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Title

Assessment of brain responses to innocuous and noxious electrical forepaw stimulation in mice using BOLD fMRI

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Introduction

Processing of noxious stimuli involves different levels and structures of the neural system. In spite of substantial progress in understanding the molecular mechanisms underlying pain, many aspects are still poorly understood. Genetically engineered mouse lines displaying altered or pathological pain states may help to elucidate the physiological and molecular basis of normal and pathological pain processing [21; 7; 17; 34]. Classically, sensory responsiveness in animals is characterized using behavior tests such as the hot plate, von Frey filaments or tail flick test [54]. These analyses, however, depend on the skills of the experimenter and require undisturbed motor function of the animal. Electrophysiological recordings of neuronal activity provide high spatial and temporal resolution. However, the method is invasive and does not allow recording signals over extended brain areas in a limited time period. An objective readout of neuronal signal processing that is non-invasive, independent of the observer performance and capable of covering the entire brain would be highly desirable. Functional magnetic resonance imaging (fMRI) has been widely used to assess changes in brain activity evoked by noxious stimuli. Noxious-evoked activation patterns revealed by fMRI correspond well with the structures of the pain processing pathway both in humans and animals [26; 11; 30; 6; 28]. There are two peripheral nerve types which process sensory input: low threshold fibers, mainly Aβ, primarily mediate touch, while high threshold fibers, mainly Aδ and C, conduct nociceptive signals [8]. The response to peripheral sensory or noxious stimulation in rats has been studied in depth [23-24; 39]. In contrast, only few fMRI studies in mice using electrical stimulation paradigms have been reported to date [2; 31; 33; 1]. This is mainly due to experimental challenges related with the small size of mice, putting high demands on spatial resolution and thus sensitivity.

Another challenge for robust fMRI measurements in mice is the maintenance of stable physiological conditions. While human fMRI experiments are carried out in awake subjects, the large majority of animal fMRI studies are performed in anesthetized animals. Therefore, anesthesia should neither interfere with brain activity nor act analgesic when investigating the response to noxious stimulation paradigms. Unfortunately, there is no such ideal anesthetic suitable for longitudinal studies e.g. for studying functional changes over time. In this work we used isoflurane, the advantages of which are ease of administration and controlled dosing. Despite these challenges, the development of robust mouse fMRI protocols is highly desirable for investigating mechanistic aspects of signal processing under normal and pathological conditions.

The aim of this study was to develop a reliable stimulation paradigm to analyze the somatosensory and nociceptive system in mice under isoflurane anesthesia. The high sensitivity of a cryogenic surface coil enabled detailed analysis of the BOLD response in activated brain regions elicited by electrical stimulation of the mouse forepaw as a function of time. BOLD signal intensities were found to correlate well with the amplitudes of the electrical stimulation applied. The quantitatively assessed dynamics of the temporal profile of the BOLD response yielded further insight into the hemodynamic response to electrical stimulation.

Materials and Methods

Animal preparation

All experiments were performed in accordance to the Swiss law of animal protection. 15 female C57Bl/6 mice weighting 22 ± 3 g were anesthetized with Isoflurane (induction 2-3 %, maintenance 1.2 % in a 70 % air – 30 % oxygen mixture; Abbott, Cham, Switzerland), endotracheally intubated and mechanically ventilated throughout the entire experiment to ensure stable physiology (90 breaths/minute, respiration cycle: 25 % inhalation, 75 % exhalation; Maraltec, Alfos Electronics, Biel-Benken, Switzerland). Animals were paralyzed by intravenous (i.v.) administration of a neuromuscular blocking agent (Pancuronium bromide, 1.0 – 1.5 mg/kg; Sigma-Aldrich, Steinheim, Germany), which avoided interference by spontaneous breathing and prevented movement artifacts during the fMRI experiments despite the low isoflurane levels. A rectal temperature probe (MLT415, AD Instruments, Spechbach, Germany) was inserted to keep the animal at 36.5 ± 0.5 °C. Body temperature was maintained using a warm-water circuit integrated into the animal support (Bruker BioSpin AG, Fällanden, Switzerland). A transcutaneous electrode (TCM4, Radiometer, Copenhagen, Denmark) was placed on the shaved upper hind limb of the mouse to measure blood gas levels (pCO_2). In some animals, heart rate and blood oxygenation was monitored using a MR-compatible infrared sensor (MouseOx® Pulse Oximeter, Starr Life Sciences, Oakmont, PA, USA). For accurate and reproducible positioning, the head of the animals was fixed with stereotactic ear bars and eye cream was applied to prevent the eyes from becoming dry. For the electrical stimulation a pair of needle electrodes (Genuine Grass instruments, West Warwick, USA) was inserted subcutaneously into the distal and proximal end of the palm of each forepaw with a distance of 2-3 mm between the two needles. The identical setup and parameters (1.5 mA) were used to stimulate the hind paw of four animals.

The entire experiment lasted approximately 2 hours, of which 20 min were used for preparation of the mouse, and the remaining time was used for acquiring fMRI data. The mice were anesthetized throughout the duration of the experiment. Animals were used for more than one experiment.

MRI equipment and sequences

All MRI experiments were performed on a Bruker BioSpec 94/30 small animal MR system (Bruker BioSpin MRI, Ettlingen, Germany) operating at 400 MHz (9.4 Tesla). For signal transmission and reception a commercially available cryogenic quadrature RF surface probe (CryoProbe), consisting of a cylinder segment (180° coverage, radius = 10 mm) and operating at a temperature of 30 K was used (Bruker BioSpin AG, Fällanden, Switzerland) (for detailed information see [3]). The ceramic outer surface of the coil touching the mouse head was kept at 30 °C using a temperature controlled heating device.

Anatomical reference images in coronal and sagittal directions (slice orientations are given using the nomenclature of the mouse brain atlas [13]) were acquired using a spin echo (Turbo-RARE) sequence with the following parameters: field-of-view (FOV) = 20 x 20 mm^2, matrix dimension (MD) = 200 x 200, slice thickness (STH) = 0.5 mm, interslice distance (ISD) = 1.0 mm, repetition delay T_R = 3500 ms, echo delay T_E = 13 ms, effective echo time $T_{E,eff}$ = 39 ms, RARE factor (number of echoes sampled for each excitation) = 32, and number of averages (NA) = 1. Subsequently, the slices for the fMRI experiment were planned on the anatomical reference

image and BOLD fMRI data were acquired using a gradient-echo echo planar imaging (GE-EPI) sequence with the following parameters: Five coronal slices covering a range of 2 to 5 mm anterior to the interaural line were recorded with FOV = 23.7 x 12.0 mm^2, MD = 90 x 60 (acquisition) and 128 x 64 (reconstruction) yielding an in-plane resolution of 200 x 200 μm^2, STH = 0.5 mm, ISD = 0.7 mm, T_R = 2500 ms, T_E = 8.5 ms, and NA = 3 resulting in an image acquisition time of 7.5 seconds. The individual sections comprised the forelimb and hind limb areas of the somatosensory and insular cortices, and the thalamus [13]. An fMRI experiment consisted of 112 repetitions and lasted 14 min, except for the group of 2.0 mA, where the acquisition consisted of 96 repetitions and lasted 12 min.

Electrical stimulation paradigm

Electrical pulses of 0.5 ms duration at a frequency of 3 Hz were applied. Current amplitudes were 0.5 mA (n = 8 animals), 1.0 mA (n = 8), 1.5 mA (n = 7), and 2.0 mA (n = 8) [42]. The stimulus strength is determined by the local current density (electric current per unit area of cross section), which itself depends on the placement of the electrodes. Current thresholds for noxious stimulation were estimated from an experiment on the hairy skin of the wrist of a volunteer with analogous electrode placement as in mice (distance between electrodes). Stimulation amplitudes of 1.5 and 2.0 mA were experienced as being painful, while the amplitude of 0.5 mA was clearly innocuous. Although innervation of human and mouse skin may differ, a noxious threshold in the range of stimulation amplitude of 1.0 mA appears reasonable.

The stimulation paradigm consisted of a block design starting with a resting period of 120 seconds (baseline) followed by 60 seconds of stimulation. This series was repeated four times and fMRI data recording was continued for another 120 seconds after the last stimulation block. The duration between positioning of the mouse in the magnet bore and the beginning of the electrical stimulation and fMRI recording was kept constant at 40 minutes to ensure the same anesthesia conditions for all animals. This time was used for adjustment of MRI conditions as well as anatomical reference and high resolution scans. Stimulation started with the left paw in all animals. Following an 8 minute resting interval, the right paw was stimulated. These two stimulation cycles were followed by a control acquisition without electrical stimulation.

Data analysis

Four regions of the brain were evaluated in detail, including the somatosensory cortex S1 contralateral and ipsilateral to the stimulated paw, the thalamus and a control region at the ventral pallidum, a structure involved in neither the sensory nor the nociceptive pathway. In addition, we looked at the S2, insular and piriform cortex in the 1.5 mA group. Statistical t-maps were calculated using the general linear model (GLM) tool integrated in the Biomap software program (M. Rausch, Novartis, Switzerland). GLM assesses correlations on a pixel-by-pixel basis between the fMRI signal train and the stimulation paradigm. Activation was detected using a statistical threshold of $p = 0.0001$ for all experiments. With a minimal cluster size of 15 voxels, two coronal sections were analyzed, of which one slice covered the thalamus, the secondary somatosensory (S2) and insular cortex (IC) (2.8 mm anterior to the interaural line (IAL +2.8

mm)) and the other covered the forepaw areas of the primary somatosensory cortex (S1) (IAL +3.7 mm). The respective regions-of-interests (ROIs) derived from the GLM analysis were used to extract BOLD signal changes as a function of time. In cases for which the correlation analysis revealed no activated voxels at the expected locations as well as for the unstimulated scans ROIs were transferred from the mouse brain atlas [13]. For group analysis, EPI images covering the S1 area (IAL +3.7 mm) and the thalamus (IAL +2.8 mm) were normalized to the coordinate system of the mouse brain atlas [13]. The fMRI coordinates were defined as followed: the origin of the right-hand coordinate system was chosen at the ventral end of the brain midline through the coronal sections. The second reference point was the dorsal end of the same midline, while the third point was placed on the edge of the right hemisphere at its widest point. The coordinate axes were defined along the midline (y-axis) and perpendicular to it (x-axis). The axes were then scaled to fit the dimensions of the mouse brain atlas, using an IDL-based software developed in-house [48].

For a detailed analysis of the fMRI time curve, the resulting BOLD profile was segregated into two components *S* ('slow') and *F* ('fast'). Component *S* was extracted by fitting the 8 data points before stimulation onset (light gray bars, shown in Fig. 3a, b) to a gamma-variate function: $y(t) = a \cdot t^r \cdot exp(-k \cdot t)$ with a (amplitude factor), r (power of growth curve), k (rate of exponential decay) being the parameters to be optimized, while t is measured with regard to the start of the first stimulation period (t = 0). The best fit curve (solid line, Fig. 3b) was then subtracted from the original data to yield component *F* of the fMRI signal (Fig. 3c). The maximal amplitudes of the fitted curve for component *S* and of the extracted curve for the first

stimulation period of component *F* (see Fig. 3) was analyzed as a function of the stimulation amplitude. The quantitative analysis was carried out for all ROIs.

We furthermore analyzed the rates of BOLD signal increase and decay for both the first stimulation cycle and the entire stimulation period. The data points of the signal decay at the end of the stimulation interval (indicated by the dotted line in Fig. 5a) were used to calculate a decay constant k_{off} assuming a single exponential decay function, $S(t) = S(t=0) \cdot exp(-k_{off} \cdot t)$, with $S(t)$ indicating the signal amplitude during the decay at time t, and $S(t=0)$ the amplitude at the end of the stimulation period $(t=0)$. A minimum of four data points of the decay curve with an amplitude exceeding noise levels were required for each individual signal curve to allow for fitting. The BOLD signal decay rate constant was then correlated to the maximum BOLD response S_{max} of the single animals (Fig. 5c).

The constant k_{on} describing the initial build-up of the signal at the beginning of the stimulation to its maximum was calculated assuming the following relation: $S(t) = S_{max} \cdot [1 - \exp(-k_{on} \cdot t/S_{max})]$ for which the initial slope yields $(dS(t)/dt)_{t=0} = k_{on}$, i.e. the initial slope was assumed to be independent of the maximum BOLD signal.

Autoradiography and intrinsic optical imaging

Autoradiography with [^{18}F]-2-fluoro-2-deoxyglucose (^{18}F-FDG) was performed on two female C57Bl/6 mice according to published protocols [44; 55]. The left forepaw was electrically stimulated at 1.5 mA using the parameters described above. A 5 minute stimulation period was

followed by a one minute break. This was repeated for the entire time course of 45 minutes, before the animals were sacrificed and the brain extracted.

One mouse was used for intrinsic optical imaging. Reflectance from 570 nm light was measured through the exposed skull using a CCD camera. The left forepaw was stimulated with a 10 second pulse train of 0.5 msec pulses of 1.0 mA current amplitude at 3 Hz. These experiments were carried out under 1.5% isoflurane anesthesia.

Results

Animal physiology and anesthesia

Non-invasive monitoring of the mice showed stable physiology throughout the duration of the experiments. Blood gas levels of pCO_2 measured transcutaneously were in the range of 40 ± 10 mm Hg, which indicates a well adjusted ventilation of the animals [50]. Body temperature was kept stable at 36.5 ± 0.5 °C for the entire experiment. The monitored heart rate was stable around 500 beats per minute in all animals and no changes were detected during the stimulation. After completion of the fMRI investigation, the animals recovered fast and could be used for further experiments, an important prerequisite for longitudinal studies.

Signal and image quality

By exploiting the significant gain in sensitivity provided by the use of a cryogenic RF surface coil for signal detection [35; 3] BOLD fMRI data sets of high quality suitable for reproducible quantitative analysis have been obtained. Comparing the CryoProbe with a conventional room temperature coil of similar dimensions (for detailed information on the coils see [3]) using the GE-EPI sequence, a gain in image signal-to-noise ratio (SNR) of a factor of 3.1 ± 0.7 (mean ± standard deviation, unpublished data) was achieved. Using a coronal slice orientation proved advantageous as cross-sectional images recorded ≥ 3 mm anterior to the interaural line were largely devoid of geometrical distortions caused by local magnetic field inhomogeneities due to different magnetic susceptibilities of adjacent tissue compartments. In caudal brain structures, significant susceptibility artifacts have been observed due to the proximity of the air filled ear cavities. This also impaired the quality of images recorded in horizontal plane view, which would allow covering larger brain areas. Distortions caused by differences in susceptibility are experienced on an absolute scale, i.e. they affect more extended brain regions in mice than in rats due to the smaller dimensions of the mouse.

fMRI data showed good reproducibility (e.g. see error bars in Figs. 1 g, h) and allowed for assessing differences in the BOLD response during stimulation at different amplitudes.

Spatial distribution and intensity of the BOLD response

The spatial distribution of the activated areas after forepaw stimulation at 1.5 mA (threshold p=0.0001, cluster size: 15 voxels) for one representative animal is shown in Figure 1. The position of five coronal slices is indicated in the sagittal section shown in Figure 1a. Besides the forepaw S1 region activated areas are present in other S1 areas (Fig. 1c-f), the primary motor cortex (Fig. 1c-f), and several nuclei of the thalamus, including the ventral posterior nucleus which relays somatosensory information to the cortex (Fig. 1f) [13]. Figure 1i shows the distinction of the forepaw area (blue) and the hind paw area (red) after the respective stimulation as an activation map of two representative animals. As expected, hind paw somatosensory S1 areas were located median to the respective forepaw regions. The activated cluster at the brain midline reflects signal contributions from the sagittal sinus. No consistent deactivations were detected in any region of the brain.

Figure 2 shows statistical maps (threshold p=0.0001, cluster size: 15 voxels) depicted on the mouse brain atlas ([13], Fig. 2 a-f: IAL +3.7 mm, Fig. 2 g-l: IAL +2.8 mm) obtained from all animals at different stimulation amplitudes ((a, g) 0.5 mA, (b, h) 1.0 mA, (c, i) 1.5 mA, (d, j) 2.0 mA). The activated clusters of individual animals were overlaid, i.e. the intensity in the activation map corresponds to the number of animals displaying a significant BOLD signal (left and right forepaw for each animal). Activation in response to the forepaw stimulation appears in the somatosensory S1 and S2 cortices, in the thalamus and at higher amplitudes in the insular cortex (regions indicated in Fig. 2e, k). For all activated regions, the spatial extent of BOLD response exceeded the topological area defined on the basis the mouse brain atlas [13].

This is attributed to the fact that fMRI assesses the hemodynamic response elicited by neural activity and not the neural activity per se.

BOLD signal changes in correlation to the forepaw stimulation paradigm

The maximal BOLD signal intensity increased with increasing stimulation amplitude in all analyzed regions involved in sensory and nociceptive processing in a comparable manner as in the regions shown in Figures 3 and 4. Stimulation at the lowest amplitude of 0.5 mA led to a maximal BOLD signal of 0.93 ± 0.25 % (in % of baseline intensity) in the primary somatosensory cortex contralateral to the stimulated paw. For amplitudes of 1.0, 1.5, 2.0 mA, the maximal BOLD signal changes in this region amounted to 1.94 ± 0.20 %, 2.54 ± 0.22 %, and 3.52 ± 0.41 %, respectively (Fig. 3a). The maximum BOLD amplitude decreased for subsequent stimulation periods. Interestingly, the signal did not return to the initial baseline level within the two minutes resting interval following a stimulation episode, but stayed elevated until the start of the next stimulation block. The BOLD response to unilateral forepaw stimulation appeared consistently bilateral in all activated regions, including the S1 (Fig. 1, 4), thalamus, S2 and insular cortex (for 1.5 mA: Fig. 1h). The maximal BOLD signal amplitude in the regions of the S2 somatosensory and insular cortex was significantly lower as compared to the S1 area. This was observed at all stimulation amplitudes except at 0.5 mA, where the amplitudes for S1 and S2 area reached similar values (data not shown). There was no delay between ipsi- and contralateral responses within the time scale of the fMRI experiment (7.5 s temporal resolution).

The control region, which was located in the ventral pallidum, a structure not involved in sensory or nociceptive processing, showed no change in BOLD intensity for stimulation amplitudes ≤ 1.5 mA (Fig. 1g, 1.5 mA). At 2.0 mA a maximum signal increase of 0.81 ± 0.12 % was detected. This unspecific increase in the BOLD signal was observed in large parts of the brain. A second, cortical control region located in the piriform cortex, behaved comparable to the ventral pallidum (for 1.5 mA: Fig. 1h). No region-specific activation whatsoever, but only background noise was revealed by the analysis of the control fMRI data sets acquired without stimulation, indicating the stability of the fMRI setup including the physiological preparation (data not shown).

Amplitudes of the two signal components S and F as a function of the stimulation amplitude

The temporal profile of the BOLD response has been segregated into the two signal components *S* and *F* by fitting component *S* to a gamma-variate function and subtracting the best fit from the experimental data (Fig. 3b, c). Analysis of the maximal amplitude of both components for the two S1 regions and the thalamus were found to correlate with the stimulation amplitude. Linear regression analysis yielded correlation factors for component *S* (Fig. 5a) of $R^2 = 0.98$ for the contralateral, $R^2 = 0.97$ for the ipsilateral somatosensory cortex and $R^2 = 0.81$ for the thalamus, respectively. The values for component *F* (Fig. 5b) were $R^2 = 0.97$ for contralateral S1, $R^2 = 0.98$ for ipsilateral S1 and $R^2 = 0.87$ for thalamus.

Analysis of the signal dynamics of component F

Not only the amplitude but also the dynamic behavior of the BOLD response depended on the stimulation amplitude as demonstrated by the averaged temporal profile of component *F* of the first stimulation period of each animal (Fig. 5a). The maximum values for the S1 region contralateral to the stimulated paw were: 1.19 ± 0.48 %, 1.69 ± 0.21 %, 2.47 ± 0.18 %, and 2.81 ± 0.37 % for 0.5, 1.0, 1.5, and 2.0 mA, respectively. A striking feature of the observed BOLD response (Fig. 3c, 5a, b) is that the signal amplitude started to decay despite ongoing stimulation. The signal maximum was observed typically 30 seconds after stimulation onset, thereafter the amplitude decreased significantly by 5 to 15 %. The fitted curves (Fig. 5a) were computed assuming single exponential signal build-up and decay with parameters described in the Materials and Method section. The exponentially decaying vasodilatory response of the neuronal signal is characterized by a time constant k_v = 0.02 s^{-1}. For the build-up of the BOLD signal the same initial rate k_{on} = 0.002 s^{-1} was assumed, irrespective of the stimulation amplitude applied. In contrast, the value for the decay rate constant k_{off} was found to decrease with increasing stimulation amplitude. This is also apparent when normalizing individual BOLD signals to the respective maximum intensity value (Fig 5b). Single exponential fitting for the averaged curves yielded first-order rate constants of k_{off} values of 0.040 s^{-1} for 2.0 mA, 0.051 s^{-1} for 1.5 mA and 0.063 s^{-1} for 1.0 mA, displaying a linear dependence on the stimulation amplitude with a correlation factor of R^2 = 1.00 (data not shown). In a next step we tested whether the decay rate k_{off} depended on the maximal BOLD change. We therefore used the average BOLD signals of all stimulation cycles (4 stimulation blocks per stimulation amplitude),

which fulfilled the criteria with at least four data points exceeding the noise level used for the fitting procedure (Fig. 5c): a significant negative correlation has been found with $R^2 = 0.76$.

Autoradiography and intrinsic optical measurements

The autoradiography data of the two animals (Fig. 6b) showed a clear bilateral increase in FDG uptake in the thalamus, consistent with the observed fMRI activation pattern (Fig. 6a). Cortical activation was found to be weak. Bilateral local increases in cortical blood volume indicative of neuronal activation were detected using intrinsic optical imaging (Fig 6d), which is in line with our fMRI finding of bilateral cortical activation.

Discussion

fMRI in rodents, predominantly in rats, has become an important tool in biomedical research e.g. to phenotype animal models of CNS disorders [36; 32; 37; 31]. In view of the many genetically engineered mouse lines the development of robust procedures for mouse fMRI protocols should be rewarding. By exploiting the significant gain in sensitivity provided by the use of a cryogenic RF surface coil for signal detection [35; 3], BOLD fMRI data sets of high quality suitable for reproducible quantitative analyses have been obtained. The CryoProbe enabled fMRI at a spatial resolution of 200x200x500 μm^3, which is sufficient to resolve the major cerebral structures of the mouse brain and allows for detailed anatomical and functional studies.

The obtained fMRI data were highly reproducible both with regard to spatial extent and temporal profile. This allowed reliable detection of even small changes in the BOLD amplitude in response to stimulations at different current amplitudes. The BOLD intensity increased significantly at each stimulus onset, though there was a net decrease of BOLD amplitude for subsequent stimulation periods across the cycle consisting of four blocks. This was observed before [18; 42] and might be due to adaptation or habituation mechanisms, occurring either peripherally in the stimulated paw, or centrally in the brain. These mechanisms may also contribute to the signal decrease observed during ongoing stimulation (Fig. 3a, b).

Due to the lack of clear evidence whether stimulation was noxious or not, the parameters used were tested on a human subject under the presumption that the threshold to activate C- or Aδ-fibers is similar in humans and mice. However, as the innervation pattern differs, the human values cannot be translated directly to the mouse but should rather be used as an estimate of the noxious threshold. This stands in contrast to a study by Nair and Duong, which report hind paw stimulation with amplitudes up to 7 mA to be somatosensory only [33]. To our experience, the stimulation used in that study was likely to be noxious, at least at higher amplitudes.

Data analysis revealed a robust activation of the S1 cortical forelimb area. However, the signal was not confined to the S1 region contralateral to the stimulated paw, but was also observed on the ipsilateral side with essentially the same amplitude and spatial extent. This stands in contrast to the majority of fMRI studies in healthy rats which report strictly unilateral responses during unilateral electrical stimulation [53; 41; 15; 20] including our own study using isoflurane

anesthesia [48]. We performed additional experiments to modulate the laterality of the fMRI response: varying anesthesia depths, male instead of female mice, or preparing only one paw with electrodes to prevent possible crosstalk between the leads carrying the needle electrodes. None of these interventions affected the bilateral symmetry of the activation pattern observed (data not shown). In addition, electrical forepaw stimulation at 1.5 mA in another strain (HsdWin:NMRI) showed the same bilateral activation pattern (data not shown). Bilateral activation of the areas responsible for pain processing has also been observed in humans [45; 52; 47; 49; 46]. These bilateral signals may be conveyed by fibers of the corpus callosum [29] or by commissural neurons of the spinal cord [38]. The occurrence of bilateral activation has also been reported previously for rat studies [25]. However, due to our relatively slow temporal resolution of 7.5 s, we were not able to resolve a possible delay between the onset of activation in the two hemispheres. Even increasing the temporal resolution to 1 s was not sufficient to reveal a potential delay of the ipsilateral versus the contralateral activation (data not shown).

Although carried out with a small number of animals, autoradiography and intrinsic optical imaging experiments support our fMRI findings. Autoradiography revealed a distinct bilateral activation of the thalamus, most pronounced in the ventral posterior nuclei, structures known to relay nociceptive information. One mouse also showed bilateral cortical activation in the regions of S2, insula and motor cortex. The intrinsic optical measurement showed a clear bilateral activation of similar amplitudes in both hemispheres. These experiments, which were performed independently from our fMRI setup, are in line with the results obtained with BOLD fMRI.

Besides the S1 region, bilateral activation was observed in the thalamus, the motor, S2 and insular cortex. These regions are known to be part of the nociceptive network. Motor cortex activation might originate from antidromic stimulation of the efferent motor fibers as seen in a study by Cho and colleagues [9]. These activated areas are in accordance with those reported for similar studies in rats [53].

Anesthesia is a recurring issue in animal imaging, in particular when investigating nociception. Isoflurane is an attractive anesthetic as it is easy to administer and control; however, there are also drawbacks. Isoflurane is a potent vasodilator causing a global increase of cerebral blood flow (CBF) in a dose dependent manner [27]. The basal energy level as derived from the cerebral metabolic rate of glucose consumption (CMR_{glc}) is lower than in the awake state. A reduction by approximately 40% was reported for an isoflurane concentration of 1.4% [22]. In comparison, α-chloralose was found to reduce baseline CMR_{glc} by approximately 60% [25]. The higher energy consumption, the high CBF and concomitantly the dilated vessels in isoflurane anesthetized animals during baseline conditions reduce the dynamic range of the hemodynamic response as compared to α-chloralose anesthesia [10; 19]. The vasodilatory effects however are dose dependent and can therefore be significantly reduced by using relatively low isoflurane levels (at around 1%). At this low level we assume reduced antinociceptive efficacy of isoflurane. Deady et al. showed that the hypnotic effects of isoflurane occurred at lower concentrations than the antinociceptive effects [12]. Also, low concentrations of isoflurane appear to exhibit minimal neuro-suppressive effects, as the flow-metabolism coupling was shown to remain preserved [16]. The robust BOLD response reported in this study as well as

data from other studies performed under isoflurane anesthesia prove it to be a useful anesthetic for fMRI studies in rodents [36; 32; 37; 40; 33; 43].

A recent study [1] performed on mice using electrical forepaw stimulation claims the α2-adrenergic receptor agonist medetomidine to be better for long time studies than other anesthetics. However, the occurrence of BOLD activation in less than 60% of all scans performed (our study: > 95%) and the noisy temporal BOLD profiles do not clearly show the superiority of medetomidine anesthesia.

When analyzing the temporal BOLD profile, it became obvious that it consists of two components, of which one is in phase with the stimulation (component *F*), while the other one is much slower, starting with the onset of the first stimulation (component *S*). The two components might be explained in terms of the underlying physiological processes: Component *F* being in phase with the stimulation episodes, is probably associated with the peripheral neuronal input of the A and C fibers, while the underlying signal described by component *S* might reflect a slow vascular response.

The BOLD signal amplitude of both components depended linearly on the stimulation amplitude. A similar linear dependence has been demonstrated using cerebral blood volume (CBV) [31] and CBF [42] measurements in mice and rats, respectively. Torebjörk et al. also showed that nociceptor responses and individual pain ratings in humans both linearly correlated with the applied heat stimulation [51].

By averaging and normalizing the BOLD signal curves of all animals, it became apparent that the rate of the signal decay following a stimulation episode decreased with increasing stimulation amplitudes. Stimulation at high amplitudes led to a larger BOLD response and thus to a higher content of oxyhemoglobin (and correspondingly a lower concentration of deoxyhemoglobin) in the vessels, as compared to stimulations at lower amplitudes. This is in line with the hemodynamic model described by Friston and colleagues [14], which combines the balloon model with a linear dynamic model of changes in CBF as caused by neuronal activity. The balloon model describes the link between CBF and the BOLD signal and is able to predict nonlinear effects of the BOLD signal, which contrast the linear relationship of CBF and synaptic activity. The central concept of the model is to treat the venous compartment as an expandable balloon, which is inflated by an increase in CBF, leading to a dilution of the deoxygenated blood and an increased expelling rate of the blood [4]. The model predicts the recovery rate following a stimulation episode to be proportional to the amount of deoxyhemoglobin in the vessel [5; 14; 4], which is in line with our experimental results (Fig.5). In contrast, the initial rate of the build-up of the BOLD response was found independent on the current amplitude within error limits.

A further aspect that becomes apparent from the temporal profile of the BOLD response is that the BOLD amplitude decays during stimulation despite ongoing peripheral input. This can be explained by a decaying vasodilatory signal, which is also subject to feedback regulation by CBF, in response to a prolonged neuronal stimulus [15, 45]. The neuronal input is described as an initial peak followed by a decay to a lower level [14; 4].

We could demonstrate that reproducible mouse BOLD fMRI data can be obtained following sensory stimulation. The high quality of the data and the use of isoflurane make longitudinal studies to study e.g. functional changes in the brain feasible.

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Figure Captions

Figure 1: Spatial distribution of the BOLD activation. **(a)** Sagittal reference image indicating the positions of the coronal EPI slices covering a large section of the mouse forebrain. **(b) – (f)** Spatial distribution of the activated clusters (p = 0.0001) of one representative animal after unilateral forepaw stimulation. The left side of the image corresponds to the left hemisphere. Nominal distance to the interaural line (IAL) is given for each slice. Scale bar indicates 5 mm. Activated areas include the S1 forelimb area **(c, d, e)**; motor cortex M1 **(c, d, e)**; and several nuclei of the thalamus, including the ventral posterior nucleus which relays somatosensory information **(f)**. Activated clusters are also observed at the sagittal sinus **(e, f)**. **(g)** Time course of the BOLD signal after unilateral electrical stimulation of the forepaw at 1.5 mA for S1 contralateral to the stimulated paw (pink), S1 ipsilateral (dashed black), thalamus (gray), and the control region (light gray). Gray bars indicate the stimulation periods. The BOLD signals of the contralateral and ipsilateral S1 are almost identical. **(h)** Maximal signal amplitude of different regions (S1, S2, insular cortex (IC), piriform cortex (PC, control region)) ipsi- (black circles) and contralateral (pink squares) to the stimulated paw at 1.5 mA. All values are given as mean + SEM. **(i)** Activation map of two representative animals showing activation of the forepaw (blue) and hind paw (red) S1 area after stimulation of the respective paw at 1.5 mA, overlaid on an EPI image. Scale bars indicate t-values.

Figure 2: Activation maps of the cortex after left and right forepaw stimulation at **(a,g)** 0.5 mA, **(b,h)** 1.0 mA, **(c,i)** 1.5 mA, **(d,j)** 2.0 mA. Data show activated clusters derived from GLM analysis

(p = 0.0001, cluster size: 15 voxels) for all animals overlaid on the mouse brain atlas at IAL +3.7 mm **(a-f)** and IAL +2.8 mm **(g-l)** [13]. **(e-k)** Mouse brain atlas with relevant regions (SI: primary somatosensory cortex, forepaw region; SII: secondary somatosensory cortex; IC: insular cortex; MI: primary motor cortex; TN: thalamic nuclei) overlaid on the anatomical image. **(f,l)** Representative EPI image revealing relatively little distortions. **(g-j)** Activation maps; intensity indicates the number of stimulation periods displaying significant BOLD activation at a given location (scale bar).

Figure 3: (a) Relative change of the BOLD signal intensity as a function of time during electrical forepaw stimulation for the different stimulation amplitudes. Dark grey bars indicate the stimulation periods, light gray boxes mark data points used for fitting the component *S* curve **(b). (c)** Signal component *F* obtained by subtracting **(b)** from **(a).** Values are given as mean + SEM.

Figure 4: The amplitudes of the two components *S* **(a)** and *F* **(b)** as a function of the stimulation amplitude. **(a)** Linear regression analysis yielded correlation factors of R^2 = 0.98 for the contralateral and R^2 = 0.97 for the ipsilateral S1 somatosensory cortical area. The corresponding value for thalamus was R^2 = 0.81. **(b)** For the fast component the correlation factors were: R^2 = 0.97 for contralateral S1, R^2 = 0.98 for ipsilateral S1 and R^2 = 0.87 for thalamus. Values are given as mean ± SEM.

Figure 5: Analysis of component *F* of the fMRI response to electrical stimulation of the forepaw in the contralateral S1 cortical region. **(a)** Mean fMRI signal response for the first stimulation episode as a function of time and current amplitude. Solid lines indicate the BOLD response modeled as described in the text. The dashed line illustrates the vasodilatory stimulus, which displays a single exponential decay with a rate constant $k_v = 0.02\ s^{-1}$. **(b)** The mean normalized fMRI response of the first stimulation period (normalized to 1) as a function of time and stimulation amplitude. The reduced rate of signal decay with increasing stimulus amplitude (and correspondingly higher maximum BOLD intensity) becomes apparent. For sake of clarity error bars have been omitted. **(c)** The decay rate constant of the BOLD signal of the single animals decrease with increasing maximal intensity of the BOLD signal. Linear regression yielded a correlation coefficient of $R^2 = 0.76$. Values are given as mean $\pm$ SEM.

Figure 6: Autoradiography and intrinsic optical imaging. **(a)** fMRI activation map of a representative animal. **(b)** ^{18}F-FDG autoradiographies of two mice after unilateral forepaw stimulation show bilateral thalamic activation and some bilateral cortical activation (blue arrows). **(c)** Reflection of 570 nm light used for intrinsic optical imaging reveals the vascular anatomy at both sides of the sagittal sinus. **(d)** Activation map of intrinsic optical imaging shows bilateral activation of the somatosensory area. Regions with increased cerebral blood volume are recognized by a decrease in signal intensity (blue). Color bar indicates changes in signal intensity in [%].

Summary

The development of a robust BOLD fMRI protocol using electrical forepaw stimulation allowed studying somatosensory and nociceptive processing in mice.

Figure 1

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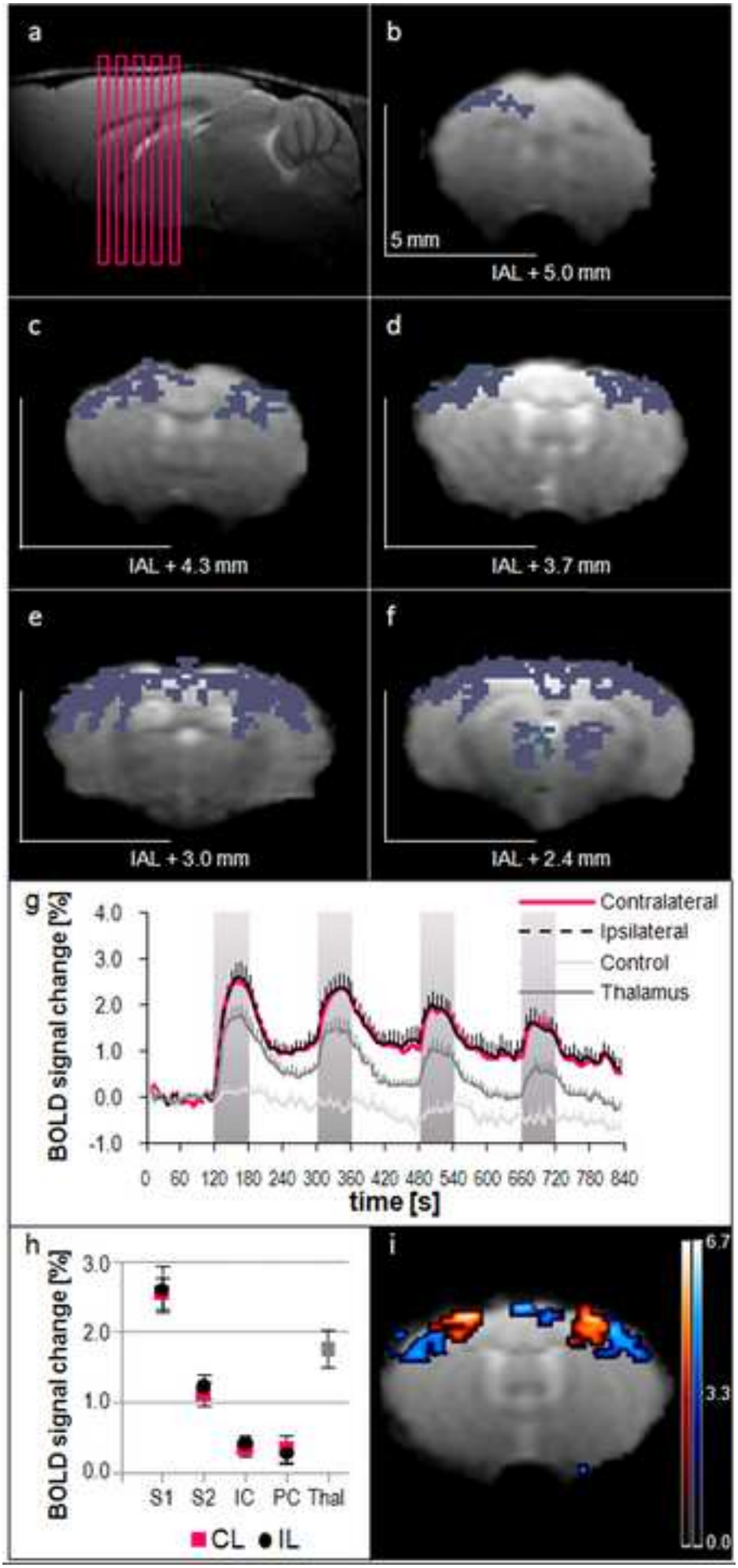

Figure 2

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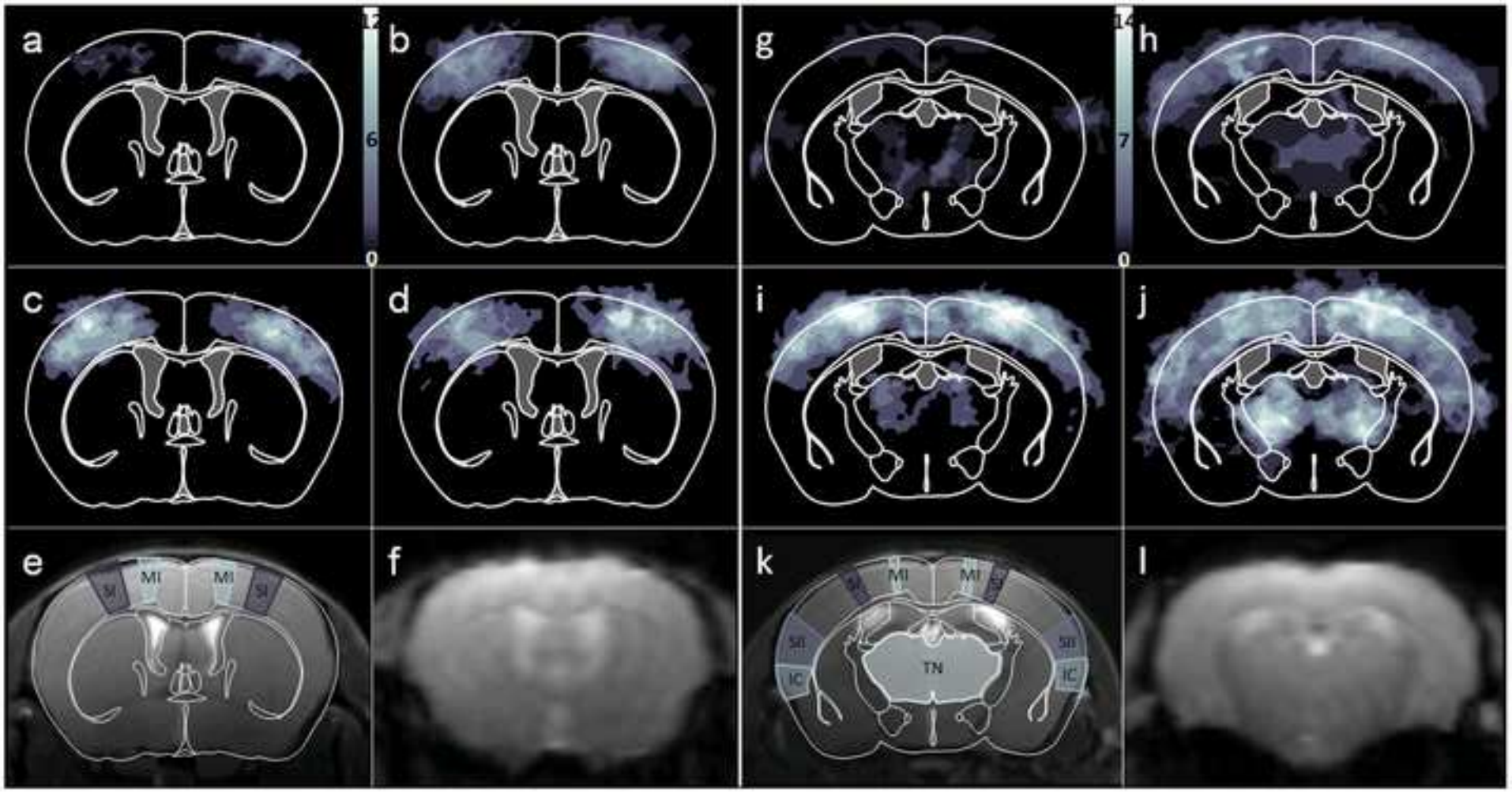

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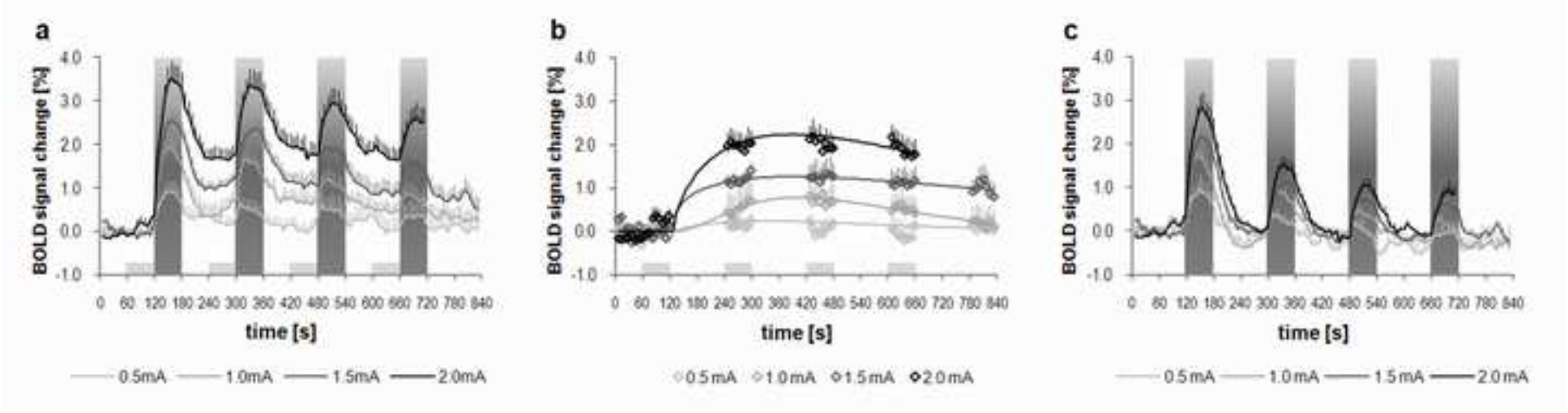

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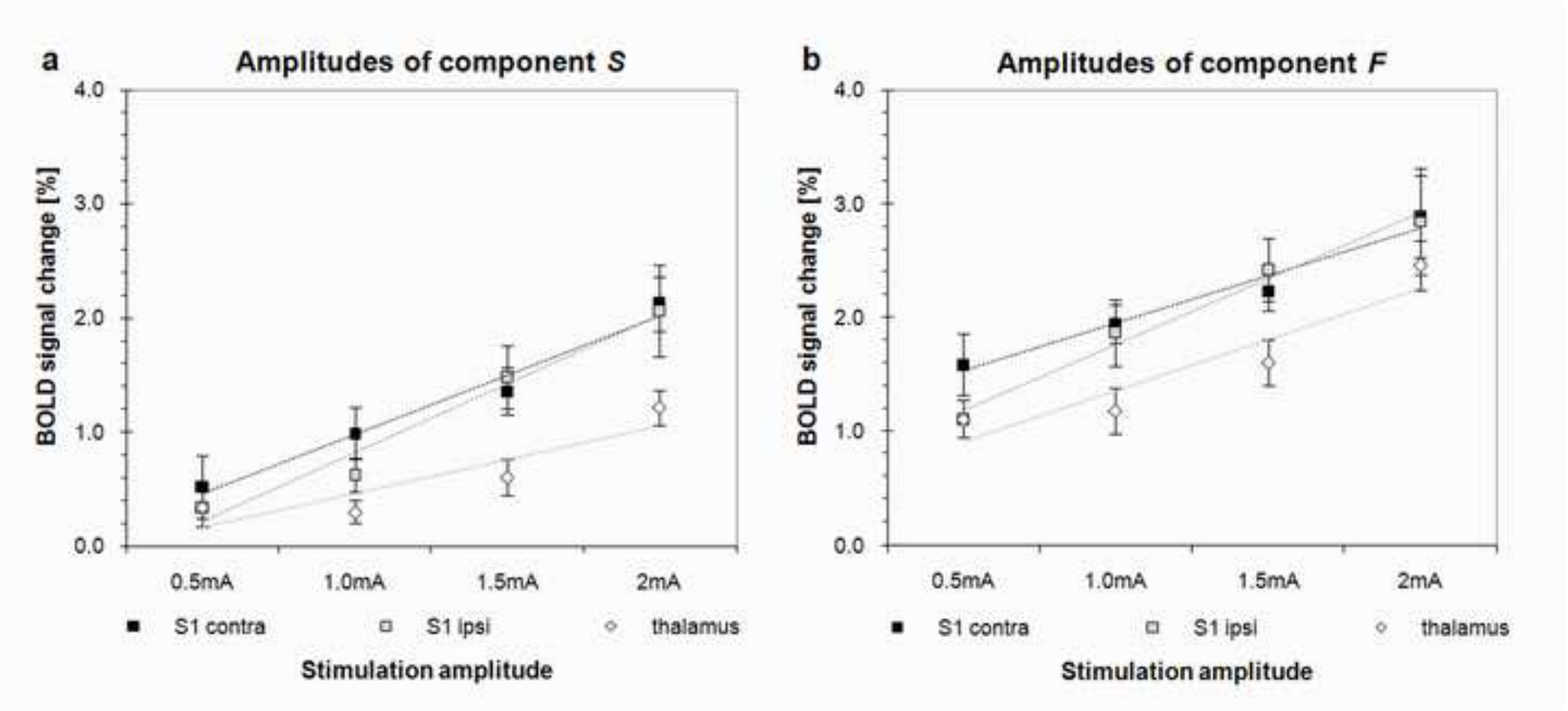

Figure 5

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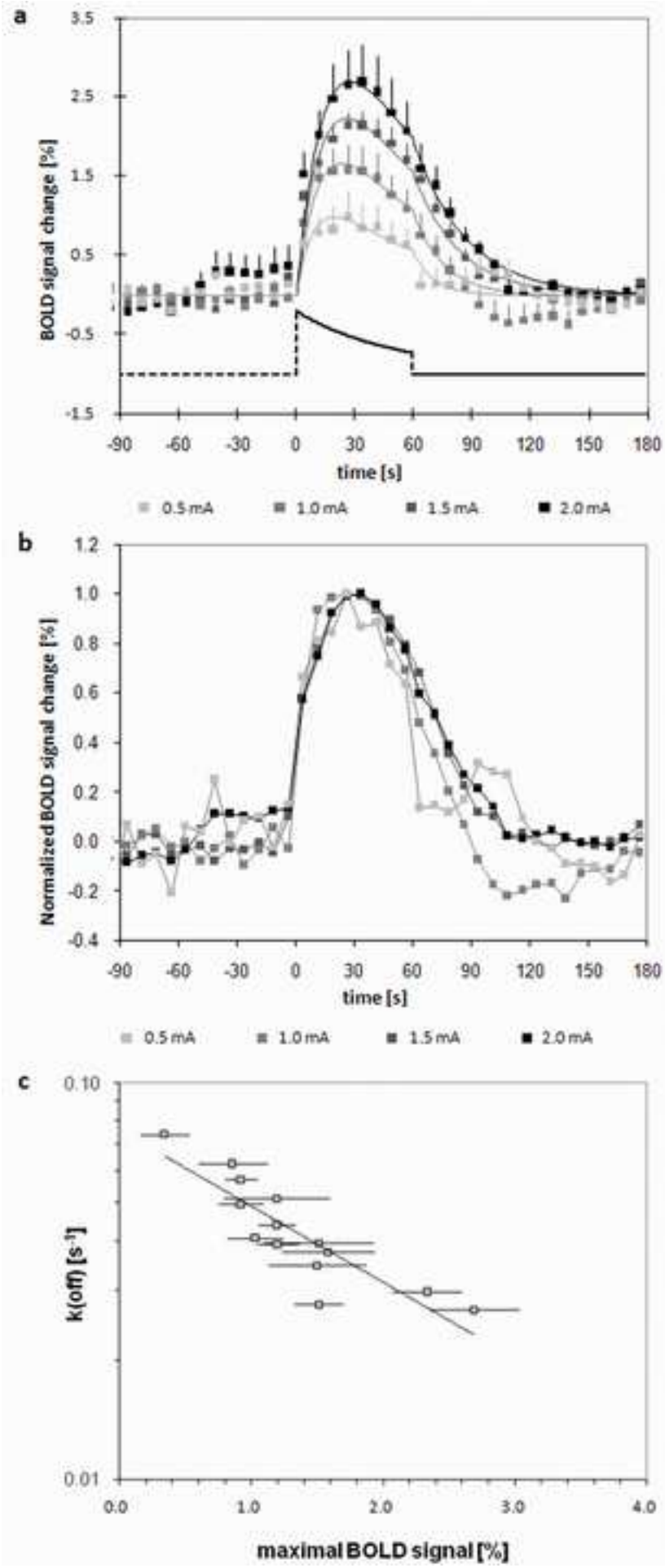

Figure 6

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