47] Phosphorylated ERK1/2 was visualized by immunoblotting with a phospho-ERK antibody (top panels). Equal loading was verified by stripping and reprobing using an anti-ERK antibody (bottom panels). Results are representative of three independent experiments.

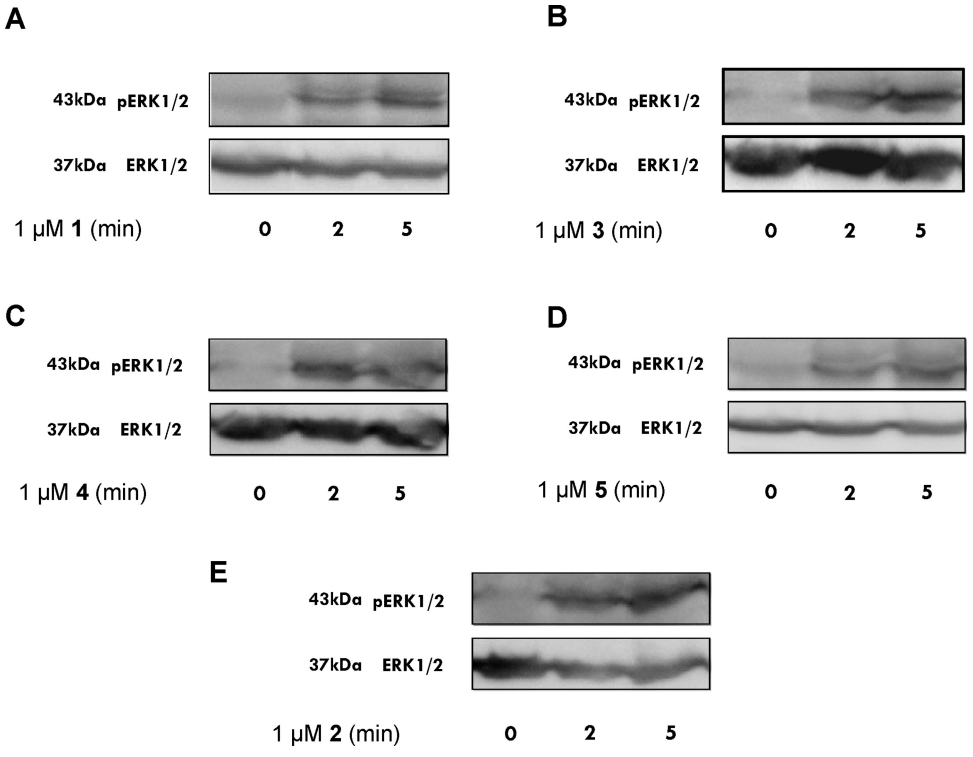

Figure 3. Effect of **1** (A), **3** (B), **4** (C), **5** (D), and **2** (E) on δ-opioid-receptor-mediated ERK phosphorylation. Stably transformed HEK293 cells expressing the δ-opioid receptor were challenged with the appropriate ligands for the indicated time, and cell lysates were resolved in SDS−PAGE (10%) as described by Morou and Georgoussi.[47] Phosphorylated ERK1/2 was visualized by immunoblotting with a phospho-ERK antibody (top panels). Equal loading was verified by stripping and reprobing using an anti-ERK antibody (bottom panels). Results are representative of three independent experiments.

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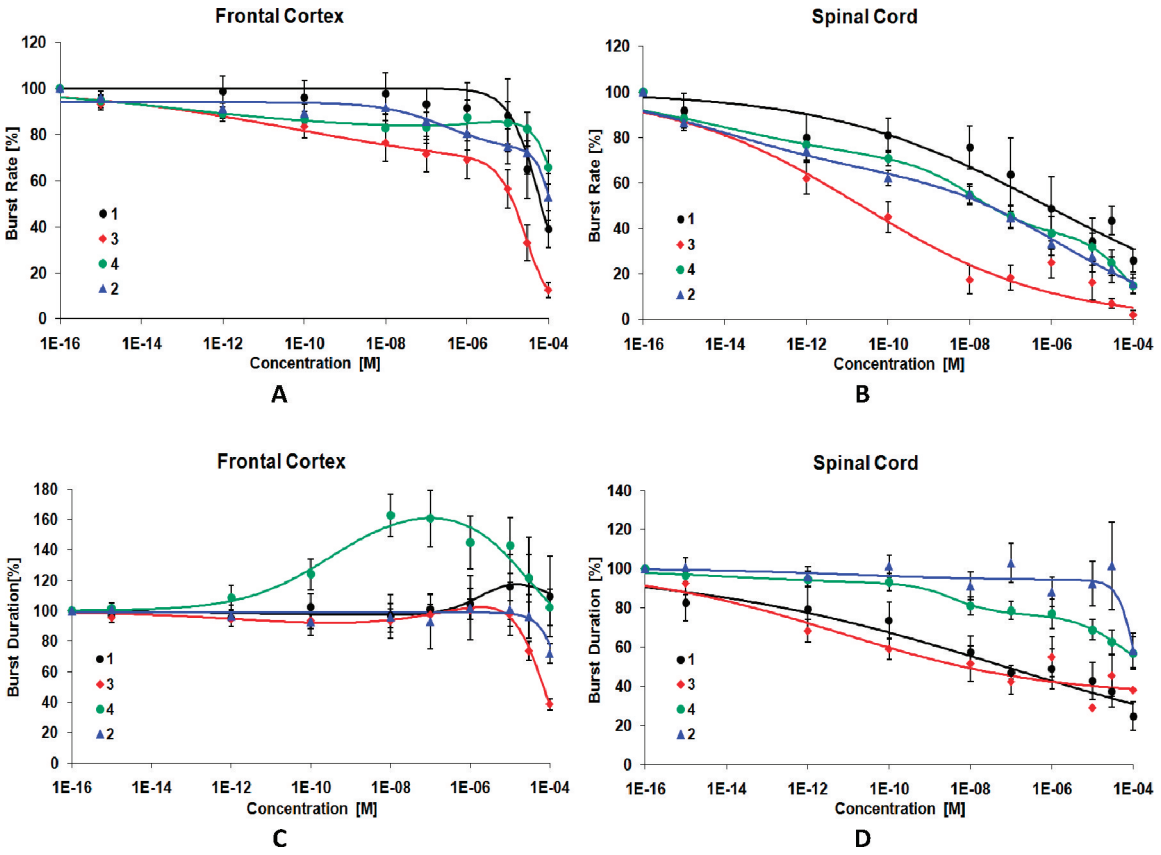

Figure 4. Comparison of the acute effects of the four tetrapeptides **1**, **3**, **4**, and **2** on the cortical (A, C) and spinal cord (B, D) network activity in vitro. Displayed are the activity describing parameters spike rate (general activity) and burst duration (burst structure) for treatment of nine accumulating concentrations in the range of 1 fM to 100 μM (mean ± standard error).

The removal of the *N*-methyl group at D-Ala^2 in **5** resulted in a decreased affinity for the μ- and δ-OR. In the functional assay **5** was 48 times less potent than **4**, whereas at the δ-receptor a 10-fold loss of potency was observed. Despite this, **5** remains a very potent μ- and δ-opioid agonist.

The incorporation of the Tyr^1 side chain into an azepinone ring to give the previously reported analogue **2**[32,33] resulted in a large decrease in μ- and δ-affinity compared to **4**, indicating that this conformational constraint is not optimal for recognition at both receptors. The functional assay of **4** displayed subnanomolar potency for both μ- and δ-opioid receptors, thus suggesting that analogue **4** behaves as full agonist for both μ, δ-opioid receptors.

Effect of Dermorphin Tetrapeptide Analogues on ERK1/2 Activation by the μ- and δ-Opioid Receptors. It has previously been demonstrated that μ- and δ-opioid receptors expressed in HEK293 cells or rat C6 glioma cells stimulate ERK1/2 activity via pertussis toxin-sensitive G protein signaling mechanisms.[47,51]

To examine whether stimulation of the μ- and δ-opioid receptors by the tetrapeptides (**1**−**5**) has a downstream effect for μ- and δ-opioid receptors, we measured alteration of ERK1/2 phosphorylation mediated by these compounds upon activation of the δ- and μ-opioid receptor. Serum-starved HEK293 cells stably expressing the μ- or the δ-opioid receptor were challenged with the opioid tetrapeptide analogues **1**, **3**, **4**, **5**, and **2**. As shown in Figures 2 and 3, Western blotting with a specific phospho-ERK1/2 antibody revealed an increase in ERK1/2 phosphorylation by μ- or δ-opioid receptor within 2 min of the respective agonist stimulation. The MAPK/ERK1,2 signaling cascade mediates a variety of cellular functions like cell differentiation and proliferation, and it has been implicated in synaptic plasticity, memory formation, and long-term gene expression changes underlying drug tolerance and dependence.[52,53] The fact that all dermorphin tetrapeptide analogues (**1**−**5**) enhanced ERK1/2 phosphorylation is a clear indication of the similar agonistic properties displayed by them on the two opioid receptor subtypes (μ-OR and δ-OR).

Functional Screening in Neuronal Cell Cultures. As an alternative approach to define in more detail the pharmacological profile of tetrapeptide analogues **1**, **3**, **4**, and **2**, functional screening of these compounds in neuronal cell cultures was performed using brain-region specific primary network cocultures and microelectrode neurochip technology. The coculture system consists of both neuronal and glial cell populations forming an intricate network with in vivo like properties. Neuronal activity in the system is monitored using modern multichannel recording techniques and characterized using multiparametric data analysis. To clarify the mode of action of the new tetrapeptide analogues (**1**−**4**), their acute dose-dependent effects on the activity of cortical and spinal cord networks were studied. We further analyzed the frontal cortex experiments using methods of pattern recognition. We carried out a classification against 77 substances in the NeuroProof database (Table S1).[42,43] Proprietary procedures of pattern recognition are applied for a multiparametric data analysis. Thus, the effects of CNS drug candidates can be examined directly, which was not possible before. A high reproducibility of data is ensured, for example, the same

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substance can be identified by its inducing specific activity changes even after years.

Electrophysiological Screening Results. To characterize the electrophysiological properties of the four tetrapeptides (**1**–**4**), we first measured well-characterized reference compounds to establish a database with substance-specific activity "fingerprints" for reference compounds. Besides compounds characterizing major receptor systems in neurotransmission (e.g., glutamatergic, GABAergic, gap junction) and specific clinical applications (e.g., antipsychotic, anticonvulsive, antidepressive, analgesic, anesthetic), we especially focused on those related to the opioid analgesic system. Here we characterized the μ-, δ-, and κ-opioid receptors and compounds related to nociception such as neurotensin and substance P. As a control series, we measured untreated network activity for 12 h and analyzed the activity pattern on a hourly basis, related to the accumulating compound concentrations.

For the acute electrophysiological evaluation of the dermorphin tetrapeptide analogues (**1**–**4**) primary neuronal networks from either frontal cortex or spinal cord were acutely treated with accumulating concentrations of the peptides in the range from 1 fM to 100 μM.

Of the four tetrapeptides, **4** was the most potent in frontal cortex in terms of activity inhibition and the effects on the burst structure, followed by **3**, **2**, and **1** (Figure 4, Table 3). Analogue **4** induced biphasic changes in the cortical activity, where the first minor activity decrease ($\leq$1 μM) was accompanied with an increase in the burst duration (Figure 4A,C). A higher concentration caused a strong activity decrease, accompanied by a decrease in the burst duration. A similar effect on the frontal cortex network activity was induced by **1**, **2**, and **3**. The activity decrease combined with an increase in the burst duration seemed to be characteristic for these four compounds.

Table 3. Electrophysiological Activity and Similarity Analysis Data[a]

		EC_{50} (M)			
compd	*N*	frontal cortex	spinal cord	GBP	SUF
1	8	5.00×10^{-12}	1.06×10^{-6}	3	2
3	8	1.29×10^{-13}	2.87×10^{-10}	1	11
4	10	1.61×10^{-15}	1.30×10^{-9}	21	20
2	7	5.67×10^{-7}	6.70×10^{-9}	14	12

[a]EC_{50} for frontal cortex: effective concentration to induce a 50% reduction in the frontal cortex activity. EC_{50} for spinal cord: effective concentration to induce a 50% reduction in the spinal cord network activity. GBP, similarity to gabapentin effects: The numbers give the percentage of activity data sets of a given newly synthesized substance that have the highest similarity to gabapentin activity patterns. SUF, similarity to sufentanil effects: The numbers give the percentage of activity data sets of a given newly synthesized substance that have the highest similarity to the frontal cortex activity pattern of sufentanil.

In spinal cord, **3** induced the largest inhibition in spike rate activity and burst duration, followed by **4**, **2**, and **1**. The different tissue specific effects induced by the tetrapeptides are ascribed to the different receptor composition and colocalized distribution in the cortex and spinal cord.

With the data derived from pattern recognition we tried to fit the 200 activity-describing parameters and combinations thereof to the analgesic properties of the reference compounds. However, we were unable to define a set of activity parameters, which change in a specific way, so that they can be correlated to the analgesic potency in general of the whole variety of reference compounds. Neither in vitro binding affinities nor in vitro EC_{50} effective concentration correlated well to the analgesic potency of all the different compounds. As result of this situation, we performed correlations by comparing the activity pattern "fingerprint" of the four tetrapeptides to those of 77 substances in the NeuroProof database (Supporting Information).

Similarity Analysis and Classification. To specify the anticonvulsive and analgesic properties of compounds **1**–**4**, we used the similarity of the activity pattern changes of these compounds to those induced in a concentration-dependent manner by sufentanil (SUF, for analgesic properties) and gabapentin (GBP, for anticonvulsive properties against seizure and antineuroleptic properties against neuropathic pain). The results of the similarity analysis to these activity profiles are shown in Table 3. Analogue **4** showed the highest similarities to sufentanil and to gabapentin. The second best similarities were found for **2**, followed by **3** and **1**. Analogue **4** also showed a high similarity profile to the neuroleptic drugs clozapine and haloperidol and the antidepressant fluvoxamine. Analogue **2** also showed a high similarity to sufentanil and gabapentin. In the top five compounds in the similarity analysis for **2** were endogenous neuropeptide orexin A, the δ-opioid receptor agonist enkephalin, and the analgesic and μ-opioid receptor agonist L-Polamidone. For analogue **3** the top five similarities were found to be the $GABA_AR$ agonist muscimol, the selective δ-opioid receptor agonist DPDPE, the selective μ-opioid receptor agonist DAMGO, the selective δ-opioid receptor agonist deltorphin, and the antipsychotic haloperidol. Analogue **1** showed the highest similarity to nalorphine, the analgesic and μ-opioid receptor agonist fentanyl, the antidepressant amitriptyline, the immunosuppressant tacrolimus, and the unspecific κ-opioid receptor agonist dynorphin A.

DISCUSSION

The highly constrained H-Hba-D-Ala-Aba-Gly-NH_2 **2** was reported to be very potent in the GPI and MVD assay and to display in vivo antinociception after intravenous administration.[32] The latter property was not related to the conformational constraint but rather to the double N-alkylation at the D-Ala and Gly residues. Indeed, the "ring opened" analogue H-Tyr-*N*Me-D-Ala-Phe-Sar-NH_2 **1** displayed similar in vitro and in vivo potencies.[32] Changing Tyr^1 in **1** by Dmt resulted in the expected increase in μ- and δ-opioid affinity and in μ-, but not δ-, functional potency in **3**. As a consequence, ligand **3** becomes a highly selective μ-OR agonist.

Constraining the Phe^3 side chain into the Aba^3 ring in **4** resulted in the expected increase in δ-affinity, giving a compound with extremely high μ- and δ-agonist potency. The Aba^3 ring fixes the phenylalanine side chain into the gauche(+) or trans rotameric state, the latter being proposed as required for opioid affinity.[30,33] In dermorphin it is responsible for the enhancement in selectivity toward the δ-OR without dramatically affecting μ-OR affinity. This is reflected in the very high potencies of analogue **4** toward both opioid receptors.

N-Methylation, such as present in **1**–**4**, restricts the conformations accessible to the C_α–C(O) bond of the preceding amino acid residue, increases metabolic stability, and decreases the capacity to form hydrogen bonds with water molecules, which results in an improved bioavailability.[54,55] Moreover, in an N-methylamide, the *cis*-amide bond conformation is energetically more favored than in the non-

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methylated one, leading to the discussion whether the *cis*- or the *trans*-amide isomer represents the bioactive conformation.[55,56] The incorporation of *N*Me-D-Ala^2 at the second residue of dermorphin (H-Tyr^1-D-Ala^2-Phe^3-Gly^4-Tyr^5-Pro^6-Ser^7-NH_2) and endomorphin-2 (H-Tyr^1-Pro^2-Phe^3-Phe^4-NH_2) was reported to result in an increase of μ-OR affinity by a factor of 6 and 3, respectively, and an increase of 13 and 354 times in μ-OR mediated biological activity.[57,58]

Therefore, analogue **5** was compared to its N-methylated analogue **4**. The removal of the *N*-Me group at the second position resulted in a somewhat larger drop in μ-affinity (11-fold) than in δ-affinity (4 fold), making **5** somewhat more δ-selective (K_i^{δ}/K_i^{μ} = 0.106) than **4** (K_i^{δ}/K_i^{μ} = 0.34) in the binding assay. In the functional assay **4** and **5** show μ-selectivity. Despite a 48-fold drop in functional potency at the μ-receptor, **5** remains a very potent μ- and δ-agonist. This suggests that the Dmt-D-Ala peptide bond adopts a trans bioactive conformation.

The double Aba constrained reference tetrapeptide **2** is a somewhat lower potent analogue for both receptors compared to the other dermorphin tetrapeptide analogues. Hence, the introduction of the Tyr^1 constraint Hba^1 is not the optimal replacement for recognition by the μ- and δ-ORs.

In the electrophysiological studies, **4** was the most potent in frontal cortex, followed by **3**, **2**, and **1** (Figure 4), whereas in spinal cord **3** induced the largest inhibition in spike rate activity and burst duration, followed by **4**, **2**, and **1**. The different tissue specific effects induced by the tetrapeptides are ascribed to the different receptor composition and colocalized distribution in the cortex and spinal cord. These data confirm that **4** behaves as an extremely potent opioid agonist.

The ranking in the electrophysiological potency in the induced activity changes were also confirmed with the similarity analysis (Table 3). Here, **4** showed the highest similarities to sufentanil and to gabapentin. The second best similarities were found for **2**, followed by **3** and **1**. Compound **4** also showed a high similarity profile to the neuroleptic drugs clozapine and haloperidol and the antidepressant fluvoxamine. These similarities suggest that **4** has a very interesting profile as a potential analgesic for neuropathic pain. Compound **2** also showed a high similarity to sufentanil and gabapentin. In the top five compounds in the similarity analysis for **2** were endogenous neuropeptide orexin A, the δ-opioid receptor agonist enkephalin, and the analgesic and μ-opioid receptor agonist L-Polamidone. The similarity to orexin A could reflect the interplay of the hypocretic/orexin and nociceptin/orphanin peptidergic system in the regulation of stress-induced analgesia.[59]

CONCLUSION

Novel highly potent dermorphin tetrapeptides **3**, **4**, and **5** were obtained by combining Dmt^1 substitution, N-alkylation of D-Ala^2, and conformational constraint of Phe^3. The most potent analogue H-Dmt^1-*N*Me-D-Ala^2-Aba^3-Gly^4-NH_2 **4** behaves as mixed μ-/δ-agonist with subnanomolar potency. In the cAMP assay, analogue H-Dmt^1-*N*Me-D-Ala^2-Phe^3-*N*Me-Gly^4-NH_2 **3** is a selective μ-opioid agonist with subnanomolar potency. All compounds stimulated ERK1/2 phosphorylation, and these analogues might be of great importance in future studies on the role of MKPs in the development of opioid dependence and tolerance.

A novel electrophysiological model assay with similarity analysis was applied at this stage, which could contribute to reduce the use of animal testing for the determination of the analgesic effect. The four peptides **1**, **3**, **4**, and **2** were studied by a functional electrophysiological in vitro screening using primary cortical and spinal cord networks, which indicated that analogue **4** is the most potent compound in the series followed by **3**, **2**, and **1**. The classification of the electrophysiological cortical network changes induced by these compounds revealed the highest similarity of **4** to sufentanil and gabapentin. Considering the best five similarity hits for **4** and since gabapentin is known as an effective drug against neuropathic pain, these results emphasize its potential application as a drug candidate as analgesic for neuropathic pain.

These highly potent compounds will be pursued further in the testing of their antinociceptive effects in vivo after systemic administration.

EXPERIMENTAL SECTION

General Methods. The following amino acids, bases, solvents, and reagents were purchased from Aldrich (Bornem, Belgium): Fmoc-*N*-Me-D-Ala-OH, Fmoc-*N*-Me-Gly-OH, Boc-*N*-Me-D-Ala-OH, Boc-D-Ala-OH, triethylamine, piperidine, ^{i}PrOH, acetonitrile, anisole, triethylsilane, acetic acid, and hydrazine hydrate. Fmoc-Dmt-OH was obtained from RSP Amino Acids (Shirley, MA, U.S.). Fmoc-Tyr(O^{t}Bu)-OH, diisopropylcarbodiimide (DIC), 1-hydroxybenzotriazole (HOBt), trifluoroacetic acid (TFA), and dichloromethane were provided by Fluka (Bornem, Belgium). The Rink amide resin and Fmoc-Phe-OH were obtained from NovaBiochem (Läufelfingen, Switzerland). *N*,*N*-Diisopropylethylamine (DIPEA), trifluoromethanesulfonic acid (TFMSA), and dimethylformamide (DMF) were obtained from Acros (Geel, Belgium). The MBHA resin was purchased from Neosystem (Strasbourg, France). 2-1*H*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) was purchased from Senn Chemicals (Dielsdorf, Switzerland).

All the compounds were analyzed using a Waters Breeze analytical HPLC on a reversed phase C-18 column DiscoveryBIO SUPELCO wide pore C18 column (25 cm × 4.6 mm, 5 μm) with a linear gradient (3−100% CH_3CN in H_2O, containing 0.1% TFA, in 20 min) and a Grace Vydac, Everest C_{18} column (25 cm × 4.6 mm, 5 μm) with a linear gradient (10−100% MeOH in H_2O, containing 0.1% TFA, in 20 min) at a flow rate of 1 mL/min with UV detection at 215 nm.

Purification was carried out on a Gilson semipreparative HPLC system with Gilson 322 pumps on a reversed phase C-18 column (DiscoveryBIO SUPELCO wide pore C18 column, 25 cm × 2.21 cm, 5 μm) with a linear gradient (20−80% CH_3CN in H_2O, containing 0.1% TFA, in 20 min) at a flow rate of 20 mL/min with UV detection at 215 nm.

Thin layer chromatography (TLC) was performed on a plastic sheet precoated with silica gel 60 F_{254} (Merck, Darmstadt, Germany).

Mass spectrometry (MS) data were recorded on a VG Quattro II spectrometer (Micromass, Manchester, U.K.) using electrospray (ESP) ionization (cone voltage, 70 V). Data collection was performed with Masslynx, version 2.22, software.

Synthetic Procedures. Synthesis of H-Dmt-*N*Me-D-Ala-Phe-*N*Me-Gly-NH_2 3. Solid phase peptide synthesis was performed in a 10 mL polypropylene syringe with a PE frit (MultiSynTech, Germany) which was shaken during coupling, deprotection, washing, and cleavage steps. Rink amide polystyrene resin (0.18 mmol, 0.60 mmol/g) was used. The resin was swollen in CH_2Cl_2 for 1 h. After filtration of the resin, the Fmoc protecting group on the Rink linker was removed by 20% piperidine/DMF (2 × 10 min). Subsequently the resin was filtered and washed (three times with DMF, ^{i}PrOH, and DMF again). Couplings were carried out by adding 3.0 equiv of the N^{α}-Fmoc-protected amino acid, together with 3.0 equiv of DIC and 3.0 equiv of HOBt in DMF ([Fmoc-AA-OH] = 0.5 M) and overnight reaction. The completeness of the couplings was checked with the NF-31 test[60] except for the coupling of Fmoc-Dmt-OH, which was checked with the chloranil test.[61] If incomplete, the coupling was repeated. When the coupling was completed, the resin was washed,

Table 4. Sequences and Physicochemical Properties of Dermorphin Tetrapeptide Analogues

compd	sequence	m/z (M + H)$^+$ [a] calcd	m/z (M + H)$^+$ [a] obsd	TLC R_f [b]	HPLC t_R [c] (min) 1	HPLC t_R [c] (min) 2	yield (%)
3	H-Dmt[1]-*N*Me-D-Ala[2]-Phe[3]-*N*Me-Gly[4]-NH_2	512.28	512.41	0.70	12.1	13.6	38
4	H-Dmt[1]-*N*Me-D-Ala[2]-Aba[3]-Gly[4]-NH_2	510.26	510.42	0.67	12.0	13.4	43

[a]Determined by ESI MS$^+$ ionization. [b]TLC system: EtOAc/*n*-BuOH/AcOH/H_2O, 1:1:1:1. [c]HPLC system 1: RP C-18 column, Supelco Discovery BIO wide pore, 25 cm × 4.6 mm, 5 μm; solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile; gradient, 3−97% B in A over 20 min; flow rate, 1.0 mL/min. HPLC system 2: RP C-18 column, Grace Vydac, Everest C_{18} column (25 cm × 4.6 mm, 5 μm); solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in MeOH; gradient, 10−100% B in A over 20 min; flow rate, 1.0 mL/min.

followed by Fmoc deprotection (20% piperidine/DMF (2 × 10 min)), with washings as described above, and coupling with the next amino acid. After completion of the synthesis and final Fmoc-deprotection, the resin was washed (three times with DMF, ^{i}PrOH, DMF again, CH_2Cl_2, and Et_2O), dried under reduced pressure (8 h), and stored at 4 °C.

The peptide was then cleaved from the resin by adding the dry resin to a mixture of 2.5% TES, 2.5% water in TFA (10 mL/g resin). After 3 h, the resin was removed by filtration and the filtrate was added dropwise to dry, cold diethyl ether. The precipitated peptide was isolated by centrifugation, and the ether was decanted. Cold ether was added two more times to the peptide, and it was decanted again after centrifugation. After this isolation process, the peptide was purified by preparative HPLC. The purified peptide **3** was isolated in 38% overall yield and was >99% pure as determined by analytical RP-HPLC. The molecular weight of the pure peptide was confirmed by electrospray ionization (ESI) (positive ion mode) mass spectrometry (Table 4).

Synthesis of H-Dmt-*N*Me-D-Ala-Aba-Gly-NH_2 4. Analogue **4** was prepared on a MBHA polystyrene resin (0.14 mmol, 0.95 mmol/g) using the Boc peptide strategy. The resin was swollen in CH_2Cl_2 for 1 h, filtered, and a 20% DIPEA/CH_2Cl_2 solution was added and subsequently filtered (3 × 1 min). After filtration and washing of the resin three times with CH_2Cl_2, couplings were carried out by adding 4.0 equiv of the $^\alpha$*N*-Boc-protected amino acid, together with 4.0 equiv of TBTU and 8.0 equiv of DIPEA in DMF/CH_2Cl_2, 1/1 ([Boc-AA-OH] = 0.5 M), for 4 h. The completeness of the couplings was checked with the NF-31 test.[60] All couplings were performed two times because of positive color tests. When the coupling was completed, the resin was washed (three times with DMF, ^{i}PrOH, and CH_2Cl_2), followed by Boc deprotection which was performed in 50% TFA/2% anisole/CH_2Cl_2 (1 × 10 min and 1 × 20 min). After that, the resin was washed with CH_2Cl_2 three times and filtered. A 20% DIPEA/CH_2Cl_2 solution was then added, and the resin was filtered (3 × 1 min). After the resin was washed three times with CH_2Cl_2, the coupling with the next amino acid was carried out.

Fmoc-Dmt-OH (2.5 equiv) was coupled overnight using HOBt (2.5 equiv) and DIC (2.5 equiv) in DMF ([Fmoc-Dmt-OH] = 0.5 M). The completeness of the couplings was checked with the chloranil test.[61] When the coupling was completed, the resin was washed (three times with DMF, ^{i}PrOH, and DMF again), followed by Fmoc deprotection and filtration (20% piperidine/DMF (2 × 10 min)). The resin was then washed (three times with DMF, ^{i}PrOH, DMF again, CH_2Cl_2, and Et_2O), dried under reduced pressure (8 h), and stored at 4 °C.

Cleavage of the peptide from the resin was performed using anhydrous HF in a Teflon made HF line (Asti, France) under vacuum. Anisole (2 mL) and HF_{liq} (10 mL) were used for 1 g of peptide−resin. After the sample was stirred for 1 h in an ice bath, the HF was removed by evaporation under vacuum. The reaction vessel was disconnected from the HF line, filled with dry ether (30 mL), and filtered on a glass filter. The precipitated peptide and the resin beads were washed with pure acetic acid. After the filtrate was lyophilized, the peptide was purified by preparative HPLC. The purified peptide **4** was isolated in 43% overall yield and was >99% pure as determined by analytical RP-HPLC. The molecular weight of the pure peptide was confirmed by electrospray ionization (ESI) (positive ion mode) mass spectrometry (Table 4).

Receptor Binding Experiments. In the experiments designed to define peptide specificity for μ- and δ-opioid receptors, membranes expressing either μ-OR or δ-OR (20 μg) were incubated at 30 °C for 45 min in buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 10 mM $MgCl_2$ as described by Megaritis et al.[62] The ability of peptides **1**−**5** to displace [^{3}H]diprenorphine (3.75 nM) binding to the opioid receptors was assessed. Nonspecific binding was measured in the presence of 10 μM naloxone. The reaction was stopped by rapid filtration and three washes in ice-cold 50 mM Tris-HCl, pH 7.4, through GF/C filters (Whatman, Maidstone, U.K.) using an automated cell harvester (Brandel Inc., Gaithersburg, MD). The radioactivity was measured by liquid scintillation counting (liquid scintillation analyzer, Packard). Analysis of the binding data was performed using the Origin, version 7.5, software (OriginLab Corporation, Northampton, MA, U.S.).

Biological Activity Assays. Cell Culture. HEK293 cells stably expressing either the EE-tagged-μ-opioid receptor or the δ-opioid receptor were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/mL penicillin, and 0.1 mg/mL streptomycin, under 5% CO_2 at 37 °C as described by Megaritis et al.[62]

Measurements of cAMP Accumulation. Measurements of adenylyl cyclase activity were performed as described by Merkouris et al.[63] Briefly, HEK293 cells stably expressing the μ- or the δ-opioid receptor were seeded in 12-well plates and incubated for 24 h in a medium containing [^{3}H]adenine (GE Healthcare) (1.5 μCi/well). The generation of [^{3}H]cAMP was assessed in response to treatment of the cells with various concentrations of the appropriate ligand (10 fM to 10 μM or 1 pM to 10 μM) using 50 μM forskolin at 37 °C for 30 min. Results are calculated as the ratio of levels of [^{3}H]cAMP to total [^{3}H]adenine nucleotides (×1000), and the data are presented as percentage of forskolin-stimulated cAMP accumulation upon agonist treatment. The radioactivity was measured by liquid scintillation counting (liquid scintillation analyzer, Packard). Analysis of the data was performed using the Origin, version 7.5, software (OriginLab Corporation, Northampton, MA, U.S.).

Cell Membrane Preparations. Confluent monolayers of HEK293 cells stably expressing either μ-OR or δ-OR were harvested, collected by centrifugation at 1500 rpm for 5 min, and washed once with phosphate buffered saline (PBS) at pH 7.5. Membranes from cell pellets were carried out as described by Georgoussi and Zioudrou.[64] Briefly cell pellets were resuspended in ice-cold membrane buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA), homogenized, and centrifuged at 2000 rpm for 3 min at 4 °C. Supernatants were further centrifuged at 45 000 rpm for 30 min at 4 °C. The membrane pellet was resuspended in ice-cold membrane buffer at a protein concentration of approximately 1 mg/mL and stored in aliquots at −70 °C. Protein concentration was determined according to the Bradford assay.

Detection of MAPK Phosphorylation. HEK293 cells stably expressing the μ- or the δ-opioid receptor were seeded in 10 cm plates. Sixteen hours before the addition of the ligands, the culture medium was removed and replaced by fresh serum-free medium. The ligands were added to the cells and allowed incubate in a time-dependent manner at 37 °C. Cell monolayers were rinsed with PBS, and whole lysates were prepared by the addition of lysis buffer containing 1% Triton X-100, 10 mM Tris, pH 7.6, 5 mM Na_2EDTA, 50 mM NaCl, 50 mM NaF, and 30 mM $Na_4P_2O_7$ supplemented with 2 μg/mL

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antipain, 2 μg/mL leupeptin, 1 μg/mL benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, protease (Complete Mini, EDTA-free; Roche), and phosphatase inhibitor cocktails (P5726, Sigma-Aldrich) for 30 min. Soluble proteins were separated by centrifugation at 3000 rpm for 10 min. Protein concentration was determined according to the method of Bradford.[65] The proteins were prepared for SDS−polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membranes. Membranes were exposed to mouse monoclonal antibodies that are specific to phosphorylated MAPK (1:1000 dilutions in blocking solution, p-ERK1, Santa Cruz Biotechnology Inc.). Immunoreactive proteins were visualized using a horseradish-peroxidase-sensitive ECL chemiluminescent Western blotting kit (Pierce, Thermo Scientific, U.S.).

Functional Screening of Dermorphin Tetrapeptide Analogues with Neuronal Cell Cultures. Primary Cortical Cell Cultures. Frontal cortex tissue were harvested from embryonic day 15 and day 14 crl:NMRI mice (Charles River, Sulzfeld, Germany). After ethyl ether anesthesia, mice were sacrificed by cervical dislocation in accordance with the German Animal Protection Act, Section 4. Frontal cortex tissue was dissociated enzymatically in DMEM 10/10 (10% horse and 10% fetal calf serum), including papain (10 U/mL) and DNase I (8000 units/mL), and mechanically with transfer pipettes. The cells were resuspended in DMEM 10/10 at a density of 1.0×10^6 cells/mL, and an amount of 300 μL was seeded onto microelectrode array neurochip (MEA) surfaces. Cultures were incubated at 37 °C in a 10% CO_2 atmosphere until used, typically 4 weeks to 3 months after seeding. Culture medium was replenished three times a week with DMEM containing 10% horse serum. The neuronal networks are composed of a mixture of neurons and glial cells comparable to the tissue of origin. The developing cocultures were initially treated with 5-fluoro-2′-deoxyuridine (25 μM) and uridine (63 μM) for 48 h to prevent further glial proliferation and overgrowth. Electrical activity starts spontaneously after approximately 3−4 days in vitro in the form of random spiking. After 4 weeks in culture, the activity pattern stabilizes and is composed of one coordinated main burst pattern with several coordinated subpatterns.[37−39] For this study, cultures between 28 and 53 days in vitro were used.

Microelectrode Array Neurochips. MEAs were provided by the Centre for Network Neuroscience (CNNS) at the University of North Texas. These 5×5 cm^2 glass chips have a central recording matrix with 64 passive electrodes and indium tin oxide conductors. The hydrophobic insulation material surface was activated by a brief butane flaming pulse through a stainless steel mask and coated with poly-D-lysine (25 μg/mL, 30−70 kDa) and laminin (16 μg/mL) to ensure cell attachment within a confined adhesive region (5 mm diameter centered on the electrode array).

Multichannel Recording and Data Analysis. For extracellular recording, MEAs were placed into sterilized constant-bath recording chambers and maintained at 37 °C. Recordings were made in DMEM/10% horse serum. The pH was maintained at 7.4 with a continuous stream of filtered, humidified airflow with 10% CO_2. Recording was performed with a computer-controlled 64-channel MEA workstation acquisition system (Plexon, Inc., Dallas, TX, U.S.) providing amplification, filtering, and digital signal processing of microelectrode array signals. The total system gain used was 10K with a simultaneous 40 kHz sampling rate. The signals routinely recorded by these neurochips are located in the range of 15−1800 μV.

The neuronal networks were acutely treated with accumulating concentrations of the new dermorphin tetrapeptide analogues **1**−**4** in the range from 1 fM to 100 μM (1 fM, 1 pM, 100 pM, 10 nM, 100 nM, 1 μM, 10 μM, 30 μM, 100 μM). The network response (spike rate) was observed online. To obtain quantitative data from the 1 h recordings, we analyzed for each of the nine concentrations a stable activity phase of the last 30 min.

The multichannel signal acquisition system delivered single neuron spike data including action potential waveforms. Spike identification and separation were accomplished using a template-matching algorithm in real time. This permitted the simultaneous extracellular recording of action potentials from a maximum of 256 neurons.

The action potentials, or "spikes", were recorded as spike trains; they are clustered in so-called bursts. Bursts were quantitatively described via direct spike train analysis using the program NeuroEXplorer (Plexon Inc., Dallas, TX, U.S.) and in-house programs. Bursts were defined by the beginning and end of short spike events. Maximum spike intervals defining the start of a burst were adjusted from 50 to 150 ms, and maximum intervals to end a burst were from 100 to 300 ms.

High content analysis of the network activity patterns provides a multiparametric description characterizing the changes in four categories: overall activity, burst structure, synchronicity, and oscillatory behavior. We quantify the substance-specific activity changes by extracting a total of 35 activity-describing spike train parameters for these four categories as described previously[37−39] Synchronicity and oscillatory behavior were captured through the temporal and network coefficients of variation (CV_{TIME} and $CV_{NETWORK}$) of the burst rate, burst period, and spike rate parameters. The variation coefficients therefore quantify the spatiotemporal behavior, reflecting temporal dynamics and the fundamental interactions within the networks. Here, CV_{TIME} characterizes the periodic behavior of a single neuron activity pattern. $CV_{NETWORK}$ describes the degree of coordination between different neurons in the activity patterns and is a measure of firing synchronicity. Other activity-describing parameters quantify the dose response kinetics in their course: number of phases, slope (Hill coefficient), and 10%, 50%, and 90% effective concentrations. For direct comparison, all parameters were normalized for each experiment and each experimental treatment with regard to the corresponding values of the native reference activity. Values were derived from 60 s bin data from 30 min after the stabilization of activity. From each network 21−158 separate neurons were simultaneously recorded.

Pattern Recognition and Classification. To clarify the mode of action of the dermorphin tetrapeptide analogues **1**−**4** on the activity of cortical networks, we further analyzed these experiments using methods of pattern recognition. For each stable activity phase after substance application, we calculated 200 spike train parameters normalized by the native activity using Squid (NeuroProof GmbH, Rostock, Germany). These data records were computed for the tetrapeptides **1**−**4** and the reference substances. Using feature selection algorithms as a widely accepted method in bioinformatics and a wide range of different approaches proposed,[66] we calculated rankings of features using various score methods based on the respective class decomposition. The 40 most suitable parameters of all 200 spike train parameters were selected, and their total correct predictions were compared. In this manner we obtained the best result for a MDL (minimal description length) modified algorithm. A train data set with these 40 spike train parameters was established in the form using the data records from the reference substances. We then trained an artificial neuronal network, a multilayer feed forward network, and back-propagation algorithm without hidden units. The respective data records of the tetrapeptides were all subsequently classified using PatternExpert (NeuroProof GmbH, Rostock, Germany).

The database was used for training of an artificial neuronal network. The machine learning algorithm is implemented in our pattern recognition software platform. We use a multilayer feed forward perceptron and a resilient-propagation learning algorithm. We do not use a hidden layer. We use as many input knots as we have features and one output knot for each class which has to be classified. The nonusage of hidden layers is justified by the relatively high variation of our data.

Validation of Classification. To validate our machine learning algorithms, we performed the validation in the form of cross-validation. For this we left out the data records of one experiment (one culture on one MEA neurochip) and trained the artificial neuronal network with all other data records. Then the left out data record was classified with the trained artificial network. This process was repeated until each data record was used once as a left out data record for classification.

Since we were interested in the mode of action, we did not use the concentration as an input parameter for the classification. Although this decreases the correctness of our classification experiments, we obtained assignments dependent solely on the mode of action. A given substance can be identified correctly by its changes of activity patterns if most of the data records are classified correctly.

With methods of pattern recognition and classification algorithms the electrical activity patterns of all newly synthesized compounds that we received from partners were compared to those of the reference compounds in the database and the similarities were calculated.

The new compounds underwent a further specific classification protocol to determine modes of actions for these compounds such as unspecific binding, agonist vs antagonist behavior, analgesic potency, and anticonvulsive properties.

Statistical Analysis. Results are expressed as series mean ± SEM. The distributions of the absolute parameters were tested for normality. By use of the SPSS statistical software, significant changes induced by substance application were tested by analysis of variance (ANOVA) followed by Dunnett's multiple comparison post hoc test with the native activity as the common control. $P < 0.05$ was considered statistically significant.

To determine the half effective concentration (EC_{50}), standard logistic dose−response curves (either one or the sum of two, depending on the data) were fitted to the data points using the sum of least squares algorithm of the Solver module in Microsoft Excel.

■ ASSOCIATED CONTENT

Supporting Information

List of substances in the NeuroProof database used in the similarity analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

Aba, 4-amino-1,2,4,5-tetrahydro-2-benzazepin-3-one; BBB, blood−brain barrier; Boc, *tert*-butyloxycarbonyl; BVD03, H-Dmt-*N*Me-D-Ala-Aba-Gly-NH_2; cAMP, cyclic adenosine monophosphate; CNS, central nervous system; DAMGO, H-Tyr-D-Ala-Gly-*N*Me-Phe-Gly-ol; DIC, 1,3-diisopropylcarbodiimide; DIPEA, *N*,*N*′-diisopropylethylamine; DMF, *N*,*N*′-dimethylformamide; Dmt, 2′,6′-dimethyltyrosine; DPDPE, *c*[D-Pen^2,D-Pen^5]enkephalin; ERK, extracellular regulated kinases; ESI, electrospray ionization; FC, frontal cortex; Fmoc, 9-fluorenylmethyloxycarbonyl; GPI, guinea pig ileum; HEK293, human embryonic kidney; GBP, gabapentin; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; MAPK, mitogen activated protein kinases; MBHA, 4-methylbenzhydrylamine; MVD, mouse vas deferens; OR, opioid receptor; PAGE, polyacrylamide gel electrophoresis; RP, reversed phase; SAR, structure−activity relationship; SC, spinal cord; SDS, sodium dodecyl sulfate; SUF, sufentanil; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TLC, thin layer chromatography

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