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ARE UNCONVENTIONAL NMDA RECEPTORS INVOLVED IN SLOWLY ADAPTING TYPE I MECHANORECEPTOR RESPONSES?

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Abstract—Specific immunohistochemical staining for NMDA receptor NR2A/B subunits was found in the outer root sheath layer of rat sinus hair (whisker) follicle. Co-localization with CK 20 confirmed that Merkel cells were stained. The NR2A/B staining seen on Merkel cells was pericellular. In addition it appeared that NF70-positive staining was in close proximity to, but did not colocalise with NR2A/B immunoreactivity, indicating that NR2A/B was only expressed by Merkel cells and not their adjacent nerve terminals. Merkel cells and the nerve terminals have previously been associated with electrophysiological recordings from slowly adapting type I (St I) mechanoreceptor unit activity. Pharmacological experiments with isolated sinus hairs using a wide range of ionotropic glutamate receptor antagonists found that only certain NMDA receptor blockers depressed St I unit responses to mechanical stimuli. AMPA/kainate receptor antagonists (CNQX and NBQX, 100 μM) had no effect, nor did classical competitive NMDA receptor antagonists, D-AP5 (600 μM) and R-CPP (100 μM), nor the NMDA glycine site antagonist 5,7-dichlorokynurenic acid (100 μM). The only effective NMDA receptor blockers were those selective for the polyamine site: ifenprodil (IC_{50} 20 μM) and Ro 25–6981 (IC_{50} ≈50 μM), and the associated ion channel: MK 801, ketamine and (±)-1-(1,2-diphenylethyl)piperidine (IC_{50} <100 μM). The two enantiomers of MK 801 were equipotent. All effects were long lasting, consistent with their non-/uncompetitive actions. The most potent drug tested, ifenprodil, at an effective dose of 30 μM, had a mean recovery time of 74 min. A three-fold increase in drug concentration was required to depress St II units (associated with non-synaptic lanceolate endings). Changes in Zn^{2+} did not affect St I unit responses. These data suggest that unconventional NMDA receptors are involved in St I unit responses, but question the notion of a glutamatergic synapse between the Merkel cell and nerve terminal. © 2005 Published by Elsevier Ltd on behalf of IBRO.

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; CK 20, cytokeratin 20; CNQX, 6-Cyano-7-nitroquinoxaline-2,3-dione; COV, coefficient of variation; D-AP5, D-(-)-2-amino-5-phosphonopentanoate; FITC, fluorescein isothiocyanate; NBQX, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide; NF70, neurofilament 70; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; PVA, polyvinyl alcohol; R-CPP, 3-((R)-2-carboxypiperazin-4-yl)-propyl-1-phosphonate; SIF, synthetic interstitial fluid; St I, slowly adapting type I; TPEN, *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)-ethylenediamine.

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Merkel cells in mammalian skin make close, synapse-like, contacts with slowly adapting type I (St I) mechanosensory nerve terminals (Iggo and Muir, 1969). The specific role of Merkel cells remains a controversial issue (Iggo and Findlater, 1984; Tachibana, 1995; Ogawa, 1996; Halata et al., 2003). One view is that they provide physical and/or chemical support to their associated nerve terminals (Diamond et al., 1988). A different view is that they operate as mechanotransducers and transmit via a synapse to nerve terminals (Baumann and Senok, 2003). According to this view a chemical transmitter is involved, and this is supported by the observation of granular vesicles in Merkel cell cytoplasm proximal to synapse-like contacts with nerve terminals. Furthermore a pharmacological study found that a broad spectrum ionotropic glutamate receptor antagonist blocked responses from St I rat sinus hair mechanoreceptors (Fagan and Cahusac, 2001; Cahusac, 2003). Finally, recent histological work has revealed essential components necessary for a glutamatergic synapse between Merkel cells and nerve terminals (Hitchcock et al., 2004).

The recent evidence therefore strongly points to the involvement of glutamatergic transmission, and the present study aims to identify the nature of the specific ionotropic glutamate receptors involved. To this end, immunohistochemistry was used to visualize the expression of ionotropic glutamate receptors in the sinus hair capsule. This histology was complemented by pharmacological tests of a range of selective glutamate receptor antagonists on St I unit responses.

EXPERIMENTAL PROCEDURES

Stirling University Department of Psychology's Ethics Committee approved the use of animals for this study. All experimental procedures followed UK Home Office regulations as well as the European Directive 86/609/EEC. The minimum number of animals necessary to demonstrate scientifically important effects was used. The suffering of animals was minimal since they were fully anaesthetized before killing.

Electrophysiology

The isolated rat sinus hair preparation developed by Baumann et al. (1996) was used. Briefly, sinus hairs with a length of the deep vibrissal nerve still attached were micro-dissected from excised whisker pads of 76 Wistar-derived rats. Each removed sinus hair was immediately immersed in synthetic interstitial fluid (SIF) bubbled with medical 95% O_2/5% CO_2 gas, which maintained a pH of 7.4. The capsule of each sinus hair was slit open longitudinally,

and then mounted on a Sylgard (184 silicone elastomer, Dow Corning, Wiesbaden, Germany) platform in a custom made organ bath (obtained from Professor K. Baumann, Hamburg, Germany). Fine insect pins were used to hold open the slit sides of the capsule so as to allow easy access by SIF (and drugs) to mechanoreceptors within the sinus hair. The ~10 mm length of nerve bundle was then stripped of its outer sheath, and fine nerve strands were teased from the bundle and placed onto a silver recording wire. The recording wire was completely immersed in Fluorinert (Sigma) at the bottom of the bath.

Electrical activity was recorded from nerve fibers using Digitimer Neurolog equipment to amplify the signal, and was monitored on oscilloscopes and a loudspeaker. Mechano-responsiveness was tested by displacing the hair shaft with fine forceps, and once established, the probe of a feedback controlled mechanical transducer was attached to the hair shaft about 5 mm from the capsule. Stimulus parameters that evoked ~75% maximal response using displacements of 0.2–1.5 mm were used. Each stimulus consisted of a 500 ms onset, a 4 s plateau and a 500 ms offset ramp (total duration 5 s). Occasionally sine wave-shaped stimuli were used (5–50 Hz), especially to study rapidly adapting units. Stimulus ramps were repeated every 30 s. Discriminated action potentials were collected on computer via a Cambridge Electronic Design 1401+ laboratory interface. St I units were distinguished from St II units by their characteristic irregular firing pattern. The coefficient of variation (COV) for inter-spike intervals during 2 s of the plateau phase (from 2–4 s of the ramp) for St I units was consistently >0.05, whereas for St II units the COV was <0.05. Furthermore it was possible to distinguish the two types of unit by their response to 10 mM caffeine: St I units increased their response to stimuli, while St II units decreased their response (both types spontaneously increased).

Drugs, dissolved in 20 ml of SIF at pH 7.4, were introduced into the bath at the rate of 1 ml/min. The temperature of the bath was maintained at 31±1 °C.

Histology

Rabbit polyclonal anti-NR1 and NR2A/B (UBI, New York, USA) and mouse monoclonal anti-neurofilament 70 (NF70, Chemicon, Chandlers Ford, UK) antibodies were used. Anti-CK20 (Progen, Heidelberg, Germany) was raised in mouse and reactive to rat.

Sinus hairs were micro-dissected out from excised whisker pads, as above. Each isolated sinus hair with capsule was dipped in 10% polyvinyl alcohol (PVA; Sigma, UK), immediately frozen in chilled isopentane (−70 °C) and mounted in 10% PVA on a brass chuck. Thin sections (5–7 μm thickness) were cut using a Bright OTF500 cryostat (Bright Instrument Co., Huntington, UK), collected on polysine slides (BDH, Poole, UK) and stored at −35 °C until use.

Sections were fixed in 4% paraformaldehyde for 5 min and endogenous peroxidase activity depleted with 3% hydrogen peroxide (Sigma, Poole, UK) for 30 min. Further pre-incubation was performed with 10% normal goat serum (Vector Laboratories, Peterborough, UK) for 30 min to block non-specific antibody binding. Sections were incubated for 30 min with primary antibody; individual antibodies were titrated on each tissue to determine optimal concentration and a range of 0.5–1 μg/ml was used. This was followed by biotinylated goat anti-rabbit secondary antibody (Vector Laboratories; 1:200 dilution) for 15 min and avidin-biotinylated–peroxidase reagent (ABC Elite, Vector Laboratories, 1:50 dilution) for 20 min. Peroxidase activity was disclosed with 0.5 mg/ml 3,3′diaminobenzidine (Sigma) and 0.3% hydrogen peroxide as substrate. All dilutions were made up in phosphate-buffered saline (PBS), pH 7.4, and incubations were performed at room temperature with three PBS washes between each incubation. Some sections were counterstained with hematoxylin prior to mounting in glycerol/PBS. For co-localizations of NR2A/B with NF70, NR2A/B was localized initially using the immunoperoxidase techniques described above. The sections were then washed in PBS and incubated with mouse anti-NF70 (1:200 dilution) for 60 min at room temperature. Following further rinses in PBS, a goat anti-mouse fluorescein isothiocyanate I (FITC)-conjugated secondary antibody (Sigma, 1:200) was applied for 30 min.

For immunofluorescent co-localizations, sections were fixed in 4% paraformaldehyde for 5 min and incubated for 30 min in 10% goat serum and 1 h with primary antibody (mouse anti-CK20; 5 μg/ml and rabbit anti-NR2A/B; 1 μg/ml). Sections were then exposed to secondary antibodies (goat anti-mouse FITC; 1:100 and goat anti-rabbit tetramethyl rhodamine isothiocyanate (TRITC); 1:100, Sigma) for 30 min and mounted in Vectorshield (Vector Laboratories). All sections were viewed using a Leica DM IRB upright microscope, equipped with epifluorescence (Leica, Milton Keynes, UK). Negative controls received the same concentration of normal rabbit/goat/mouse IgGs (Vector Laboratories) in place of primary antibody.

Transmission electron microscopy through the outer root sheath allowed identification of Merkel cell-neurite complexes. Vibrissae were fixed in 4% paraformaldehyde/2.5% glutaraldehyde in 100 mM phosphate buffer (pH 7.0) for 90 min at room temperature. They were then washed three times with PBS and subjected to secondary fixation in 1% osmium tetroxide for 1 h on ice and washed 2× in PBS. This was followed by dehydration through graded ethanols, dried over a molecular sieve and washed 2× in epoxypropane for 5 min. Embedding was done using 60% epoxypropane/40% epon araldite for 30 min and left to desiccate with silica gel overnight. Fresh epon araldite was then added and allowed to polymerize for 48 h at 60 °C before sections were cut and stained with saturated uranyl acetate in 50% ethanol with Reynolds lead citrate and viewed using a transmission electron microscope (JLJEM 1200 EX; Tokyo, Japan).

RESULTS

Expression of ionotropic glutamate receptors

NR1. There was selective staining in the monolayer of cells of the external root sheath below and above the ringwulst. However, staining was uniform for all cells in this layer, see Fig. 1a.

NR2A/B. Intense staining was restricted to sections of the external root sheath cell layer commonly associated with the distribution of Merkel cells, in proximity to the ringwulst; see Fig. 1b–d. Figs. 1d and 2c–e indicate pericellular staining. There was no staining of lanceolate nerve endings, although it is possible that finer nerve endings, such as reticular and club-like endings at the level of the cavernous sinus or ringwulst, escaped detection. Staining for the well-known Merkel cell marker, CK 20, was shown in the same area as NR2A/B staining, Fig. 2a. Co-localization studies revealed that there was a close overlap between NR2A/B and CK 20 staining, Fig. 2a, b. In addition it appeared that NF70-positive staining was in close proximity to, but did not colocalise with NR2A/B immunoreactivity (Fig. 2c–e). Transmission electron microscopic images taken through the same areas of the outer root sheath reveal Merkel cells and associated nerve terminals; see Fig. 3.

NMDA receptor pharmacology

A range of competitive NMDA receptor antagonists was tested. In apparent agreement with the histology, the prototypical NR2B subtype receptor antagonist ifenprodil was

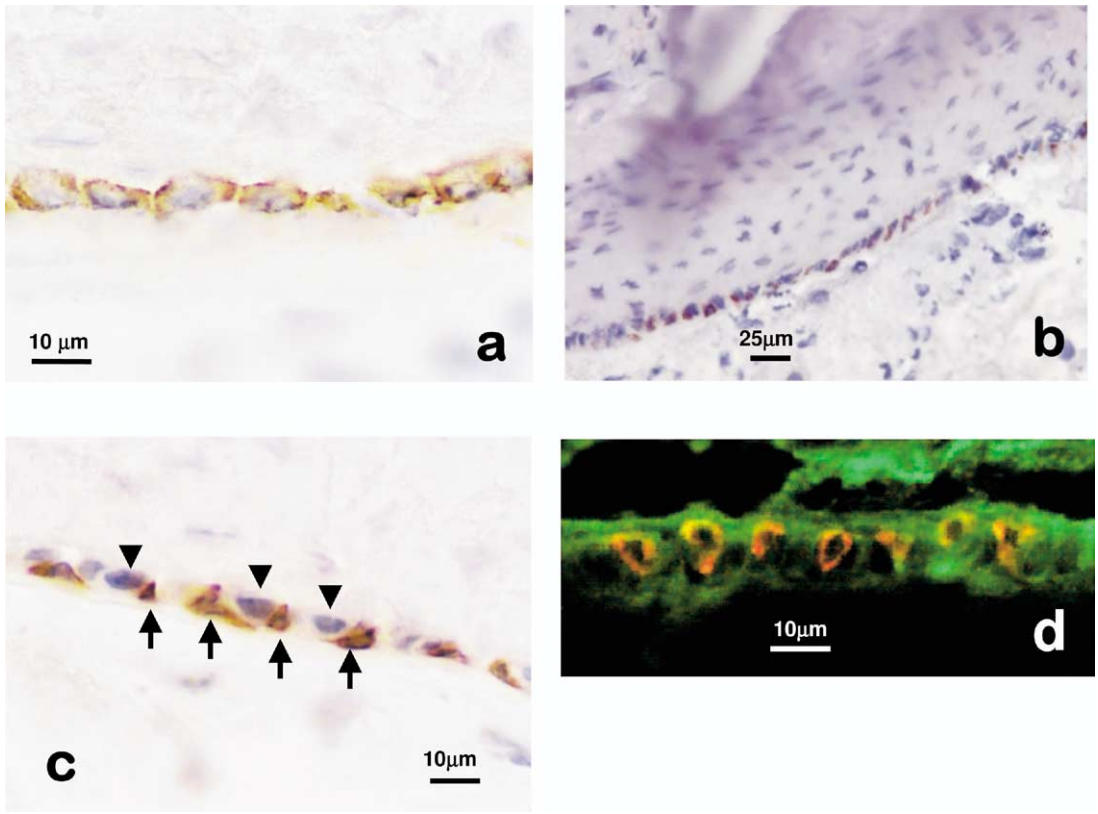

Fig. 1. Immunolocalization of NR1 and NR2A/B subunits in the rat sinus hair follicle. Staining for NR1 (a) was distributed evenly through the monolayer of the external root sheath. This contrasts with staining for NR2A/B (b, c) where there was discrete and intense staining within the monolayer (brown reaction product in b, c). In c four of the seven stained elements are indicated with upright arrows, while inverted triangles indicate cellular elements obviously clear of any staining. In d the more discrete immunofluorescence for NR2A/B (red) shows it to be pericellular. All of these images are oriented so that the basement membrane is situated below the layer of stained cells.

the most potent compound tested in these experiments; see Fig. 4 for the results of one such experiment. Reliable depressant effects occurred at concentrations as low as 10 μM; see Figs. 5 and 6a. St II unit activity was less affected by the drug, requiring three-fold higher concentrations to obtain the same level of depression; see Fig. 6a. An analysis of covariance showed that ifenprodil depressed St I responses significantly more than St II responses across the range of doses used ($F(1,45)=5.81$, $P=0.02$). A related samples *t*-test showed that the static component (3.5–4.5 s of the ramp stimulus) was more affected than the dynamic component (0–0.5 s) in St I units ($t(27)=3.25$, $P=0.003$); see Fig. 6b, but not in St II units ($t(19)=.773$, $P=0.449$); not shown. The IC_{50} for the static component of the response was ≈20 μM; see Fig. 6b. At an effective dose of 30 μM the mean recovery time ($N=3$) was 74 min. The more potent NR2B receptor antagonist Ro 25–6981 had no effect on St I responses at 10 μM, though did reliably cause depression at 50–100 μM ($N=6$). Zinc is known to block NMDA receptors (Westbrook and Mayer, 1987) through an action at the NR2A receptor subtype (Paoletti et al., 1997). As a further test to define the receptor subtype, Zn^{2+} 100 μM ($N=3$) and the zinc chelator *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN) 10 μM ($N=3$) were applied to St I units without effect.

The uncompetitive ion channel blocker (+)-MK 801 at 100 μM had clear depressant effects on St I responses compared with St II responses. Data combined across four experiments for each St type are shown in Fig. 7. Similar results were obtained using (−)-MK 801 at 100 μM. Other ion channel blockers ketamine and (±)-1-(1,2-diphenylethyl)-piperidine were also effective at 100 μM ($N=3$), and selectively affected St I responses. As observed above with ifenprodil, the static was more affected than the dynamic response component by these compounds. At higher doses of these drugs, 300 μM or more, St II responses were also depressed.

The widely used NMDA antagonist D-AP5 was completely without effect (10–600 μM) in six experiments. The potent antagonist R-CPP (10–100 μM) also had no effect in three experiments. Similarly, the NMDA glycine site antagonist 5,7-dichlorokynurenic acid ($K_i=79$ nM) was without effect at doses of 10–100 μM.

AMPA/kainate receptors

The AMPA/kainate receptor antagonist CNQX was tested on St I responses using concentrations of 1–100 μM ($N=7$) without effect; see Fig. 5. Similarly, DNQX and NBQX were tested 10–100 μM, without effect.

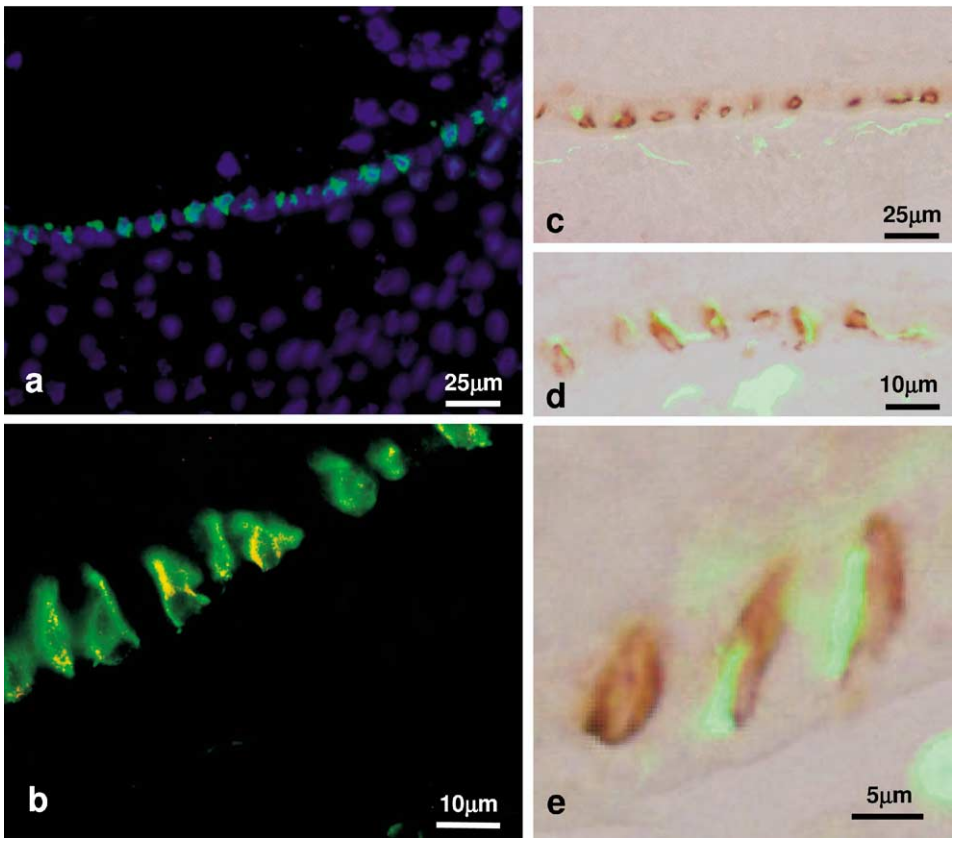

Fig. 2. Immunostaining for NR2A/B, the Merkel cell marker CK 20 and Neurofilament 70 (NF-70). Merkel cell marker CK 20 shown as green fluorescence (a, blue fluorescence indicates DAPI-stained nuclei). Co-localization showed the proximity and overlap between NR2A/B (red) and CK 20 (green) staining as a yellow fluorescent signal (b). Positively stained NF-70 elements (c–e, green fluorescence) were in close association with NR2A/B immunoreactivity (c–e, brown reaction product) but did not co-localize. In a and b the images are oriented so that the basement membrane is above the layer of fluorescent-stained cells. In c–e the basement membrane is below the NR2A/B-stained cell layer.

DISCUSSION

Histology and pharmacology

Functional NMDA receptors require both NR1 and at least one type of NR2 subunit (Cull-Candy et al., 2001), therefore the staining for NR1 alone is not indicative of NMDA receptors. Using immunohistochemistry we identified NMDA receptors within the external root sheath layer, the cell layer containing Merkel cells. While staining for the NR1 subunit was uniform throughout this cell layer, staining for the NR2A/B subunit was closely associated only with cells generally identifiable as Merkel cells. Their identity was confirmed by double-staining for CK 20, an acknowledged marker for Merkel cells. Staining for NR2A/2B was identified with CK 20-stained Merkel cells. Staining for NF70 allowed selective visualization of nerve terminals. NF70-positive nerve terminals were found to be in close association with the NR2A/B-labeled Merkel cells, but NF70 and NR2A/B expression patterns did not appear to overlap. The expanded nerve terminals are coextensive with Merkel cells (Ebara et al., 2002), and the intimacy of the relationship between them is shown in the electron micrographs of Fig. 3. Our data suggest that the nerve terminals do not express NR2A/B, supporting an intrinsic glutamatergic signaling mechanism in Merkel cells. The expression of glutamatergic vesicle loading proteins VGLUT1 and VGLUT2 together with vesicle recycling proteins synaptogyrin and syntaxin-6 in cells of the outer root sheath indicate that glutamatergic transmission occurs between cells (Hitchcock et al., 2004). Although the identity of the cells involved was not determined, it is likely that they were Merkel cells which contain vesicles adjacent to contacts with nerve terminals; see Fig. 3. Other recent studies have now confirmed the presence of vesicular glutamate transporters in Merkel cells, and show that their immunoreactivity is most intense on the nerve terminal side of the cell (Haeberle et al., 2004; Nunzi et al., 2004).

In several respects our pharmacology appears to nicely complement the histological findings. First, AMPA/kainate receptor antagonists were without effect on St I unit activity, ruling out AMPA/kainate receptors in Merkel cell-nerve terminal transmission. This is consistent with earlier histological work showing that AMPA receptor subunits were not present in the external root sheath layer (Senok et al., 2003). Second, NMDA receptor antagonists, notably those associated with an action at receptors containing the NR2B subunit, depressed St I unit activity. NMDA receptor blockade may occur in a number of different ways on the NMDA receptor complex: the NMDA site,

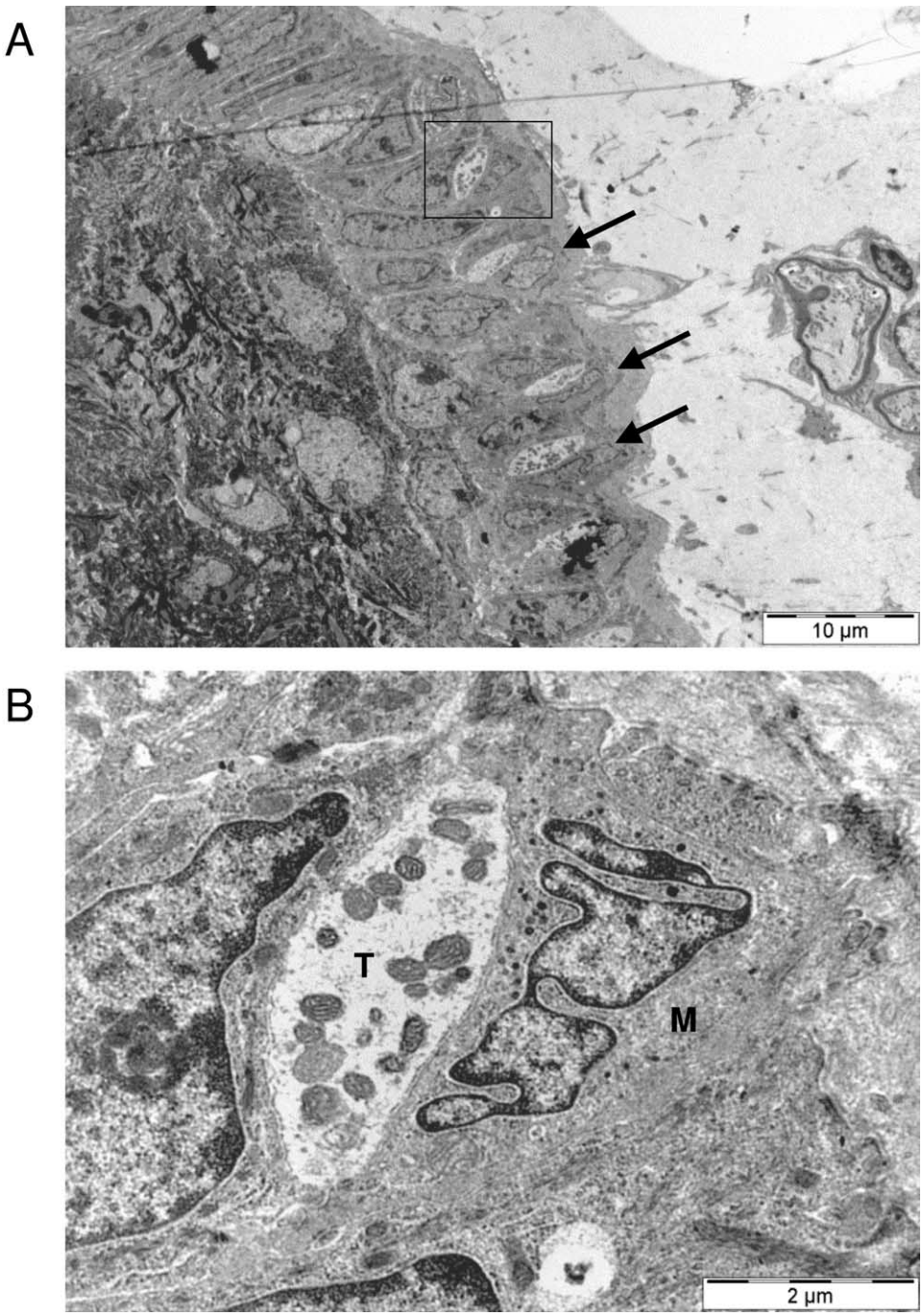

Fig. 3. Transmission electron microscopy imaging of the outer root sheath layer of a rat sinus hair follicle. Merkel cell–nerve terminal complexes are shown in A (arrows). One Merkel cell (M) and associated nerve terminal (T) enlarged from A (rectangle) is shown in B. To the right and top of the Merkel cell, microvilli can be seen penetrating the basal lamina (glassy membrane). Dense core granules in the Merkel cell's cytoplasm are adjacent to the nerve terminal. The basement membrane is to the right of the line of Merkel cells.

the glycine site, the polyamine site and in the associated ion channel. Surprisingly, the classical competitive NMDA receptor antagonists, D-AP5 and the more potent R-CPP, were completely without effect in our experiments. In addition glycine site antagonists, 5,7-dichlorokynurenic acid and CNQX, were ineffective. Only compounds acting at the ion channel and polyamine sites were effective in blocking St I responses. Ifenprodil, the prototypical NR2B receptor and polyamine site antagonist, was most potent (IC_{50} for the static phase was $\approx$20 μM). Ion channel blockers, MK 801, ketamine and ($\pm$)-1-(1,2-diphenylethyl)piperidine were also effective at micromolar concentrations, though they were clearly less potent than ifenprodil. Furthermore the two enantiomers of MK 801 were equipotent in con-

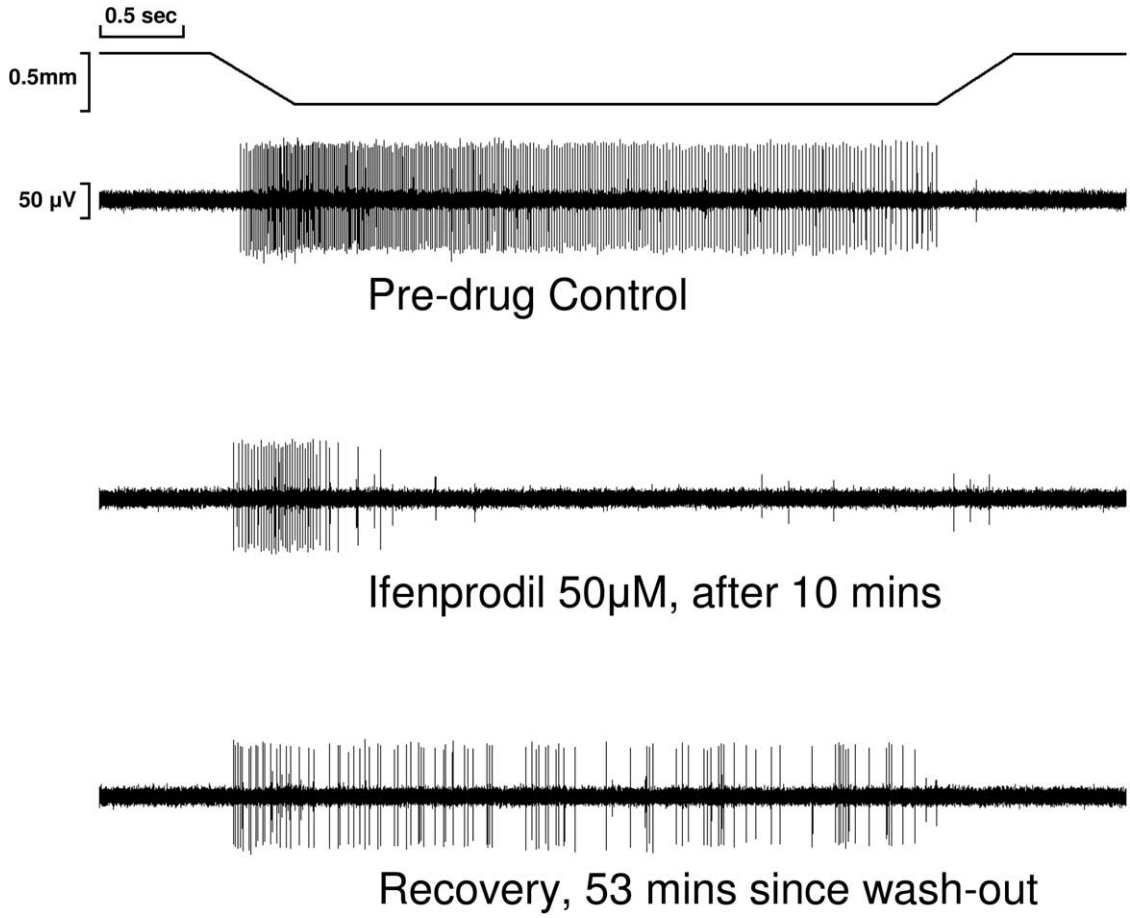

Fig. 4. Oscilloscope spike record showing the effect of ifenprodil on a St I unit. Spike record showing the depressant effect of ifenprodil (50 μM) on a type I unit. The top trace shows the stimulus ramp applied to the vibrissa (time from left to right). Below this there are three oscilloscope traces of the signal showing spikes from the gated unit. (A smaller unit with unrelated activity is also visible, but was small enough not to be gated.) The top oscilloscope trace shows the response before application of drug. The middle trace shows the effect after 10 min from the start of the perfusion. The unit lost all responsiveness after 20 min of exposure to the drug. Following wash-out the characteristically weak recovery was obtained after 53 min.

tradiction to the 10-fold difference reported previously (Genovese and Lu, 1991). The two enantiomers were also found to be equipotent (IC_{50}=14 μM) in inhibiting nicotinic receptors, probably via a mechanism involving channel blockade (Briggs and McKenna, 1996). All these drugs (ifenprodil, MK 801 and related compounds) *selectively* depressed St I unit responses, in that a three-fold greater concentration was required to similarly depress St II unit responses. St II unit responses are believed to be mediated by non-synaptic lanceolate nerve terminals, situated on the outer surface of the glassy membrane (Rice et al., 1993).

Drugs acting at the ion channel and polyamine sites tend not to act competitively. Hence, ion channel blockers (such as MK 801) act uncompetitively, by blocking activated receptors only (a so-called "use-dependent" action). Polyamine site blockers (such as ifenprodil) act non-competitively in a voltage-independent manner (Dingledine et al., 1999). The slow recovery of St I responses following the actions of both classes of blockers is consistent with their non-/uncompetitive actions.

NR2 subunits

Ifenprodil has been reported to have a 160-fold greater selectivity for NR2B over NR2A-containing NMDA receptors (NR2B 250 nM; NR2A 40 μM (Trube et al., 1996)). We tested the highly potent and selective NR2B receptor antagonist Ro 25–6981, which is reported to have >5000-fold selectivity (IC_{50} NR2B 9 nM; NR2A 52 μM (Fischer et al., 1997)). We found Ro 25–6981 to be, if anything, less potent than ifenprodil (IC_{50}>50 μM). This result suggested that these compounds might be acting at the NR2A receptor in general agreement with their IC_{50}'s given above (40 μM and 52 μM). However this was ruled out by our finding that both Zn^{2+} and the zinc chelator TPEN (Paoletti et al., 1997) had no effect on St I responses.

An action by ifenprodil at NR2C receptors appears to be ruled out because the IC_{50} is >100 μM (Chenard and Menniti, 1999). There is a possibility (though in apparent contradiction to our histology) that they act at NR2D receptors, which have so far only been found extrasynaptically and have prolonged deactivation kinetics (Cull-Candy et al., 2001). Ifenprodil enhances proton block of NMDA

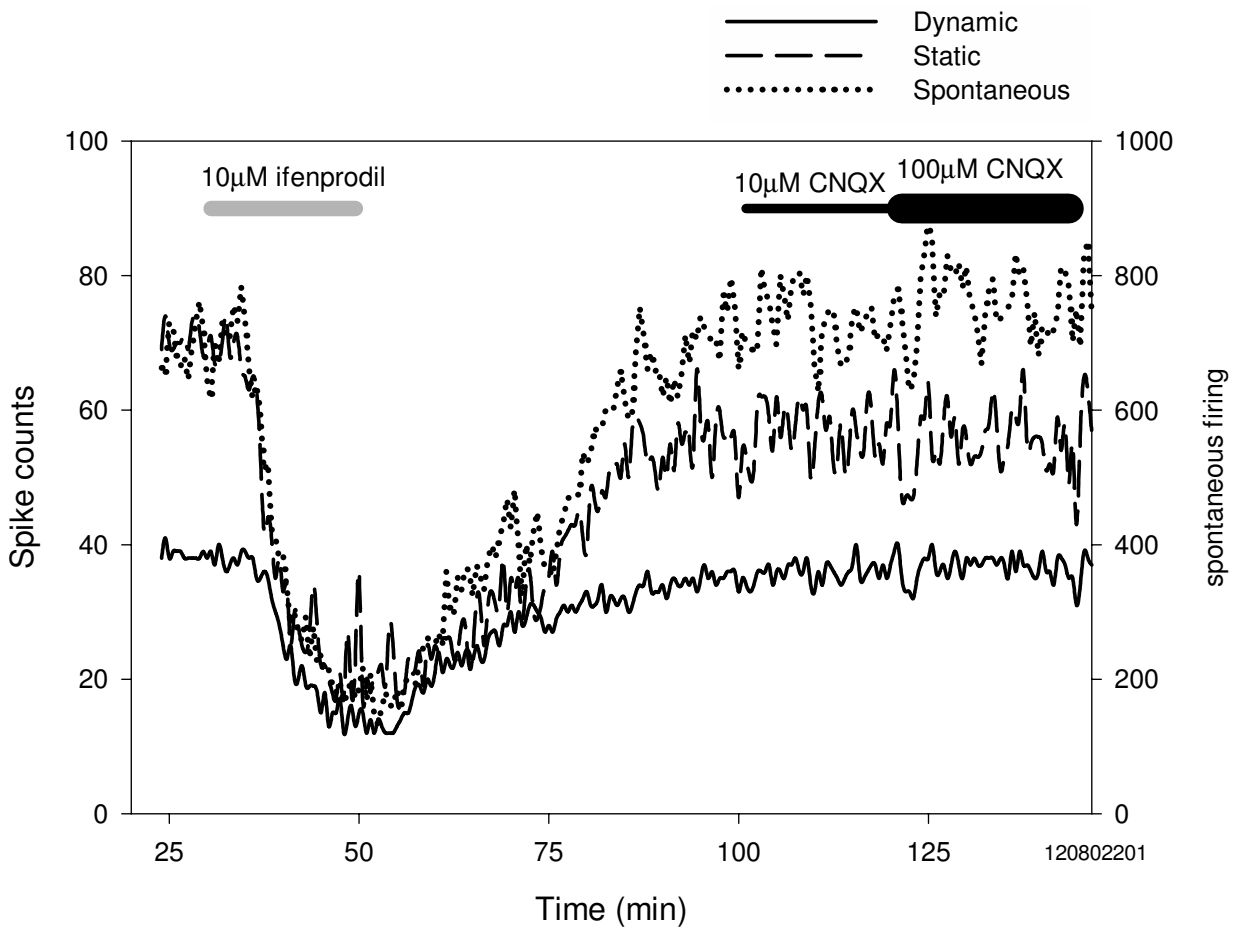

Fig. 5. The effects of ifenprodil and CNQX on St I unit responses to mechanical ramp stimuli. Ifenprodil (10 μM, gray bar) produced a clear depression of firing which almost recovered to pre-drug control levels after 45 min. The subsequent application of CNQX (10 μM, thin black bar) had no effect, nor did an additional higher concentration (100 μM, thick bar). The spike counts shown here represent the firing during the different components of the response (solid line is dynamic; dashed is static; dotted is spontaneous). The spontaneous rate vertical scale is shown on the right.

receptors, and is also known to act at calcium channels, 5-HT receptors, σ opiate receptors and α_1 adrenoceptors (Jane, 2002; Bath et al., 1996). Studies implicating 5-HT receptors in type I responses have not been conclusive, and at best support a modulatory rather than transmitter role (He et al., 2003). Since Ro 25–9681 has a low relative affinity for 5-HT, σ and α_1 receptors (Mutel et al., 1998), it suggests that ifenprodil is not achieving its effect through these receptors (presuming that ifenprodil and Ro 25–9681 are acting at the same site in our experiments).

The site of action

The evidence from this study strongly suggests that the NMDA NR2A/B subunit receptors are situated on Merkel cells, rather than postsynaptic on the apposed nerve terminals.

If ifenprodil, MK 801 and their related compounds are acting at a presynaptic site (i.e. on Merkel cells) then this would provide compelling evidence that Merkel cells are mechanotransducers. The microvilli of Merkel cells (shown in Fig. 3) are known to contain filaments, probably composed of actin. In a manner similar to that seen in auditory cochlear hair cells, the distortion of these filaments (either by stretching or compression) may initiate transduction by opening Ca^{2+} channels (Iggo and Findlater, 1984). NMDA receptors may be mechanosensitive (Casado and Ascher, 1998; Paoletti and Ascher, 1994) through the binding of an actin protein to the cytoplasmic tail of both NR1 and NR2B subunits (Wyszynski et al., 1997). In addition, it has been noted that the NMDA ion channel shares structural features with other receptor ion channels (Ramoa et al., 1990). It has been suggested that both Merkel cells and terminals are mechanosensitive, as proposed by the two-receptor-sites hypothesis (Yamashita and Ogawa, 1991). Briefly, this hypothesis states that high frequency dynamic responses are largely mediated by the nerve terminals, and the lower frequency static responses are largely mediated by Merkel cells. Our pharmacological data would be consistent with this in that the antagonists (including kynurenate (Fagan and Cahusac, 2001)) had a relatively greater depressant effect on the static than the dynamic component of the response (see Figs. 4 and 6b).

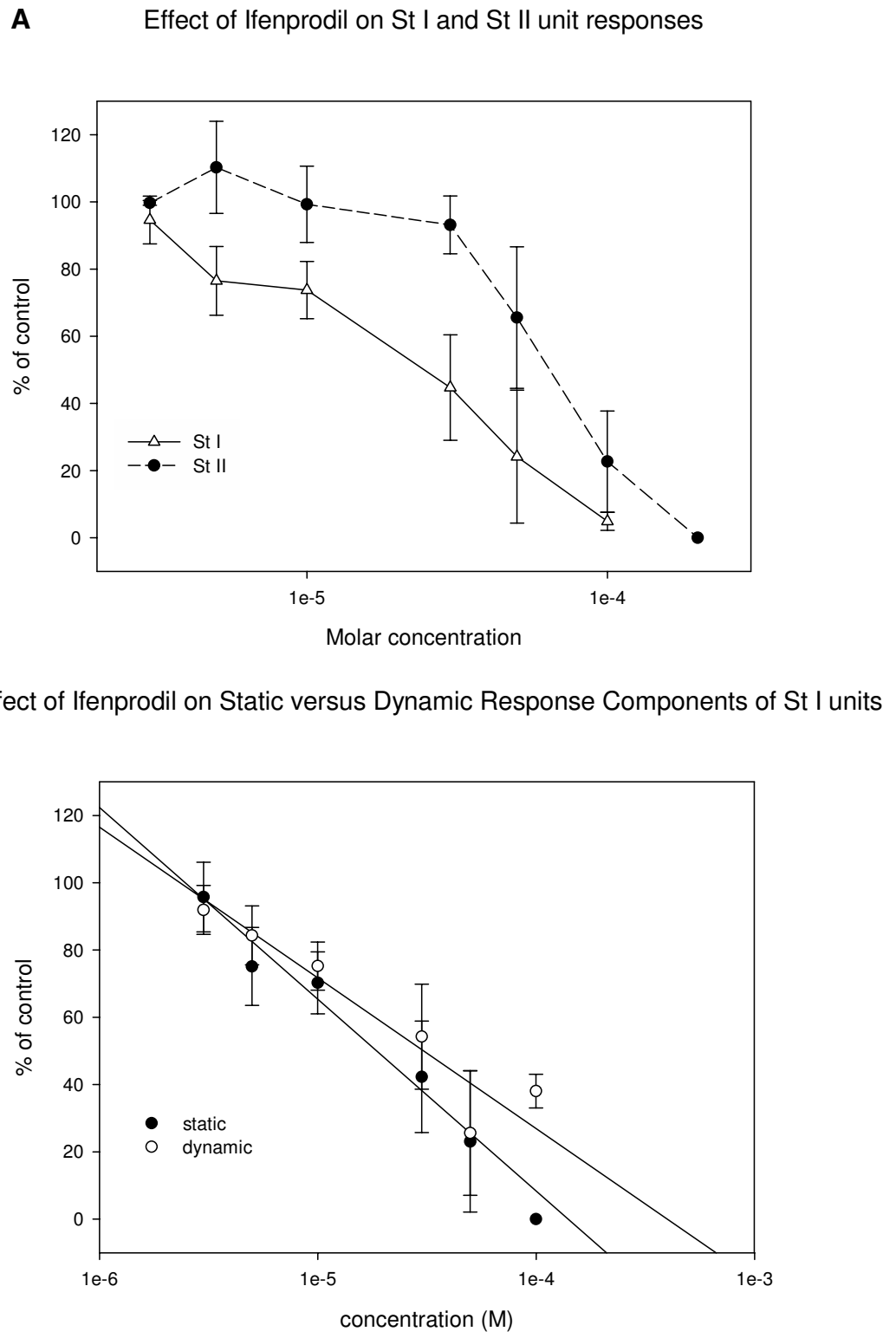

Fig. 6. Dose-response plots for ifenprodil. In 6a is shown the relative potency of ifenprodil for St I (triangles) vs St II (circles) units. The St I units were affected at a lower concentration than St II units. In contrast St II units showed a steeper decline in responses with concentrations exceeding 30 μM. (The plot includes data from five experiments from rapidly adapting units among 17 experiments from St II units). In 6b is shown the relative effect of ifenprodil on static (filled circles) compared with dynamic responses (open circles). The static component was relatively more affected than the dynamic component. Standard error bars are shown for each point in the plots.

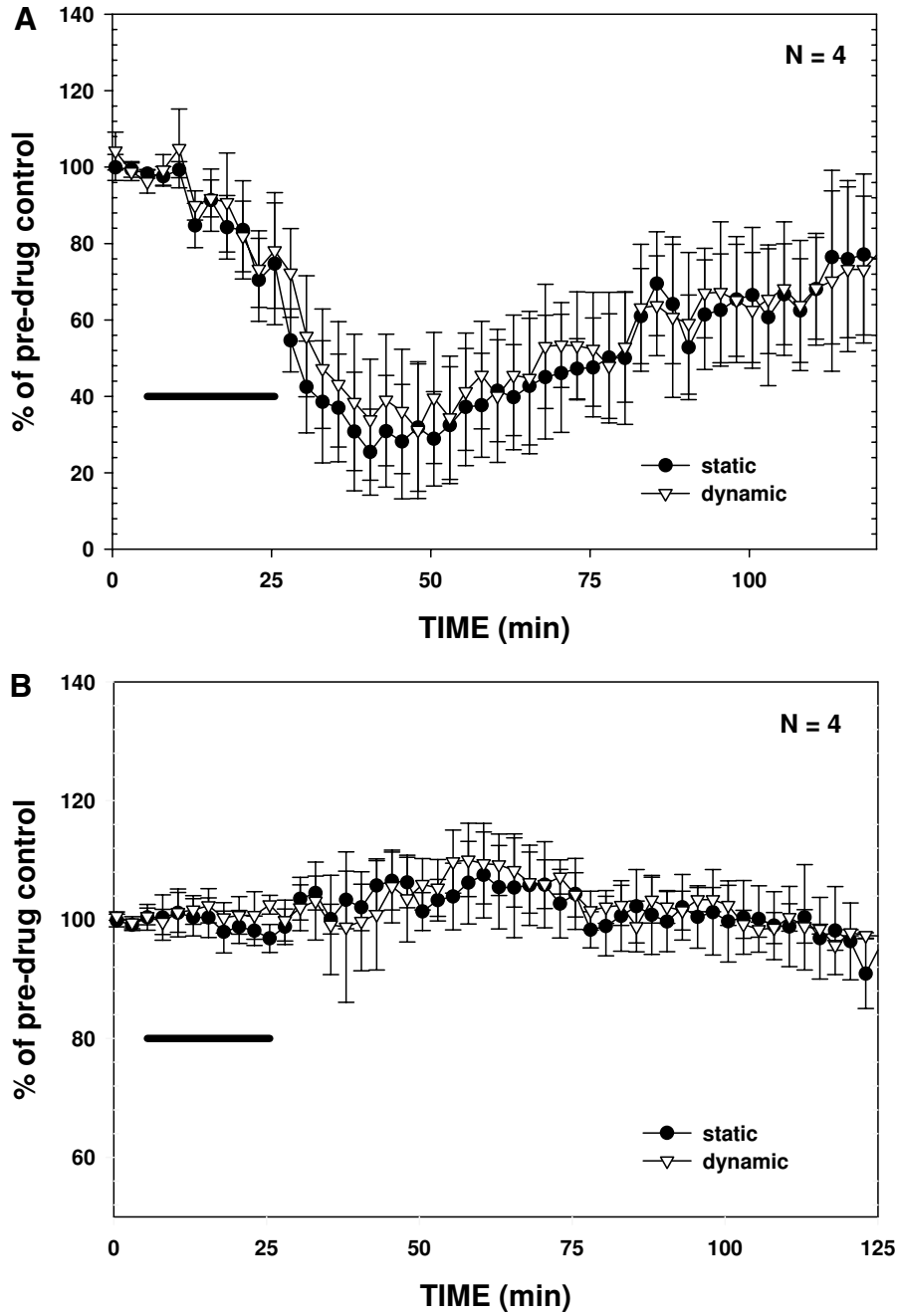

Fig. 7. Effect of MK 801 on St I and St II unit responses. MK 801 at 100 μM consistently depressed St I units (a), but had no consistent effect on St II units (b). Results from four separate experiments on St I and St II units are combined in each of the figures. The mean % of control (pre-drug) response is plotted for static (filled circles) and dynamic (triangles), with error bars representing 1 standard error. Incomplete recovery from the effects of MK 801 on St I units was observed during the time course shown (and the degree of recovery was variable across units).

If synaptic transmission is envisaged from the Merkel cell, then a postsynaptic site might implicate receptors with NR2A subunits since they are specifically associated with a synaptic location, unlike NR2B (and NR2D) which are located extrasynaptically (Cull-Candy et al., 2001). However this seems unlikely in view of the lack of effect of changes in Zn^{2+} ions on St I responses. The restricted (pericellular) localization of NR2A/B receptors on Merkel cells and the lack of effect of conventional NMDA and non-NMDA receptor antagonists (such as D-AP5 and CNQX) do not support the simple hypothesis that glutamate is the neurotransmitter at the Merkel cell–neurite junction. Instead it might suggest that glutamate released by a Merkel cell modulates its own excitability via an autoreceptor. The identity of the neurotransmitter remains to be determined, but the necessary molecular machinery in Merkel cells for the production and release of amino acid and neuropeptide transmitters has been identified recently (Haeberle et al., 2004).

It is also conceivable that the effective NMDA antagonists (e.g. ifenprodil) used in this study were acting at a "postsynaptic" site on the nerve terminals and/or on fibers, at sites which were not labeled using our immunohistochemical methods. Furthermore, it is possible that these antagonists, which are all known as channel blockers, were acting at mechanogated channels (on Merkel cells and/or nerve terminals) rather than at neurotransmitter receptors. Some support for this possibility comes from a pharmacological study of gastric mechanoreceptors (Sengupta et al., 2004), where responses were dose-dependently attenuated by MK 801 and memantine (*and* CNQX) but not D-AP5.

In summary we have shown selective staining of NMDA receptor NR2A/B subunits in the outer root sheath, an area rich in Merkel cells. Only NMDA receptor antagonists with an action at the polyamine site and the NMDA ion channel selectively depressed St I units associated with Merkel cells. The profile of actions from the range of selective NMDA receptor antagonists used indicates that conventional NMDA receptors are not involved. Further work must identify the way in which these antagonists are blocking St I unit activity, and so provide insight into the functioning and functional importance of Merkel cells.

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